CHARACTERIZING AN EARLY LIFE MODEL OF GLUTEN SENSITIVITY

CHARACTERIZING GLUTEN IMMUNOPATHOLOGY IN DR3-DQ2 TRANSGENIC MICE SENSITIZED TO GLUTEN IN EARLY LIFE

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DESCRIPTIVE NOTE

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LAY ABSTRACT

Celiac disease is the destruction of the upper gut lining by an immune reaction caused by gluten in people with genetic risk. Celiac patients cannot absorb nutrients well and have many complications. While it can occur at any age, its onset in children is associated with the HLA-DQ2 gene. Because not every child with the HLA-DQ2 gene will develop celiac disease, additional factors are suspected. Understanding these factors could help prevent disease, as the only treatment – a life-long gluten-free diet – is not always effective. Thus, an animal model that mimics early life disease onset would be useful. Therefore, I characterized signs of celiac disease in young mice with the HLA-DQ2 gene. I determined that gluten and a microbial toxin given in early life induces inflammation and positive celiac blood tests. This model constitutes a useful tool to test the role of environmental factors in celiac disease in early life.

ABSTRACT

The gastrointestinal tract specializes in digestion and nutrient absorption via its mucosal surface. Through this large mucosal surface, interactions between the host and its environment, including food antigens and microbes, occur. Therefore, it is imperative that the gut discriminates between innocuous food components, and potential threats such as infections. On some occasions, this fine-tuned discrimination fails, leading to chronic inflammation. Celiac disease (CeD) is an autoimmune enteropathy triggered by gluten, the name given to a family of storage proteins (prolamins) that are naturally found in wheat, barley, and rye. To develop CeD, an individual must carry the susceptibility genes, the HLA-DQ2 and/or HLA-DQ8 alleles and consume gluten. However, this is not sufficient to cause disease, indicating that environmental co-factors are at play. Individuals homozygous for the HLA-DQ2 allele are at high risk to developing CeD in infancy. Currently, there is no existing transgenic animal model that addresses early life exposure to gluten, co-factors, and their effects on CeD development. Therefore, the overall goal of my thesis is to characterize a mouse model transgenic for the HLA-DQ2 allele with exposure to gluten in early life.

I first studied the physiological and immunological responses to gluten at the time of solid food introduction using DR3-DQ2 transgenic mice. I determined that after sensitization to gluten before weaning, mice developed moderate enteropathy and some developed both anti-tissue transglutaminase 2 and anti-gliadin antibodies. I then evaluated the recovery of gluten immunopathology after gluten was removed for an extended period. After 6 months on gluten-free food, enteropathy and intestinal anti-gliadin and anti-TG2

antibody levels improved. These findings show pre-weaning sensitization of DR3-DQ2 transgenic mice reproduces key features of CeD, which can be used in future studies to assess environmental triggers and mechanisms that are of importance during early life.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
AMP	Anti-microbial peptide
APC	Antigen-presenting cells
ASF	Altered Schaedler flora
ATI	Amylase-trypsin inhibitor
BSA	Bovine serum albumin
CCK	Cholecystokinin
CDGEMM	Celiac Disease Genomic, Environmental, Microbiome, and Metabolomic cohort
CeD	Celiac disease
CFA	Complete Freund's adjuvant
CT	Cholera toxin
DAB	diaminobenzidine
DC	Dendritic cell
DGP	Deamidated gluten peptides
DNase	Deoxyribonuclease
DOHaD	Developmental origins of health and disease
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
ENS	Enteric nervous system
FAE	Follicle-associated epithelium
GALT	Gut-associated lymphoid tissue
GCED	Gluten-contamination elimination diet
GFD	Gluten-free diet
GI	Gastrointestinal
GIP	Glucose-dependent insulinotropic peptide
GLP	Glucagon-like peptides
H&E	Hematoxylin & eosin
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cells
HSP	Heat-shock protein
ICC	Interstitial cells of Cajal
IEC	Intestinal epithelial cells
IEL	Intraepithelial lymphocytes
IFN	Interferon
IgA	Immunoglobulin A
IL	Interleukin
ILF	Isolated lymphoid follicle
IP	Intraperitoneal
LP	Lamina propria
LT	Long-term
M cell	Microfold cell
MHC	Major histocompatibility complex
MICA	MHC class I chain related gene A
MLN	Mesenteric lymph nodes
NK	Natural killer
NKG2D	Natural killer group 2D
NOD	Non-obese diabetic
NS	Non-sensitized
P	Post-natal day
PBS	Phosphate-buffered saline
PBS-T	0.05% Tween 20 in PBS
PCR	Polymerase chain reaction
pIgA	Polymeric immunoglobulin A
pIgR	Polymeric immunoglobulin receptor
PMSF	Phenylmethanesulfonyl fluoride
PP	Peyer's patches
PRR	Pattern-recognition receptor
PT-gliadin	Pepsin-trypsin digested gliadin
PYY	Peptide YY
RNase	Ribonuclease
rRNA	Ribosomal ribonucleic acid

RT	Room temperature	TEDDY	The Environmental Determinants of Diabetes in the Young cohort
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2		
SD	Standard deviation	TG2	Tissue transglutaminase 2
SEM	Standard error mean	Th2	T helper type 2
SIgA	Secretory IgA	TJ	Tight junctions
SPF	Specific-pathogen free	TMB	Tetramethylbenzidine
ST	Short-term	Treg	Regulatory T cell
TCR	T cell receptor	V/C	Villus-to-crypt

DECLARATION OF ACADEMIC ACHIEVEMENT

I, Julie K. Godbout, declare that I am the sole author of this document. The work presented in 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 Verdú, Dr. Heather Galipeau and Dr. Marco Constante.

1. CHAPTER 1: INTRODUCTION

INTRODUCTION

1.1 Anatomy and physiology of the small intestine

The gastrointestinal (GI) tract's primary function is the breakdown of food and the absorption of nutrients which is mainly completed in the small intestine, with the exception of complex fibres which can be metabolized in the colon (König et al., 2016; Liao et al., 2009). To accomplish this, the small intestine has specific structural and functional characteristics.

The walls of small intestine are composed of four layers: the mucosa, the submucosa, the muscle, and the serosa (Liao et al., 2009). The exterior layer of the intestinal wall combines two muscle types; the outer longitudinal muscles and the inner circular muscles, which allows both the mixing of foodstuffs and their motility through the GI tract (Liao et al., 2009). Layered over the muscle layers of the intestinal wall are the submucosa and subsequently, the mucosa, followed by another thin layer of muscle known as the muscularis mucosa (Figure 1.1) (Gourevitch, 2005; Liao et al., 2009).

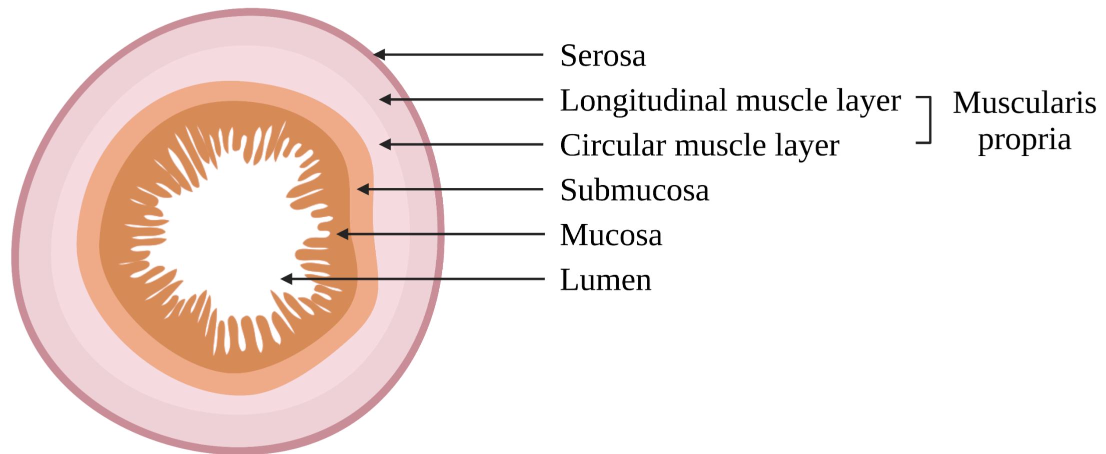

Figure 1.1 Anatomy of the small intestine

The small intestine is composed of several layers. Starting with the outer layer towards the inside, the layers are as follows: serosa, muscularis propria composed of a longitudinal muscle layer and circular muscle layer, submucosa, mucosa, and finally, the lumen. Created with BioRender.com

Structurally, the small intestine must have a large surface area that maximizes absorption. To do so, it displays long, thin folds named villi, lined with a single layer of intestinal epithelial cells (IECs) with apical microvilli (Gourevitch, 2005). Many different cells fall under the term IEC, amongst them: enterocytes, goblet cells, Paneth cells, stem cells and enteroendocrine cells (Kong et al., 2018).The resulting surface area dedicated to absorption in the small intestine is 200 m^2 (DeSesso & Jacobson, 2001).

Within the intestinal wall, goblet cells are responsible for producing mucus (Gourevitch, 2005). Intestinal crypts are found at the base of the villi, where enteroendocrine cells, goblet cells and other IECS are produced by intestinal stem cells (Gourevitch, 2005). Enterocytes, simple columnar epithelial cells, compose the majority of IECs (Kong et al., 2018). Enterocytes play an important role in the absorption of nutrients

and the secretion of immunoglobulins (or antibodies) (Kong et al., 2018). Enteroendocrine cells are chemoreceptors, triggered by stimuli in the lumen to secrete hormones such as gastrin (G cells), ghrelin (P or X cells), somatostatin (D cells), cholecystokinin (CCK, I cells), serotonin (enterochromaffin cells), glucose-dependent insulinotropic peptide (GIP, K cells), glucagon-like peptides (GLPs) and peptide YY (PYY, L cells) (Sternini et al., 2008). Enteroendocrine cells can be classified as open where their microvilli extend into the lumen or closed where they do not (Sternini et al., 2008). When new cells exit the intestinal crypt, they will slowly move towards the tip of the villi where they will be shed as they mature (Gourevitch, 2005; Sternini et al., 2008). This allows for constant renewal of the villi lining every two to seven days (Crosnier et al., 2006).

To further accomplish its primary function, the gut harbors a complex neural apparatus, the enteric nervous system (ENS), which independently from the brain coordinates secretion, absorption, smooth muscle function as well as a vast neuroendocrine system that modulates appetite, digestion, and post-prandial events (Furness et al., 2014; Liao et al., 2009). The ENS is composed of both the myenteric plexus (also known as the intermuscular plexus) and the submucosal plexus (Liao et al., 2009). The regulation of muscle contractions is done by small ganglia, a network of nerves within the myenteric plexus which are embedded in a collagen matrix within the muscle layer in the GI tract (Furness et al., 2014; Liao et al., 2009). Special cells present in nerve endings and the smooth muscle, named interstitial cells of Cajal (ICCs) have also been found to play an important role in the contractions of the smooth muscle of the GI tract (Liao et al., 2009). ICCs do so by both generating and propagating the electrical activity (electrical slow waves) responsible for

the regulation of the motility of the smooth muscle (Liao et al., 2009). ICCs also mediate the neurotransimission from enteric motor neurons to surrounding smooth muscle cells (Liao et al., 2009). Furthermore, in terms of blood flow regulation in the gut, the sympathetic and parasympathetic nervous systems, as well as exogenous nerves are at play (Liao et al., 2009). These are responsible for sensations such as satiety (gut feeling full), needing to defecate, and pain (Liao et al., 2009).

1.1.1 Small intestinal barrier function

The intestinal barrier (mucosa) is composed of a physical barrier, a layer of mucus and a monolayer of IECs, as well as immunological processes occurring at the barrier and in the underlying lamina propria (LP) (König et al., 2016). The mucus found in the lining of the small intestine is produced by goblet cells, which contain large mucus-filled organelles and express the MUC2 gene (McDermott & Huffnagle, 2014). The generated mucus can be divided into two layers: the outer layer which is in contact with the intestinal microbiome, and the inner layer which is in direct contact with the intestinal barrier (McDermott & Huffnagle, 2014). This inner layer of mucus also contains anti-microbial peptides (AMPs) which are also produced chiefly by Paneth cells and also by goblet cells (Kong et al., 2018; McDermott & Huffnagle, 2014).

The mucus layer also contains secretory immunoglobulin A (SIgA), one of the most abundant antibodies secreted within this layer, making it a key component for the immune parameters of the intestinal barrier (Pietrzak et al., 2020; Schoultz & Keita, 2020). Polymeric immunoglobulin A (pIgA) is produced by plasma cells in the LP before binding to polymeric immunoglobulin receptors (pIgRs) found on secretory IECs and being

endocytosed into said IECs (Brandtzaeg, 2013; Pietrzak et al., 2020). The pIgR-pIgA complex will pass through the IEC to be secreted into the lumen where proteolysis will remove a portion of the pIgR, leaving behind a secretory component-immunoglobulin A (IgA) complex referred to as SIgA (Brandtzaeg, 2013). In fact, the secretory component in SIgA will protect it from further proteolytic degradation (Brandtzaeg, 2013). SIgA is secreted in response to contact with luminal antigens and microbes, and contributes to the intestinal barrier by restricting host bacteria and potential pathogens to the intestinal lumen and keeping them out of the LP (Pietrzak et al., 2020).

The intestinal barrier is also composed of IECs and the tight junction (TJs) proteins which connect them (König et al., 2016; Schoultz & Keita, 2020). TJs regulate paracellular permeability, allowing for the passage of moderate-sized hydrophilic molecules across the barrier, between IECs (König et al., 2016; Schoultz & Keita, 2020). TJs are composed of transmembrane proteins like occludin, tricellulin, and Marvel D3 (Schoultz & Keita, 2020). It is important to note that this method of permeability is impervious to proteins, providing protection from antigens present in the intestinal lumen (Schoultz & Keita, 2020).

Transcellular permeability, a process which allows small hydrophilic lipid-soluble compounds to pass through IECs into the LP passively (does not require energy), also contributes to the intestinal barrier (Schoultz & Keita, 2020). Larger particles and molecules such as proteins and bacterial products will be engulfed in the plasma membrane of IECs via endocytosis, as a way to mediate the uptake of antigens for immunological assessment and response (Schoultz & Keita, 2020). After endocytosis, antigens will be

actively transported (requires energy) through the IEC's cytoplasm by transcytosis (Schoultz & Keita, 2020).

The intestinal mucosa is where most immunological processes occur in the small intestine (König et al., 2016). When the intestinal barrier is disrupted, intestinal permeability increases, allowing harmful substances and micro-organisms to translocate directly into the bloodstream (König et al., 2016). In addition, immune cells, intestinal microbiota, and AMPs will also contribute to intestinal barrier function by protecting the host from pathogenic microbes present in the lumen (König et al., 2016).

1.1.2 Mucosal immune system

The GI tract's immunity is a delicate balance between reacting to substances and micro-organisms that are truly harmful and need to be eradicated, and harmless compounds or commensal micro-organisms which need only be ignored as they pass through (König et al., 2016; Macpherson & Harris, 2004). The mucosal immune system present in the small intestine contributes to intestinal barrier function and protecting the host (Wershil & Furuta, 2008).

Immune responses in the body can be classified under two categories: adaptive or innate. Adaptive immunity is an antigen-specific immune response (Bonilla & Oettgen, 2010). In adaptive immunity, antigen-presenting cells (APCs) such as dendritic cells (DCs), along with T lymphocytes, produced in the thymus and also known as T cells, and B lymphocytes (B cells), produced in the bone marrow, will lead to the development of antigen-specific effector pathways, immunologic memory, and maintaining immune homeostasis (Bonilla & Oettgen, 2010). When an antigen is first encountered and sensed

via pattern-recognition receptors (PRRs, found on a variety of adaptive and innate cells), both long-lived memory T and B cells will be primed and differentiated in the mesenteric lymph nodes (MLN) and gut-associated lymphoid tissue (GALT) against said antigen, allowing for rapid reactivation during antigen re-exposure (Bonilla & Oettgen, 2010; Jain, 2020; Medzhitov & Janeway, 2000). However, it will take three to five days to generate a sufficient quantity of cells to contain an ongoing infection (Medzhitov & Janeway, 2000). This means that although adaptive immunity may be effective in the long term at addressing specific antigens, in the case of unknown antigens and the need for immediate response, it is not as effective.

Therefore, the immediate and non-specific immune response to antigens is carried by innate immunity, for which it specializes (Medzhitov & Janeway, 2000). In innate immunity, AMPs, phagocytes and the alternative complement pathway will be activated as soon as the host is infected (Medzhitov & Janeway, 2000). This allows for rapid control of the infection by limiting the replication of the infecting microbe (Medzhitov & Janeway, 2000).

The body has two different intestinal immune compartments: inductive sites such as the GALT and the MLN, and effector sites such as the LP and epithelium (Mörbe et al., 2021). The LP is rich in both B and T cells, important components of the immune response (McDermott & Huffnagle, 2014). In the inductive sites, the GALT are key in the intestinal adaptive immune response, as antigen sampling sites (Mörbe et al., 2021; Wershil & Furuta, 2008). Both GALT and MLNs are sites for immune cell priming and differentiation (Mörbe et al., 2021). These primed immune cells will then be released into effector sites to

contribute towards the homeostasis of the intestinal barrier (Figure 1.2) (Mörbe et al., 2021).

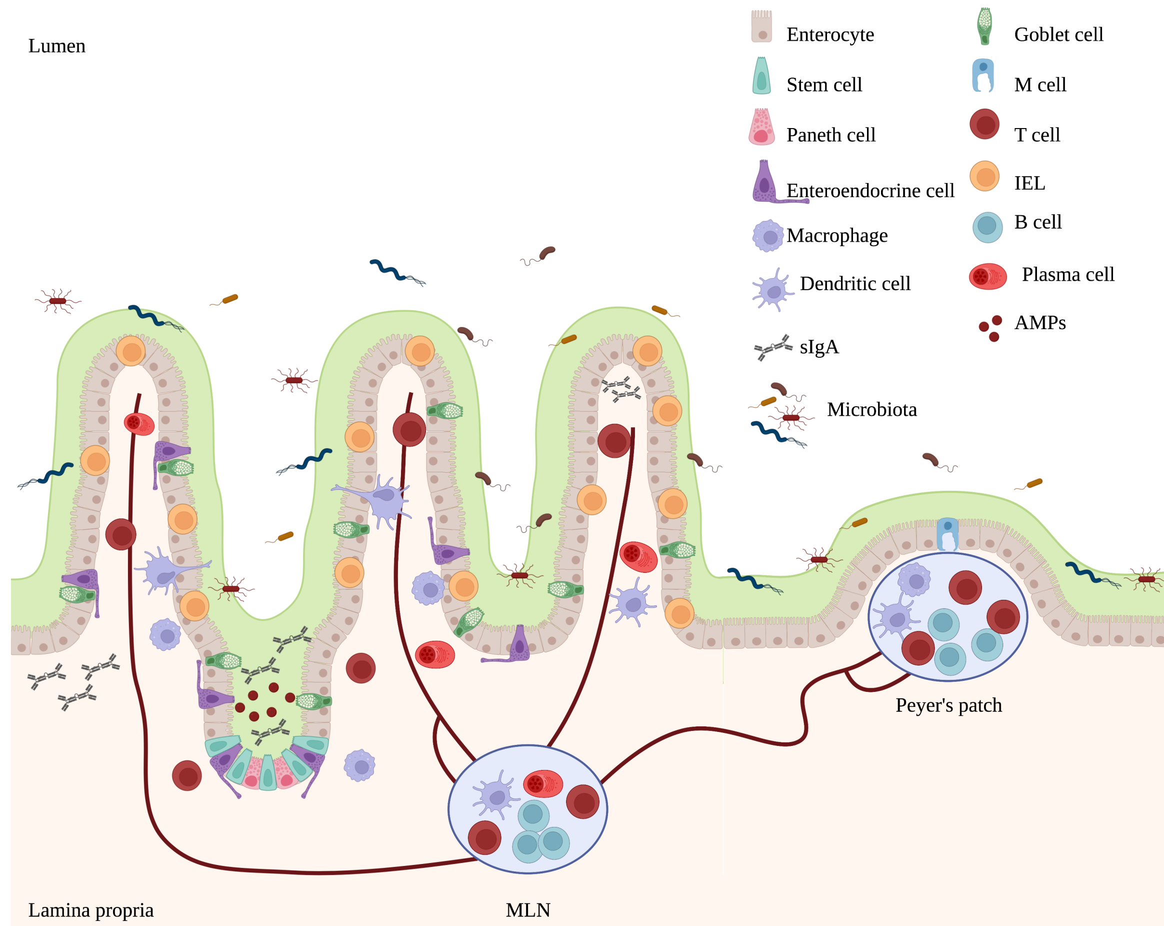

Figure 1.2 Overview of the mucosal immune system

Within the mucosal system are effector sites (lamina propria and epithelial cells) and inductive sites (Peyer's patches and mesenteric lymph nodes (MLN), responsible for the immune responses within the small intestine. A single layer of intestinal epithelial cells, secreted molecules, intraepithelial lymphocytes (IELs), the microbiota and other immune cells work in together to protect the host and maintain intestinal homeostasis. Created with BioRender.com
AMPs, antimicrobial peptides; SIgA, secretory immunoglobulin A.

1.1.2.1 Gut-associated lymphoid tissues and mesenteric lymph nodes

GALT are found within the intestinal wall as Peyer's patches (PPs) and isolated lymphoid follicles (ILF) (Mörbe et al., 2021; Wershil & Furuta, 2008). PPs are found

specifically on the anti-mesenteric wall and will contain between 10 to 100 individual follicles (Mörbe et al., 2021). PPs are covered by specialized follicle-associated epithelium (FAE) which contain microfold (M) cells, a specialized type of epithelial cell (Mörbe et al., 2021). Through these M cells, antigens are passed to APCs such as DCs to activate naïve T lymphocytes in the MLNs and initiate an immune response (Houston et al., 2016; Mörbe et al., 2021). Activated T cells will in turn activate B cells found in PPs or ILFs to secrete antigen-specific antibodies (Feldman et al., 2021; Mörbe et al., 2021).

1.1.2.2 Intraepithelial lymphocytes

Intraepithelial lymphocytes (IELs) are found between enterocytes within the intestinal epithelium and mostly consist of antigen-experienced "innate-like" T cells (Abadie et al., 2012; Feldman et al., 2021). The placement of IELs in the intestinal mucosa allows them to interact with their neighbouring enterocytes to maintain barrier function and halt pathogens from entering the LP (Abadie et al., 2012). These T cells can be divided into two classifications: induced (also called type A or conventional) and natural (also called type B or unconventional) and will express one of two T cell receptors (TCR): $\alpha\beta$ or $\gamma\delta$ (McDermott & Huffnagle, 2014; Olivares-Villagómez & Van Kaer, 2018). Type A IELs will develop in the periphery from antigen-experienced conventional T cells in the gut epithelium, while type B IELs will develop in the thymus and will directly migrate to the intestinal epithelium after their development (Olivares-Villagómez & Van Kaer, 2018). Natural IELs are present at birth while induced IELs will develop and expand as the host is exposed to antigens (Olivares-Villagómez & Van Kaer, 2018). The induced IEL category comprises of TCR$\alpha\beta^+$ cells, whose majority is made up of TCR$\alpha\beta^+$CD8$\alpha\beta^+$ cells (See

Table 1.1) (Olivares-Villagómez & Van Kaer, 2018). Natural IELs will express TCR$\alpha\beta^{+}$ or TCR$\gamma\delta^{+}$, but will not express CD4 or CD8$\alpha\beta$ receptors like their induced counterparts (Abadie et al., 2012). The majority of natural IELs will express CD8$\alpha\alpha^{+}$ receptors (Abadie et al., 2012).

Table 1.1 Major mouse intraepithelial lymphocyte subsets

IEL subset	TCR phenotype	Frequency	Functional role
Induced (Type A)	TCR$\alpha\beta^{+}$CD4^{+}	<15%	Development of oral tolerance Antimicrobial responses Intestinal barrier protection
	TCR$\alpha\beta^{+}$CD8$\alpha\beta^{+}$ TCR$\alpha\beta^{+}$CD8$\alpha\beta^{+}$CD8$\alpha\alpha^{+}$	20-30%	NKG2D-mediated cytotoxicity[1]
Natural (Type B)	TCR$\alpha\beta^{+}$CD8$\alpha\alpha^{+}$ TCR$\alpha\beta^{+}$CD8^{-}CD4^{-}	20-50%	Anti-inflammatory responses Immune regulation
	TCR$\gamma\delta^{+}$CD4^{-}CD8^{-} TCR$\gamma\delta^{+}$ CD8$\alpha\alpha^{+}$	40-70%	Antimicrobial responses Intestinal barrier protection

[1]Plays an important role in CeD pathogenesis.
TCR, T cell receptor; NKG2D, natural killer group 2D.

IELs also express CD103, which mediates the IELs adherence to IECs in concert with E-cadherin, expressed by IECs (McDermott & Huffnagle, 2014). After exposure to stimuli, IELs can be activated to express effector cytokines such as interferon (IFN) γ, tumour necrosis factor-α, and keratinocyte growth factor, leading to protective or pathogenic roles in immune defense (McDermott & Huffnagle, 2014). For example, the primary function of cytotoxic IELs, which express TCR$\alpha\beta^{+}$CD8$\alpha\beta^{+}$ cells, is to prevent infection of the host by producing pro-inflammatory cytokines and cytolyzing already infected cells (Pagliari et al., 2013). It is also known that the cytokine interleukin (IL)-15 can contribute to needless inflammation by acting as a proinflammatory stimulant for cytotoxic IELs proliferation

(Pagliari et al., 2013). Another example of IEL function is the role that TCR $\gamma\delta^+$ natural IEL play in maintaining the integrity of the intestinal epithelium and maintaining mucosal homeostasis (Cheroutre et al., 2011). TCR $\gamma\delta^+$ cells maintain the intestinal barrier by controlling epithelial cell growth and turnover, repairing damaged epithelial cells, and producing IgA (Cheroutre et al., 2011).

1.1.3 Immune system development in early life

During the first years of life, the immune system is still being established (Tordesillas & Berin, 2018). As the immune system matures, there are critical times where exposure to stimuli can impact a neonate's long-term health (Jain, 2020). The Developmental Origins of Health and Disease (DOHaD) hypothesis relies on the immune system's developmental plasticity such as hematopoietic stem cells (HSCs) which can differentiate to numerous blood and immune cells as they are exposed to stimuli (Jain, 2020). Plasticity seems to be a key aspect of antigen-specific immune responses where different stimuli will determine the differentiation of effector cells (Jain, 2020). Studies have shown that this is the case in other conditions (Jain, 2020). For example, if a mother is malnourished, it will decrease the transfer of her antibodies to the neonate, allowing her offspring to be more susceptible to allergies and autoimmune diseases (Jain, 2020).

The innate immune system cells of neonates will be first to respond to the boom of antigen exposure after birth (Jain, 2020). Host cells expressing PRRs help recognize both pathogens and commensal microbes in the gut to initiate the appropriate immune response (Jain, 2020). Failure to do so will cause undue inflammation of the gut mucosa and hinder the establishment of a healthy gut microbiota (Jain, 2020). The adaptive immunity of

neonates is limited but adequate (Jain, 2020). Neonatal T cells will produce T helper type 2 (Th2) cytokines such as IL-4, IL-5 and IL-13 when stimulated, establishing auto-tolerance (tolerance to the host's own cells) to maintain homeostasis (Jain, 2020). However, newborns are more susceptible to infection, as their immune system is still developing and learning which microbes and antigens pose a threat, leading to the disruption of homeostasis and immune dysfunction later on in life (Jain, 2020).

1.1.4 Small intestinal microbiota

Within the GI tract exists a diverse ecosystem of microbes referred to as the microbiota (Kastl et al., 2020). Through the immune functions present in the gut, the microbiota is identified as harmless, through mutualism (Bäckhed et al., 2005; Kastl et al., 2020). A mutualistic relationship is a type of symbiosis where both participants experience benefits from said relationship (Bäckhed et al., 2005). For example, bacteria in the gut will contribute to the digestion of food, benefiting both the host and the bacteria (Bäckhed et al., 2005; Kastl et al., 2020). The mutualism seen in the human gut is initially established by the microbiota present in the gut such as *Bacteroides*, *Clostridium* and *Eubacterium* genera (Bäckhed et al., 2005).

Colonization of the gut begins at birth, where contact with the mother's intestinal and vaginal flora begins this process, with the predominant colonization of *Lactobacillus* species (Butel et al., 2018; Neu & Rushing, 2011). During a caesarean delivery, bacteria from the environment, including those present on the mother's skin, will play a role in the colonization instead (Butel et al., 2018; Neu & Rushing, 2011). For caesarean births, infants have been found to be mostly colonized by *Staphylococcus* and *Acinetobacter* genera

instead (Butel et al., 2018; Neu & Rushing, 2011). Studies have shown that the colonisations as a result of caesarean sections leave infants at a high risk of developing immune-mediated disease, supporting what has been shown in mice (Butel et al., 2018; Neu & Rushing, 2011).

It is known that during early life, the intestinal microbiota will consistently be changing; the quantity of specific species will fluctuate as an infant is introduced to more and more antigens, leading to a bloom in microbiota variety after weaning (Kastl et al., 2020). From there, the microbiota will begin stabilizing and will likely remain the same throughout adulthood (Kastl et al., 2020). Comparing the microbial colonies throughout the gut, the small intestine hosts a relatively low number of bacteria (~10^3 bacteria cells/g) given its harsh environment (bile, pancreatic secretions, and acid bolus from the stomach) (El Aidy et al., 2015; Kastl et al., 2020).

The intestinal microbiota plays an important role in maintaining the host intestinal homeostasis by protecting the host from pathogens and aiding with digestion (Kastl et al., 2020). Germ-free animals have been shown to have several developmental defects in their vasculature, respiratory system, intestinal function and motility, metabolism and nutritional needs (Smith et al., 2007). Germ-free animals have also been found to have immune deficiencies such as reduced intestinal plasma cells, IELs, IgA and IgA^+ cells in the gut, reducing the mice's immune responses to harmful stimuli (Hapfelmeier et al., 2010; Smith et al., 2007). However, when colonized with non-pathogenic microbes such as the altered Schaedler flora (ASF, a mix of microbiota naturally present in the gut of mice) and certain strains of *Escherichia coli*, IgA mechanisms were re-established (Hapfelmeier et al., 2010).

It has also been shown that delayed microbiota colonization will lead to profound immune changes in later life, such as an increase susceptibility to inflammatory disease like colitis (Al Nabhani et al., 2019). Therefore, we know that this maturation will protect its host from being susceptible to autoimmune diseases, but must act in concert with key immune developments outlined above and oral tolerance (Kastl et al., 2020).

1.1.5 Oral tolerance

The GI tract also fulfills important immune functions, such as the development of oral tolerance to the foods we ingest, while protecting the host from harm (Friedman et al., 1994; König et al., 2016; Liao et al., 2009). Oral tolerance is best described as an active regulation of mucosal and systemic inflammatory suppression to innocuous antigens such as food proteins (Pabst & Mowat, 2012). Active suppression is mediated by forkhead box p3 (Foxp3)$^{+}$ regulatory T cells (Tregs) and occurs when a host is exposed repeatedly to low doses of antigen (Pabst & Mowat, 2012). Food antigens are taken up via transcellular or paracellular pathways, as previously described above, or by DCs which extend into the lumen for antigen sampling (Pabst & Mowat, 2012). Thereafter, antigens are taken up by APCs, which migrate to the MLNs, to present antigens to T and B cells, inducing Foxp3^{+} Tregs to migrate back to the LP to suppress immune reactions to food antigens and promote oral tolerance (Pabst & Mowat, 2012).

Oral tolerance is the immune system's default response to innocuous food antigens and is vital for the proper functioning of the small intestine (Pabst & Mowat, 2012). The disruption of oral tolerance can lead to the development of allergies or other food

sensitivities such as celiac disease, a well-defined food sensitivity to gluten resulting in villous atrophy in the small intestine (Pabst & Mowat, 2012; Tye-Din et al., 2018).

1.2 Celiac disease

Celiac disease (CeD) is a T-cell mediated enteropathy that can be developed at any point during the life span (Galipeau et al., 2015; Tye-Din et al., 2018). In CeD patients, there is a loss of oral tolerance to gluten, a group of proteins found in wheat barley and rye which results in villous atrophy in the small intestine (Galipeau et al., 2015; Tye-Din et al., 2018). CeD patients may experience symptoms from the resulting villous atrophy, such as decreased weight, diarrhea, abdominal pain, and nausea (Murray et al., 2004). However, some patients will have no symptoms at all (Rutz et al., 2002).

To diagnose a patient with CeD, the levels of serum tissue transglutaminase-2 (TG2) IgA antibodies must first be tested (Lebwohl & Rubio-Tapia, 2021). Following a positive result for TG2 IgA antibodies, a biopsy should be performed to assess the patient's Marsh-Oberhuber score, an established scoring system for CeD (See Table 1.2) (Adelman et al., 2018; Lebwohl & Rubio-Tapia, 2021). If the villi are normal, the patients will continue to be monitored as they have the potential to develop CeD in the future (Lebwohl & Rubio-Tapia, 2021).

Table 1.2 Marsh-Oberhuber histological scoring of celiac disease

Score	Histological description
0	Normal – Normal appearing villus architecture
I	Infiltrative – Normal mucosal and villus architecture; increased number of IELs
II	Hyperplastic – Similar to I score. Includes enlarged crypts and increased crypt cell division
IIIa	Partial villus atrophy – Shortened, blunt villi; mild lymphocyte infiltration, enlarged hyperplastic crypts
IIIb	Subtotal villus atrophy – Clearly atrophic villi, enlarged crypts whose immature epithelial cells are generated at an increased rate, influx of inflammatory cells
IIIc	Hypoplastic – Total villus atrophy, complete loss of villi, severe crypt hyperplasia, infiltrative inflammatory lesion

To develop CeD an individual must first consume the antigen: gluten (Tye-Din et al., 2018). Individuals must also carry specific alleles: HLA-DQ2 and/or HLA-DQ8 (Tye-Din et al., 2018). Finally, an individual must be exposed to a trigger for disease development to begin (Tye-Din et al., 2018). All three necessary aspects of CeD development will be further explained below.

1.2.1 Gluten

As previously mentioned, gluten is a group of proteins found in wheat, barley, and rye and belongs to the prolamin group (Daly et al., 2020). Prolamins are alcohol-soluble and responsible for energy storage within the tissue from different types of grain (Daly et al., 2020). The prolamins of interest for CeD are gliadins and glutenins (from wheat), hordeins (from barley) and secalins (from rye) (Daly et al., 2020). There are three major types of gliadins: α, γ, and ω (Wrigley et al., 2006). Prolamins are poorly digested by the enzymes present in the gut since they are rich in the amino acids proline and glutamine (Shan et al.,

2002). Gliadins and glutenins are especially poorly digested, due to the unique placement of their proline residues (Shan et al., 2002). This means that the majority of partially digested peptides in the gut are rich in proline and glutamine residues (Shan et al., 2002). Although both gliadins and glutenins have been shown to initiate the adaptive immune response in CeD patients, a specific peptide resulting from the digestion of α-gliadin, named 33-mer, has been found to be the most effective for the activation of naïve T cells (Shan et al., 2002). Please note that throughout this thesis, the term gliadin will be used to refer to the entire group of gliadins found in wheat.

1.2.2 Genes

The specific genes that have been linked to CeD are the HLA-DQ2 and HLA-DQ8 alleles, which encode for the major histocompatibility complex (MHC) class II molecules of the same name (Galipeau et al., 2015; Tye-Din et al., 2018). MHC class II HLA-DQ2 and HLA-DQ8 molecules play an important role in the adaptive immune response of CeD patients, as explained below (Galipeau et al., 2015; Tye-Din et al., 2018).

It has been found that the risk of both developing CeD and its severity are linked to which genes are carried. Approximately 90% of CeD patients carry the HLA-DQ2 allele, which is also the allele associated with the higher risk of developing CeD (Tye-Din et al., 2018). Furthermore, an individual that is homozygous for the HLA-DQ2 allele is at the highest risk of developing the disease (Tye-Din et al., 2018). This is due to the configuration of the MHC class II molecules and the fact that the HLA-DQ2 molecule has a strong binding affinity to bind to a variety of partially digested gliadin peptides when compared to the binding abilities of the HLA-DQ8 molecule (Kim et al., 2004).

More specifically, individuals homozygous for the HLA-DQ2.5 isoform, also known as the DR3-DQ2 haplotype, are associated with the highest risk of developing CeD (Ploski et al., 1993; Sollid & Thorsby, 1993). Heterozygotes for both isoforms, HLA-DQ2.5 and HLA-DQ2.2, known as the DR7-DQ2 haplotype, will also express high levels of HLA-DQ2.5 molecules, placing them at high risk for CeD as well (Ploski et al., 1993; Sollid & Thorsby, 1993). Throughout this thesis, the HLA-DQ2.5 isoform will be referred to as the HLA-DQ2 allele and molecule.

1.2.3 Adaptive & innate immune responses in celiac disease

Mechanisms from both the adaptive and innate immune responses of CeD have been elucidated, although the adaptive branch is better defined.

The adaptive immune response in CeD is as follows: in the lumen of the small intestine, human and microbial proteases will begin digesting gluten (Tye-Din et al., 2018). However, due to gluten's chemical structure, as previously discussed above, it cannot be fully digested resulting in partially digested gluten peptides (Tye-Din et al., 2018). These partially digested gluten peptide fragments will then translocate across IECs into the LP where they will be post-translationally modified or deamidated by TG2 to produce deamidated gluten peptides (DGPs) (Tye-Din et al., 2018). Deamidation plays an important role in enhancing the binding affinity of gluten peptides to the MHC class II molecules, converting specific glutamine residues to glutamate (Tye-Din et al., 2018). The DGPs will then bind to MHC class II molecules (HLA-DQ2 and/or HLA-DQ8) expressed by APCs such as DCs (Tye-Din et al., 2018). Deamidation at specific peptide positions and amino acid structure will determine to which MHC class II molecules the DGPs will bind (See

Figure 1.3) (Tollefsen et al., 2006). For example, deamidation at peptide position 4 is important for the HLA-DQ2 molecules whereas positions 1 and 9 are important for the HLA-DQ8 molecules. If a combination of these deamidations occur in a peptide, it could bind to either molecule (Tollefsen et al., 2006). In the MLNs, APCs will present these fragments or complexes to naïve helper T cells resulting in their priming and activation to produce IFN-γ (Tye-Din et al., 2018). These activated T cells will then go on to "help" with B cell priming, in turn leading to these plasma cells producing both anti-gliadin and anti-TG2 antibodies (Tye-Din et al., 2018). Importantly, it has been shown that TG2 and DGPs can form complexes, which allows DGPs to cross-link B cell receptors and recruit T cells, leading to the development of antibodies by B cells (Gefen et al., 2015; Jabri & Sollid, 2017; Lindstad et al., 2021).These TG2-DGP complexes are able to activate both TG2-specific and DGP-specific B cells, thus resulting in the production of anti-DGP and anti-TG2 IgA antibodies (Lindstad et al., 2021).

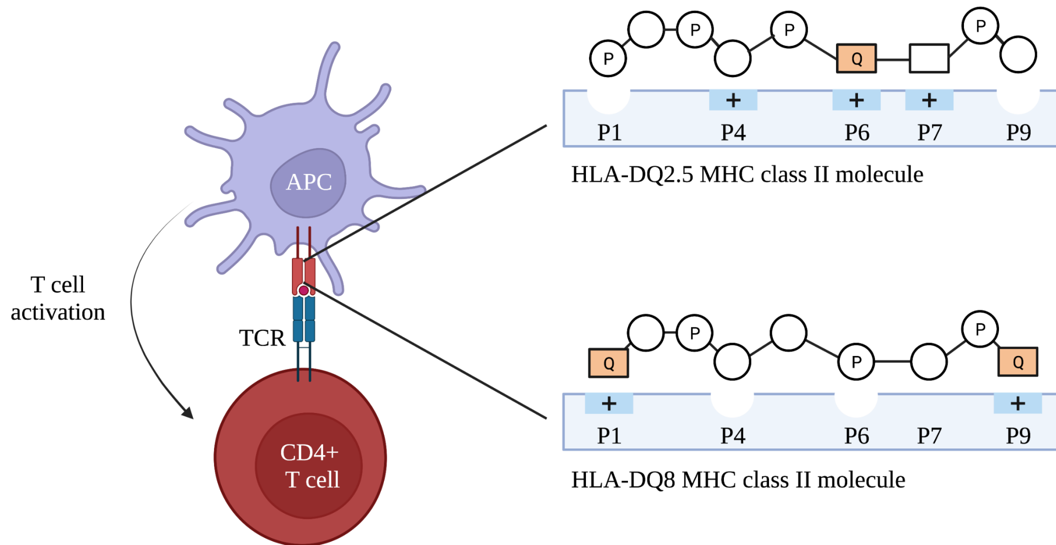

Figure 1.3 Gluten presentation and machinery

Deamidated gliadin peptides will bind to major histocompatibility complex (MHC) class II molecules on antigen-presenting cells (APCs), which will then travel to the mesenteric lymph nodes (MLNs). Binding affinity to the MHC class II molecule will vary according to its configuration, as well as the configuration of DGPs. In the MLNs, APCs will present the DGPs to naïve T cells on their T cell receptors (TCRs) to activate said T cells. Created with BioRender.com
P1, position 1 (etc.)

In terms of the innate immune response of CeD, fewer features have been elucidated. Currently, a variety of different triggers for this response have been suggested, such as gluten peptides, amylase-trypsin inhibitors (ATIs) and opportunistic pathogens (Araya et al., 2016; Caminero et al., 2019; Schuppan & Zevallos, 2015). Thus far, we know that cytotoxic IELs become activated, likely by IL-15 to mediate the innate immune response (Tye-Din et al., 2018). IEL phenotypes and their interactions with IECs involved in CeD have been extensively studied (Abadie et al., 2012; Sziksz et al., 2014). IEL phenotypes are distinguished by the expression of innate-like T-cell receptor (TCR)αβ and TCRγδ, which in turn express natural killer (NK) lineage markers (Abadie et al., 2012; Sziksz et al., 2014).

By expressing the NK lineage markers, IELs are able to recognize stress ligands expressed by IECs, such as heat-shock proteins (HSP) 27 and HSP70 which may be key inducers of epithelium directed immune responses in CeD (Abadie et al., 2012; Sziksz et al., 2014). There are also epithelial MHC class I chain related genes A and B (MICA/B) that serve as ligands for the natural killer group 2D (NKG2D) receptor found on cytotoxic $CD8^+$ IELs (Abadie et al., 2012). When activated, NKG2D receptors will induce IEC apoptosis, leading to villous atrophy (Abadie et al., 2020; Tye-Din et al., 2018).

The combination of both immune responses results in the enteropathy seen in CeD patients (See Figure 1.4) (Tye-Din et al., 2018).

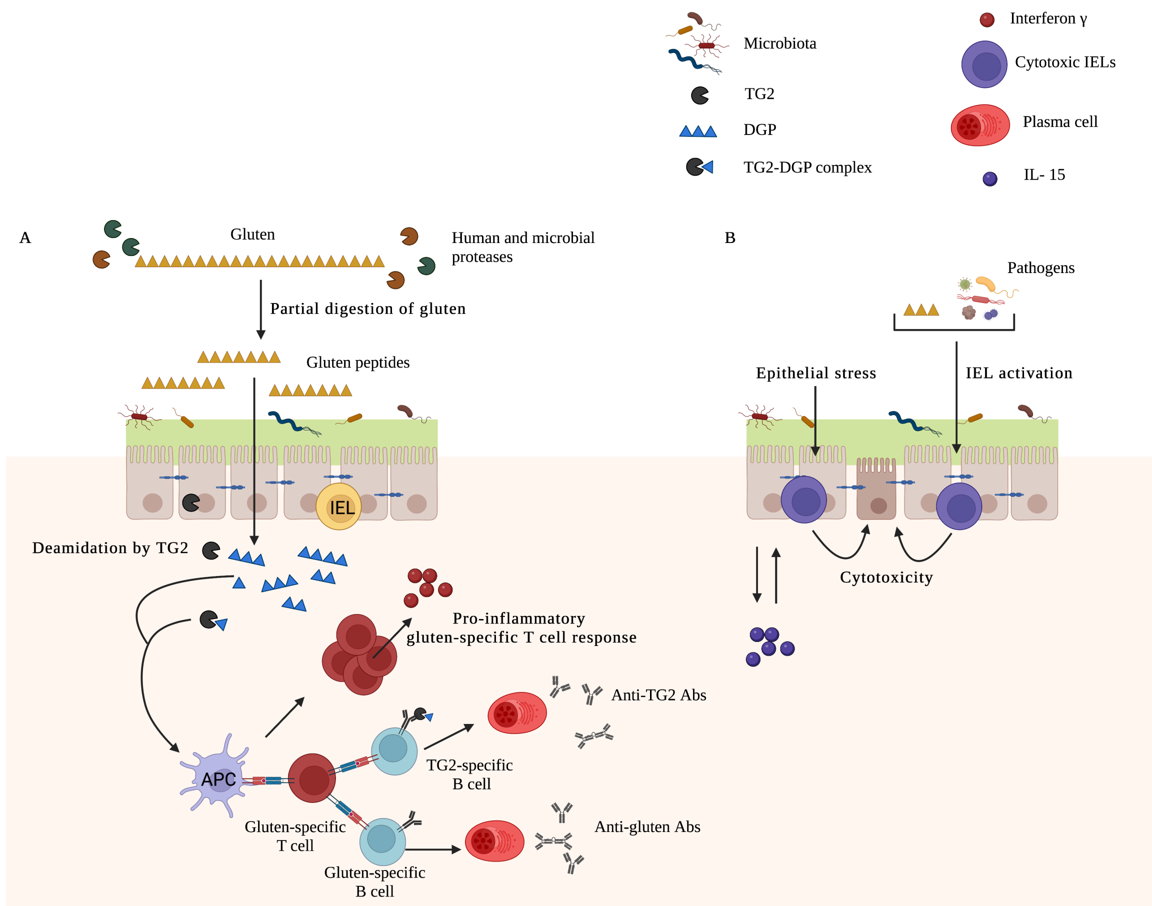

Figure 1.4 Overview of adaptive and innate immune responses in celiac disease

(A) The adaptive immune response in celiac disease is gluten specific. In the intestinal lumen, gluten is partially digested by human and bacterial proteases. From there, the partially digested gluten proteins will cross the intestinal epithelial barrier into the lamina propria where they will be deamidated by tissue transglutaminase 2 (TG2). The resulting deamidated gluten peptides (DGPs) will bind to the MHC class II molecules on antigen-presenting cells (APCs). The APCs will then travel to the mesenteric lymph nodes (MLNs) to present DGPs to naïve T cell, activating them. In turn, activated T cells will activate B cells, to lead to the production of both anti-gliadin and anti-TG2 antibodies. Activated T cells will also produce the cytokine interferon γ. **(B)** The innate immune response in CeD is non-specific. Possible triggers for this reaction are innate gluten peptides, amylase-trypsin inhibitors (ATIs) and opportunistic pathogens. Cytotoxic intraepithelial lymphocytes (IELs) will be likely activated by interleukin (IL)-15 to begin mediating the innate immune response. Specific receptors found on cytotoxic IELs will lead to the apoptosis of intestinal epithelial cells (IECs), resulting in villous atrophy. Created with BioRender.com

1.2.4 Environmental co-factors

As has been previously stated, an individual must both carry the genetics for CeD and consume gluten (Tye-Din et al., 2018). However, approximately 30% of worldwide populations carry the allele for CeD, and only 1% will go on to develop the disease (Green & Cellier, 2007; Tack et al., 2010). This implies that these two factors are not sufficient to cause disease development, and that environmental factors are needed to trigger CeD development.

Furthermore, although CeD can occur at any age, there is no doubt that early life is a key time point since it is when infants are first introduced to the antigen (gluten) which drives inflammation. As mentioned previously, the majority of CeD patients are carriers for the HLA-DQ2 allele, and those homozygous for this allele are most likely to develop the disease, especially early in their life span (Green & Cellier, 2007).

1.2.4.1 Epidemiological evidence of environmental risk factors

A variety of early life factors have been suggested as contributing factors for CeD development, such as viral and bacterial infections, the time at which gluten is introduced in an infant’s diet as well as high doses of gluten in infancy (Caminero & Verdu, 2019; Ivarsson et al., 2000; Pinto-Sánchez et al., 2016; Tye-Din et al., 2018). However, these are mere associations, and causality has not been established.

Gluten introduction to an infant’s diet is of high interest, however in both clinical studies and metanalyses, the timing of gluten introduction (i.e. before or after weaning) has not been found to be significant in increasing CeD risk in genetically susceptible children (Aronsson et al., 2019; Lionetti et al., 2014; Pinto-Sánchez et al., 2016; Vriezinga et al.,

2014). The dosage of gluten has been suggested as an important trigger, as was described during the “celiac epidemic” in Sweden in the late 1980s (Ivarsson et al., 2000). From 1984 to 1996, a surge of infants was diagnosed with CeD after commercial feeding formulas began including high amounts of gluten (Ivarsson et al., 2000). Once the gluten was removed from formulas, the number of CeD diagnoses began to decrease, indicating there was a correlation between the two (Ivarsson et al., 2000). Since this event, it is believed that introducing such high amount of this dietary antigen (gluten) while oral tolerance and the immune system are being established can cause detrimental effects. However, this still needs to be confirmed in a more controlled environment such as an animal model.

Recently, a longitudinal study using The Environmental Determinants of Diabetes in the Young (TEDDY) cohort found that high amounts of gluten in combination with the frequent use of antibiotics and/or infections may increase the risk of celiac autoimmunity in at-risk children (Kemppainen et al., 2017). This suggests that infections could also play a part in CeD development. Another longitudinal study using the Celiac Disease Genomic, Environmental, Microbiome, and Metabolomic (CDGEMM) cohort was also able to detect an increase in microbes linked to other autoimmune diseases, such as *Dialiste invisus*, *Parabacteroides* sp., and *Lachnospiraceae*, in at-risk infants that went on to develop CeD (Leonard et al., 2021). The CDGEMM study also found a decrease in microbes known to have anti-inflammatory effects such as *Streptococcus thermophilus*, *Faecalibacterium prausnitzii*, and *Clostridium clostridioforme*, in these same children (Leonard et al., 2021). This indicates that even in early life, CeD children can develop pro-inflammatory microbial

shifts. This could aid with early detection through close monitoring of children who are at-risk to develop CeD and exhibit similar microbial shifts in their gut.

To understand the mechanistic interactions of environmental co-factors in initiating CeD development, a controlled environment with well-defined parameters such as an animal model would be very useful.

1.3 The gluten-free diet (GFD)

Currently, the only existing treatment for CeD is adopting a strict and lifelong GFD (Green & Cellier, 2007; Tack et al., 2010). Unfortunately, its effectiveness is limited. After adopting the GFD, 30% of patients will remain symptomatic, leaving to question if patients are being exposed to small amounts of gluten or if the treatment is truly ineffective (Green & Cellier, 2007; Tack et al., 2010). If this is the case, gluten contamination should be ruled out by reviewing dietary intake with a registered dietician (Raiteri et al., 2022). Contamination may also be ruled out with the aid of another diet designed to address this very issue: the gluten-contamination elimination diet (GCED) (Leonard et al., 2017).

The GCED is designed as a multi-phasic diet that stretches out over 12 weeks and helps understand where gluten contamination may be occurring and if it is also occurring with CeD patients who do not respond to the GFD (Leonard et al., 2017). Unfortunately, it may be the case that gluten contamination does not occur, and that patients are truly non-responders (Raiteri et al., 2022). There are little to no options for these patients, and they will need to be monitored very closely, as celiac non-responders will be susceptible to micronutrient deficiencies, poor bone health and some cancers, namely enteropathy-

associated T cell lymphoma and intestinal adenocarcinoma (Green & Cellier, 2007; Raiteri et al., 2022; Tack et al., 2010).

There are also other negative outcomes to the GFD, such as increased cost as well as the social impact and isolation that comes with following such a strict diet (Ciacci & Zingone, 2015; Lee et al., 2007). To this day, gluten-free foods cost double of their counterparts and their availability can be quite limited (Lee et al., 2007). CeD patients have reported feeling burdened from the social impact of following a GFD and increased anxiety when not eating at home, due to the higher chance of gluten contamination (Ciacci & Zingone, 2015; Sainsbury et al., 2013).

Although the GFD is the only available treatment for CeD, there are other therapies that are currently in clinical trials for treatments to be used alongside the GFD or to replace it completely.

1.3.1 Therapies currently in clinical trials

Currently there are a few clinical trials of interest for CeD. The only treatment that is currently in phase 3 trial is larazotide, a controversial tight junction regulator which researchers believe will help restore barrier integrity, though its mechanisms of action are currently unknown (Meters Biopharma, 2021). Previous clinical trials of this drug have found it to be effective in decreasing CeD symptoms (Meters Biopharma, 2021). This oral treatment continues to be tested for symptom management and relief in CeD patients (Meters Biopharma, 2021).

A variety of other treatments are currently in the clinical trial pipeline in phase 2 such as latiglutenase and PRV-015 (Immunogenics et al., 2021; Provention Bio, 2023).

Latiglutenase is designed to be taken as a supplement to the GFD, in the hopes of lessening the effects of accidental gluten exposure including enteropathy and symptoms (Immunogenics et al., 2021). PRV-015 is also taken while on the GFD, by patients which remain symptomatic (Provention Bio, 2023). It is an antibody which targets IL-15, a cytokine thought to play an important role in CeD (Provention Bio, 2023).

There are additional therapeutics in the preclinical phase and phase 1 clinical trials. However, all these treatments address treatment for patients with CeD, and not how to prevent the development of the disease in the first place.

1.4 Preventing celiac disease

As previously mentioned, the gut is also responsible for the development of oral tolerance, an important homeostatic process consisting of the lack of immune response to dietary antigens (Wershil & Furuta, 2008). CeD is caused by the breakdown of oral tolerance to gluten, which means that to prevent CeD, we must prevent the breakdown of oral tolerance (Tye-Din et al., 2018).

To do so, we must tackle environmental factors that can trigger disease onset, which is why it is important to continue investigating CeD environmental factors to obtain mechanistic insight which can lead to prevention (Tye-Din et al., 2018). As previously discussed, early life factors such as breastfeeding, the timing of gluten introduction and gluten quantity have been investigated to assess their impact on CeD (Aronsson et al., 2019; Ivarsson et al., 2002; Lionetti et al., 2014; Pinto-Sánchez et al., 2016; Vriezinga et al., 2014). These studies have resulted in specific guidelines changes such as the removal of high amounts of gluten in baby formula in Sweden, after it was linked to the CeD epidemic

(Ivarsson et al., 2000). Essentially, the goal should be to identify and modify the environmental triggers, to in turn prevent CeD.

It is important to state that placing all high-risk individuals on the GFD may not be the most effective method of CeD prevention as it carries its own adverse effects (Niland & Cash, 2018). Gluten-free products have been found to have lower average protein, sodium, and fibre content and non-celiac adults who consume a GFD were found to not meet all nutritional requirements while on the GFD (Niland & Cash, 2018). These individuals were also susceptible to the same adverse effects as CeD patients: increased social burden, increased financial burden and increased stress (Niland & Cash, 2018).

As was previously explained, early life events are especially important to understand in the framework of individuals homozygous for the HLA-DQ2 allele, as they are at the highest risk of developing CeD in infancy (Liu et al., 2014). Given the nature of clinical studies and the variety of uncontrolled variables that occur, animal models with controlled environments that mimic features of CeD would be very useful in investigating environmental factors.

1.5 Current mouse models in celiac disease

There are many existing mouse models of CeD, each serving their purpose in modelling specific features of the disease. Existing mouse models which are transgenic for human genes playing a role in CeD development will be further discussed below.

1.5.1 HLA-DQ8 model

Mice used for this model express the human HLA-DQ8 gene, human or mouse CD4 and are deficient in mouse MHC class II molecules as well as mouse class II antigens (AB^0)

(Black et al., 2002; Verdu et al., 2008). This was the first transgenic mouse model used to measure features of CeD.

Two models exist with this genetic background. In the first, mice were injected intraperitoneally (IP) with 500 μg of crude gluten in complete Freund's adjuvant (CFA) (Black et al., 2002). After a week's rest, they were gavaged with 2mg gluten (Black et al., 2002). The mice developed anti-gliadin antibodies and had an increased production of IL-10, IL-6, and tumor growth factor β (Black et al., 2002). In the second, mice expressing human DQ8 gene were sensitized to gluten by gavage with 500 μg gliadin and 50 μg CFA (Verdu et al., 2008). Then, 2 mg gliadin was administered by oral gavage, three times a week for three weeks (Verdu et al., 2008). Sensitized mice developed anti-gliadin antibodies, as was seen in the previous paper, barrier dysfunction as well as an increase in $CD3^+$ IELs (Verdu et al., 2008).

The HLA-DQ8 mice were the first existing mouse model based on the expression of necessary genes for developing CeD. The model proved successful in mimicking some features of the disease and its mild phenotype stimulated further model development.

1.5.2 NOD AB° DQ8 model

Mice expressing the human HLA-DQ8 allele (as described above) were backcrossed with non-obese diabetic (NOD) mice to generate the NOD AB^0 DQ8 mice, deficient in mouse MHC class II molecules (Galipeau et al., 2011). These mice were crossed with the NOD background, as this background increases the mice's risk of autoimmunity, an important feature that may improve the lack of villous atrophy in the preceding model (Galipeau et al., 2011).

Eight-to-ten-week-old specific pathogen-free (SPF) mice kept on a low fat (4.4%) GFD were given IP injections of 250 μg monoclonal anti-CD25 antibodies once a week for two weeks, to induce immune dysregulation (Galipeau et al., 2011). Mice were given one week's rest before then being sensitized with 500 μg pepsin-trypsin digested (PT) gliadin and 25 μg cholera toxin (CT), once weekly for three weeks (Galipeau et al., 2011). Mice were then placed on a gluten-containing diet for the remainder of the study (Galipeau et al., 2011).

It was found that NOD AB° DQ8 mice exposed to gluten developed moderate enteropathy (villus-to-crypt ratios and $CD3^+$ IELs), a first in CeD transgenic mouse models (Galipeau et al., 2011). Researchers also found that these mice developed barrier dysfunction as well as anti-gliadin antibodies (Galipeau et al., 2011). This model provided more mechanistic insight to CeD development and its relationship with type 1 diabetes (Galipeau et al., 2011).

1.5.3 DQ8-D^d-villin-IL-15tg model

Mice expressing the human allele HLA-DQ8 were crossed with villin-IL-15tg mice, to better understand the role of IL-15 in CeD (Abadie et al., 2020). The resulting DQ8-villin-IL-15tg expressing mice were crossed with D^d-IL-15tg expressing mice to generate the triple transgenic DQ8-D^d-villin-IL-15tg mice, which overexpress IL-15 in their intestinal epithelium and LP (Abadie et al., 2020). To note, these mice also express I-A^b MHC class II molecules (Abadie et al., 2020).

Ten-week-old SPF DQ8-D^d-villin-IL-15tg mice were fed standard rodent chow (contains gluten) and were supplemented 20 mg crude gliadin or 100 μg PT-gliadin by oral

gavage, every other day for 30 or 60 days (Abadie et al., 2020). To evaluate their recovery an additional group was placed on standard rodent chow for 30 days, then on a gluten-free chow for another 30 days (Abadie et al., 2020). A proportion of mice were also given IP injections every 4-5 days with 200μg or 400 μg of anti-CD4 or 200 μg of anti-CD8α for the entire study (Abadie et al., 2020).

It was found that DQ8-D^d-villin-IL-15tg mice exposed to gluten developed partial villous atrophy, a first in mouse models of CeD, inferring that the overexpression of IL-15 was key in this development (Abadie et al., 2020). This mouse model provided insight in the role of IL-15 in CeD development for patients which carry the HLA-DQ8 allele (Abadie et al., 2020). However, this model expresses enteropathy in the ileum, and is not reproduced in two thirds of CeD cases, which are not driven by increased IL-15.

1.5.4 DR3-DQ2 model

Mice which express the human haplotype DR3-DQ2, which includes the human allele HLA-DQ2, were recently characterized as a model of CeD (Clarizio, 2020). It is important to note that this model exclusively expresses mouse CD4, unlike the further humanized hCD4 DR3-DQ2 mice which only express human CD4 and have yet to be characterized for gluten-immunopathology (de Kauwe et al., 2009). Both models are knock-outs for mouse MHC class II molecules (de Kauwe et al., 2009). The DR3-DQ2 mouse model was an important addition to the field, as it is the first to address the majority of CeD patients (Clarizio, 2020). As previously stated, over 90% of CeD patients will carry the HLA-DQ2 allele (Tye-Din et al., 2018).

Our group previously studied adult SPF DR3-DQ2 transgenic mice that were sensitized with 500 µg PT-gliadin and 25µg CT (a mucosal adjuvant) by gavage once a week for three weeks, followed by 10 mg gluten gavages, three times a week for three weeks (Clarizio, 2020). Following this protocol, sensitized mice developed moderate enteropathy (villus-to-crypt ratios and $CD3^+$ IEL quantification) as well as both anti-TG2 and anti-gliadin antibodies (Clarizio, 2020). Some of these mice were also found to have increased IL-15 expression in the intestine (Clarizio, 2020).

1.5.5 Gaps in CeD models

Currently there is no existing CeD mouse model in early life, which limits our understanding of the environmental co-factors that could play an important role in disease development at that important stage. Knowing that most patients who will develop CeD in early life are individuals homozygous for the HLA-DQ2.5 gene, DR3-DQ2 mice are the ideal transgenic mouse model for this purpose, which is why this will be the focus of my thesis.

2. CHAPTER 2: THESIS OVERVIEW

THESIS OVERVIEW

2.1 Thesis scope

CeD is an immune-mediated enteropathy with several well-defined pathogenic features such as its risk genes, its main environmental trigger (gluten), and the mechanisms explaining adaptive and innate immune system activation. There are many existing animal models that have been characterized to study specific facets of CeD pathophysiology, but none thus far that have attempted to evaluate early life sensitization and its impact on gluten immunopathology. **Therefore, the goal of this thesis was to characterize the gluten immunopathology of transgenic mice expressing the human DR3-DQ2 haplotype sensitized to gluten in early life.**

The findings for this thesis are organized in 2 aims and addressed throughout the chapters of this thesis. I first established the early life sensitization model and then proceeded to explore the long-term recovery of mice sensitized to gluten and kept on a GFD. Specific aims have been outlined below.

2.2 Thesis aims

1) To characterize gluten-induced immunopathology in DR3-DQ2 transgenic mice sensitized to gliadin, a well characterized gluten protein known to initiate adaptive immune responses in CeD patients, in early life,
2) To characterize the long-term gluten recovery of mice sensitized to gliadin in early life and kept gluten-free afterwards.

2.3 Thesis contributions

The work I performed for this thesis occurred from September 2019 to April 2022. I planned and conducted the bulk of the experiments and analyzed data with the help of my supervisor E. Verdú. Throughout this time, I received technical support and guidance from H. Galipeau (ELISAs), X. Wang (Immunohistochemistry), T. Ribeiro (Early life protocols), M. Constante (Nested statistical analysis), as well as M. Wulczynski and L. Rondeau. DR3-DQ2 transgenic mice were provided by R. Anderson.

3. CHAPTER 3: MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 Animals & protocols

All experiments were approved by the McMaster University Animal Research Ethics Board. Female and male (8-12 weeks old) SPF mice transgenic for human haplotype DR3-DQ2 on a C57BL/6 background were originally generated at the University of Melbourne (de Kauwe et al., 2009) and bred at McMaster University's Central Animal Facility. Please note, this specific breed exclusively expresses mouse CD4. All mice included in the study were fed and watered *ad libitum*.

3.1.1 Early life breeding protocol

Male and female six-to-eight-week-old SPF DR3-DQ2 mice were paired until females gained 5g of bodyweight, at which point females were labelled as pregnant and separated from males.

Females' first pregnancies were culled at post-natal day (P) three. Females were left to rest for one-week post-culling before being repaired with males from previous step. Again, females with 5g increase in bodyweight were labelled as pregnant and separated from males. The subsequent litters were sexed and standardized to two males and two females, ideally, at P3 and assigned for experimental use (Figure 1.0).

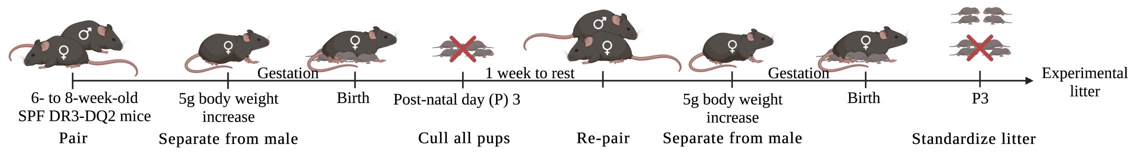

Figure 3.1 Diagram of early life study breeding protocol

Six-to-eight-week-old male and female, specific pathogen free (SPF) DR3-DQ2 transgenic mice were paired and kept together until the female showed an increase in 5g of body weight, at which point, the female was placed in her own cage and left for the reminder of her gestation. On post-natal day (P) 3 all pups from the female's first litter were culled and the female was given one week's rest. At the end of this rest, breeding pairs were placed together again, and females were monitored for pregnancy as previously described. Pups from the second litter were standardized to two males and two females, ideally and assigned for experimental use. Created with BioRender.com

3.1.2 Early life gluten sensitization protocol

Experimental litters were standardized on P3 to four mice, ideally two males and two females. At P14, mice were ear notched and then sensitized using PT-gliadin which was prepared as previously described (Thomas et al., 2006). Gliadin (Sigma-Aldrich, Milwaukee, Wisconsin) was dissolved in endotoxin-free 0.2 N HCl (Sigma-Aldrich) for 2h in a 37°C water bath with pepsin (Sigma-Aldrich). After digestion, pH was adjusted to 7.4 with endotoxin-free 2 M NaOH (Sigma-Aldrich). Trypsin (Sigma-Aldrich) was added, and solution was boiled for 30 minutes. PT-gliadin was stored at -20°C. Mice were gavaged with 1mg of PT-gliadin and 25μg of CT (Sigma-Aldrich) on P14, P16, and P18. Non-sensitized controls were gavaged with 25μg of CT only and naïve controls were gavaged with vehicle only (Phosphate-buffered saline, PBS). All control mice were kept on a low-fat GFD (TD.05620, Envigo, Madison, Wisconsin) when weaned from their mothers at three weeks old (P21). Gluten-sensitized mice were placed on either the same low-fat GFD

(TD.05620, Envigo) or an isocaloric gluten-containing diet (TD.200056, Envigo) containing the equivalent daily intake of 20g of gluten for a 70kg human (Figure 1.1). Endpoint for the study was at ten weeks old.

An additional group was added during the second aim, mice were sensitized as previously described above and kept on the same low-fat GFD (TD.05620, Envigo). The endpoint for this long-term GFD group was 27 weeks (Figure 3.2).

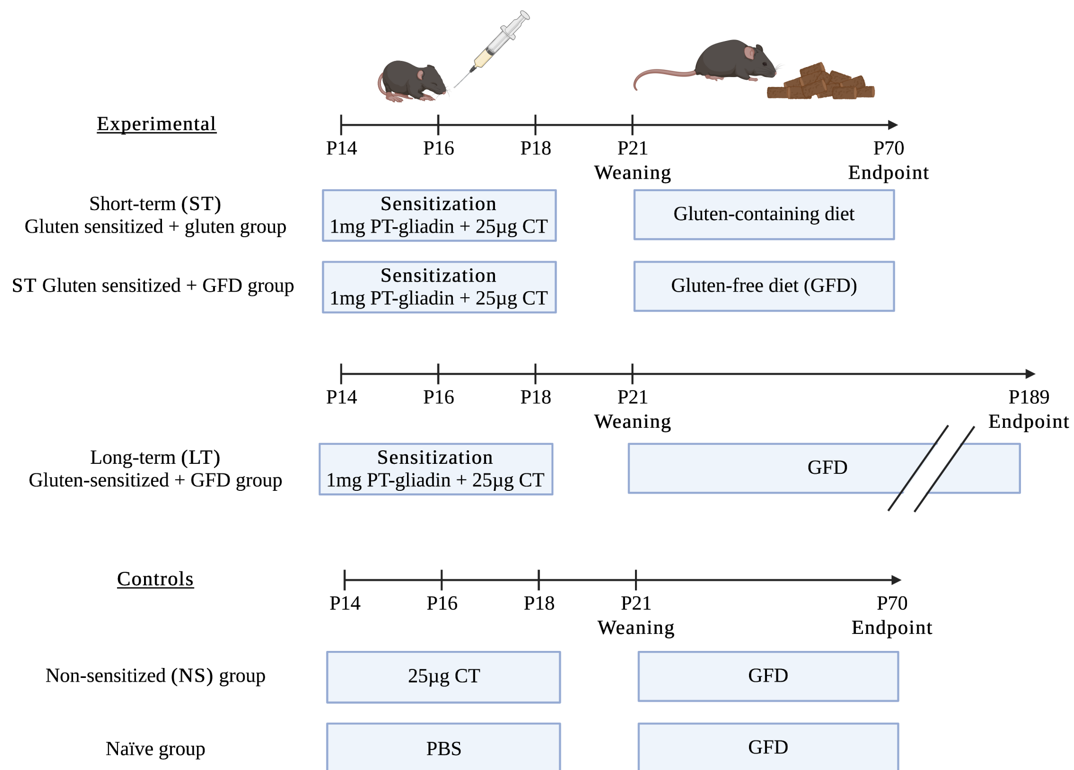

Figure 3.2 Diagram of all groups in early life sensitization study

Experimental mice were given 1mg of pepsin-trypsin digested (PT) gliadin and 25μg of cholera toxin (CT) by oral gavage on post-natal days (P) 14, 16 and 18, before being weaned from their mothers at P21. From then, mice were kept on a gluten-containing diet until their endpoint of P70. Additional experimental groups were sensitized in the same manner but kept on a gluten-free diet (GFD) for the entirety of the study, until days P70 or P189. Controls were gavaged with either 25μg CT or vehicle-only and remained on the GFD. Created with BioRender.com

3.2 Animal sample processing

3.2.1 Histology

Cross-sections of two to four pieces of proximal jejunum were fixed in 10% formalin and embedded in paraffin.

3.2.1.1 Hematoxylin and eosin stain

Paraffin-embedded proximal jejunum samples were stained with hematoxylin and eosin (H&E) for histological evaluation under light microscopy (Olympus, Richmond Hill, Ontario, Canada). Images were acquired and analyzed by QuPath version 1 (Bankhead et al., 2017). Samples were evaluated for the presence of inflammation and villus-to-crypt (V/C) ratios were measured from ten blinded villi and crypts at 40× magnification.

3.2.1.2 $CD3^+$ IEL stain

Paraffin-embedded proximal jejunum samples were stained to detect the presence of $CD3^+$ IELs as previously described (Verdu et al., 2008). Samples were incubated overnight at 4°C with rabbit anti-mouse $CD3^+$ antibody (1:1,000, Dako, Burlington, Ontario, Canada), followed by a secondary incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Dako). Visualization was accomplished using 3,3’–Diaminobenzidine (DAB, Dako) as the substrate and counterstained with Meyer’s hematoxylin (Sigma-Aldrich). Negative controls were performed in absence of primary antibody. Again, samples were evaluated under light microscopy (Olympus) and images were acquired and analyzed by QuPath version 1 (Bankhead et al., 2017). $CD3^+$ IELs were quantified by counting IELs present per 20 enterocytes in ten randomly chosen villi tips according to previously described methods, under 40× magnification (Biagi et al., 2004). Data was reported as IEL/100 enterocytes.

3.2.2 Enzyme-linked immunosorbent assays (ELISAs)

Assays were performed on seral samples collected at endpoint and stored at -80°C. Small intestinal wash samples were also measured and were collected at endpoint with 5mL of 0.05 ethylenediaminetetra-acetic acid (EDTA, Sigma-Aldrich) in PBS with soy trypsin inhibitor (Sigma-Aldrich) and phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich). Samples were spun down and supernatant was collected for measurement. Samples were evaluated at complete concentration.

3.2.2.1 Anti-gliadin IgA ELISA

Anti-gliadin antibodies were assessed using previously described methods (Galipeau et al., 2011). Gliadin (Sigma-Aldrich) was dissolved in 70% ethanol, then diluted in PBS. In a 96-well Nunc-Immuno plate (Thermo Fisher Scientific, Waltham Massachusetts), gliadin was added for a total concentration of 5μg per well and incubated overnight at 4°C. Plates were then incubated using 2% bovine serum albumin (BSA, Sigma-Aldrich) in 0.05% Tween-20 PBS (PBS-T) for 2h at room temperature (RT). Samples were added to plate for 2h at RT. Incubated with HRP-conjugated secondary anti-mouse IgA (1:8,000, Abcam, Cambridge, UK). Plates were washed with PBS-T. Substrate used was tetramethylbenzidine (TMB, Dako).

3.2.2.2 Anti-TG2 IgA ELISA

Anti-TG2 antibodies were assessed using previously described methods (Galipeau et al., 2011). TG2 derived from guinea pig liver (Sigma-Aldrich) was diluted in PBS for a total concentration of 0.1μg per well in a 96-well Nunc-Immuno plate (ThermoFisher). Plate was incubated overnight at 4°C, followed by a 2h incubation with 2% BSA (Sigma-

Aldrich) in PBS-T at RT. Samples were added to plate for 1h at RT. Plate was then incubated with HRP-conjugated secondary anti-mouse IgA (1:6,000, Abcam). Plates were washed with PBS-T. Substrate used was TMB (Dako).

3.2.2.3 Protein quantification assay

Protein quantification in intestinal wash samples was determined using protein assay reagents S, A and B (Bio-Rad Laboratories, Hercules, California) according to manufacturer's instructions. Eight-part serial dilution was done for the standard curve beginning at 8mg/mL BSA (Sigma-Aldrich) in 0.05 EDTA (Sigma-Aldrich) PBS with pH of 8.

3.2.3 Genotyping

The genotype of mice was confirmed using Phire Tissue Direct PCR kit (Thermo Fisher Scientific) according to manufacturer's instructions. Briefly, 1 square mm of tissue was incubated with DNA release agent and the solution was then used for each of the downstream PCRs. PCR mix was prepared with 8µL deoxyribonuclease (DNase)/ ribonuclease (RNase-free) water, 10µL 2× Phire mix, and 0.5µL each of 10µM forward primer and 10µM reverse primer, per 1µl DNA sample. PCR protocol used was (98°C/2min) ×1, (98°C/30s, 58°C/30s, 72°C/45s) ×30, (72°C/2min) ×1. Process was repeated twice, once for each of the following primer sequences.

To assess the presence of the HLA-DR3-DQ2 gene (680bp) the following primer sequences were used:

DRA-FOR 5'-TCCCTTGATGATGAAGATGG-3'
DRA-REV 5'-CAGAGGTAACTGTGCTCACG-3'

To assess the absence of the mouse MHC class II gene (748bp) the following primer sequences were used:

Hygro-FOR 5'-CTG AAC TCA CCG CGA CGT CT-3'
Hygro-REV 5'-CCT CCG CTC GAA GTA GCG CGT C-3'

3.3 Statistical Analysis

Linear mixed modelling was performed using the *lme4* package for R version 4.1.1, with sex and treatment as fixed effects and the litter as a random effect (Bates et al., 2015; R Core Team, 2022). Normal distribution was used for histological and serological data processing. Additionally, serological data was processed with a generalized linear mixed model with the same fixed effects and random effect as above, with a binomial distribution based on a calculated positive reactivity threshold previously described as 3 standard deviations (SD) above the mean of all control mice (naïve and non-sensitized) (Galipeau et al., 2011). Naïve and non-sensitized mice were evaluated for significant differences before being combined and compared to experimental groups. Statistical differences between groups were identified using estimated marginal means and custom contrasts using *emmeans* and *constrast* functions of the *emmeans* package for R (Lenth, 2022). Control groups (naïve and non-sensitized) were compared between each other and combined for pairwise comparison of all groups. Šidák correction for multiple comparisons was used. Additionally, normality was evaluated for all treatment group outcome variables, before testing for two-way linear correlations using the non-parametric Spearman's test.

4. CHAPTER 4: RESULTS

RESULTS

4.1 Establishment of an early life model in DR3-DQ2 mice

Before beginning our experiments, establishing a proper early life model was key. Under the guidance of Dr. Tatiane Ribeiro (Sloboda lab), I followed the standards established in previous early life studies. Importantly, first litters were culled due to high pup mortality rates from cannibalism by C57BL/6 dams, the background of DR3-DQ2 transgenic mice (Brajon et al., 2021). Furthermore, experimental litters were sexed and standardized to 2 males and 2 females ideally, assuring equal nutrient distribution across all litters (Silver, 1995). To confirm pregnancy, I established that an increase in 5g of body weight confirmed pregnancy in the DR3-DQ2 mice, which is slightly higher than the 3g used in prior studies with C57BL/6 mice.

4.2 DR3-DQ2 mice sensitized before weaning develop moderate enteropathy

We first assessed enteropathy in mice sensitized before weaning by measuring both V/C ratios and $CD3^{+}$ IELs in villi tips. It was found that mice that had been sensitized to gluten before weaning developed a moderate reduction of their V/C ratios compared to pooled naïve and non-sensitized controls, where experimental mice with lowest V/C ratios had 1.3 times lower values (Figure 4.1, A and B). Mice placed on a gluten diet after sensitization were the only ones to develop a statistically significant reduction in V/C ratios. This change in V/C ratios was characterized by a reduction of the villi height specifically, rather than the crypt height, suggesting that villi shortening occurs first in this model rather than crypt elongation (Figure 4.1, C and D). Gluten-sensitized mice on a GFD were trending for V/C ratio reduction and the reduction of their villi length.

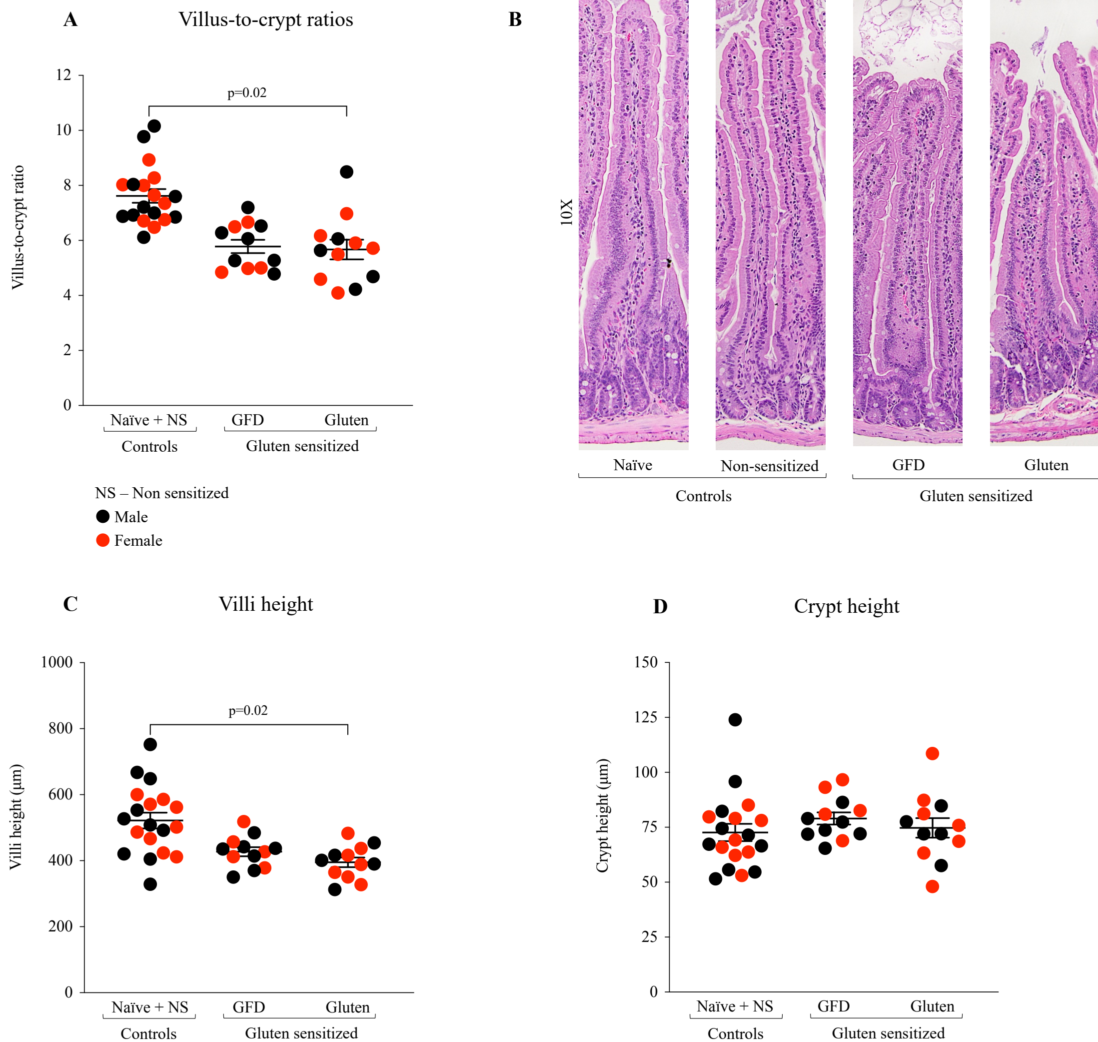

Figure 4.1 DR3-DQ2 mice sensitized to gluten before weaning develop reduced villus-to-crypt ratios

Villus-to-crypt ratios, villi height and crypt height were quantified in the proximal jejunum of DR3-DQ2 mice. **(A, C, D)** Villus-to-crypt ratios, villi height and crypt height were measured from hematoxylin and eosin-stained slides. Each dot represents an individual mouse, with females in red; data are represented as mean ± standard error mean (SEM); p-values were determined using a linear mixed model with sex and treatment as fixed effects, the mice's litter as a random effect and with normal distribution; controls were evaluated for significant differences, before pairing them to compare to both experimental groups. **(B)** Representative photos were taken at 10× magnification.

$CD3^+$ IELs counts indicated a significant increase in mice sensitized to gluten before weaning, where mice with highest levels had double the amount than pooled naïve and non-sensitized controls (Figure 4.2, A and B). Mice placed on a gluten-containing diet after sensitization were the only ones to develop statistically significant changes. Gluten-sensitized mice on a GFD had a trending increase of $CD3^+$ IEL counts.

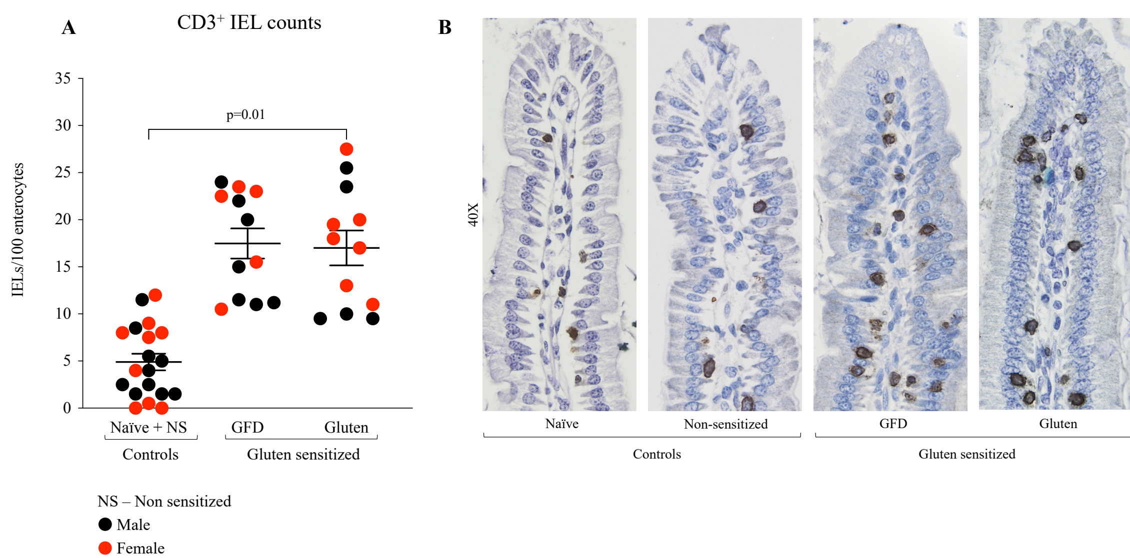

Figure 4.2 DR3-DQ2 mice sensitized to gluten before weaning develop higher intraepithelial lymphocyte counts

$CD3^+$ intraepithelial lymphocytes (IELs) were quantified in the proximal jejunum of DR3-DQ2 mice. **(A)** $CD3^+$ IELs were quantified using immunohistochemistry. Each dot represents an individual mouse, with females in red; data are represented as mean ± standard error mean (SEM); p-values were determined using a linear mixed model with sex and treatment as fixed effects, the mice's litter as a random effect and a normal distribution; controls were evaluated for significant differences, before pairing them to compare to both experimental groups. **(B)** Representative photos were taken at 40× magnification.

Together, these results showcase that this animal model develops moderate enteropathy after being sensitized to gluten in early life and placed on a gluten containing-diet after weaning. Although not significantly altered, there was also a trend for decreased

V/C ratios and increased IEL counts in mice sensitized in the same manner and kept gluten-free.

4.3 Some of DR3-DQ2 mice sensitized before weaning develop anti-gliadin and anti-TG2 IgA antibodies

To assess the ability of mice to develop antibodies used in CeD diagnosis, sera and intestinal content were used to measure anti-gliadin and anti-TG2 IgA antibodies. It was found that some gluten-sensitized mice, regardless of diet, developed both anti-gliadin and anti-TG2 antibodies in both their serum and intestinal content (Figure 4.2). Overall, these results indicate that some mice in this model produce the same antibodies measured in CeD patients.

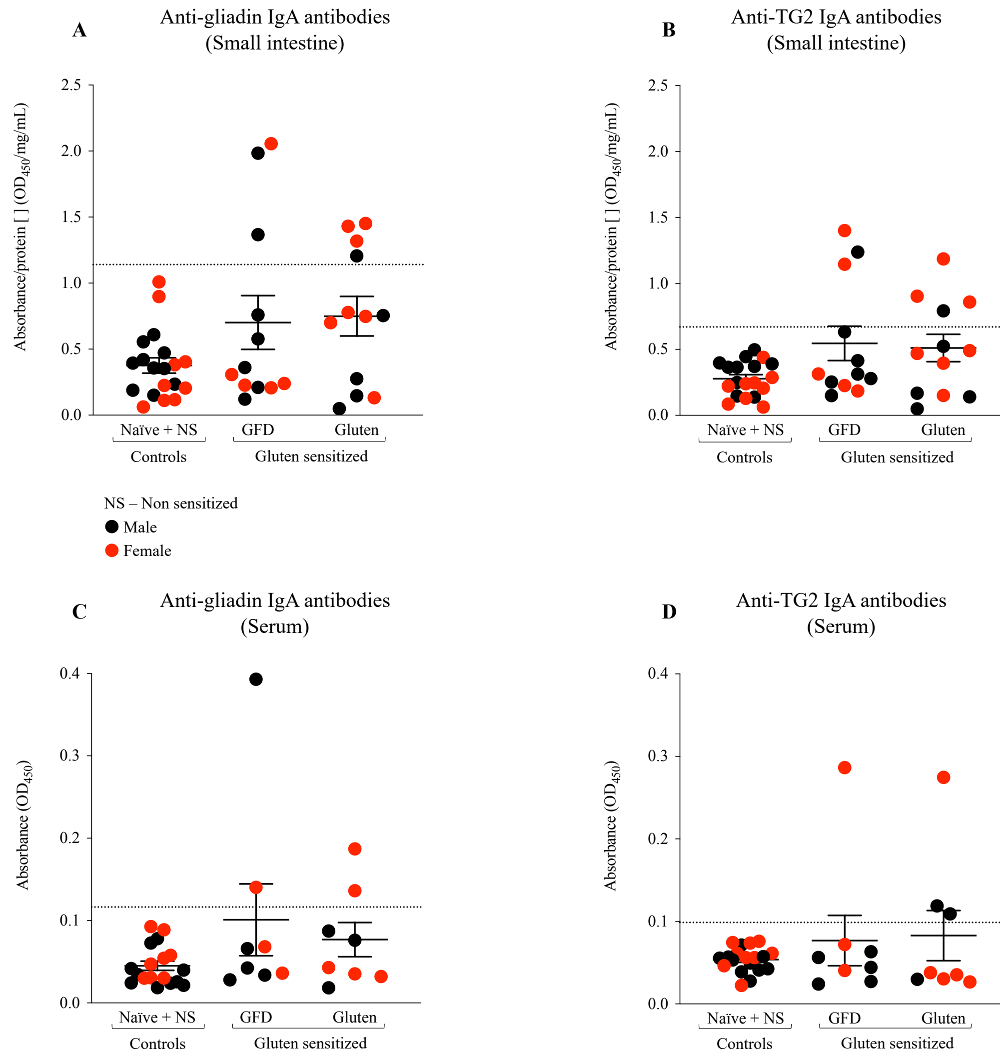

Figure 4.3 Some mice sensitized to gluten before weaning develop gliadin and TG2 antibodies

Antibodies in serum and intestinal content of DR3-DQ2 mice were measured. The presence of small intestinal anti-gliadin **(A)** and anti-TG2 **(B)** antibodies, as well as serum anti-gliadin **(C)** and anti-TG2 **(D)** antibodies were determined using ELISAs. Each dot represents an individual mouse, with females in red; positive reactivity was determined using a positive cut-off value of ≥ 3 standard deviations (SD) above the mean of control mice (dotted line); data are represented as mean ± standard error mean (SEM); p-values were determined using a generalized linear mixed model with sex and treatment as fixed effects, the mice's litter as a random effect and a binomial distribution; controls were evaluated for significant differences, before pairing them to compare to both experimental groups.

4.4 There are strong associations between specific outcome measurements

Due to the spread of antibody responses in early life gluten sensitized mice, associations between different outcome variables were investigated.

We first looked at the correlation between V/C ratios and $CD3^+$ IEL counts and found a strong negative correlation (r= -0.7) between the two variables, which achieved statistical significance (Figure 4.4, A). That is, lower V/C ratios were associated with higher IEL counts. Comparison of anti-TG2 IgA and anti-gliadin IgA in intestinal contents resulted in a very strong positive correlation (r= 0.9) which also achieved statistical significance (Figure 4.4, B). A similar association was found when comparing anti-TG2 and anti-gliadin IgA antibodies in the serum of DR3-DQ2 mice, yielding a statistically significant moderate positive correlation (r= 0.5; Figure 4.4, C). Comparison of serum and small intestinal antibodies did not yield meaningful correlations (Figure 4.4, D)

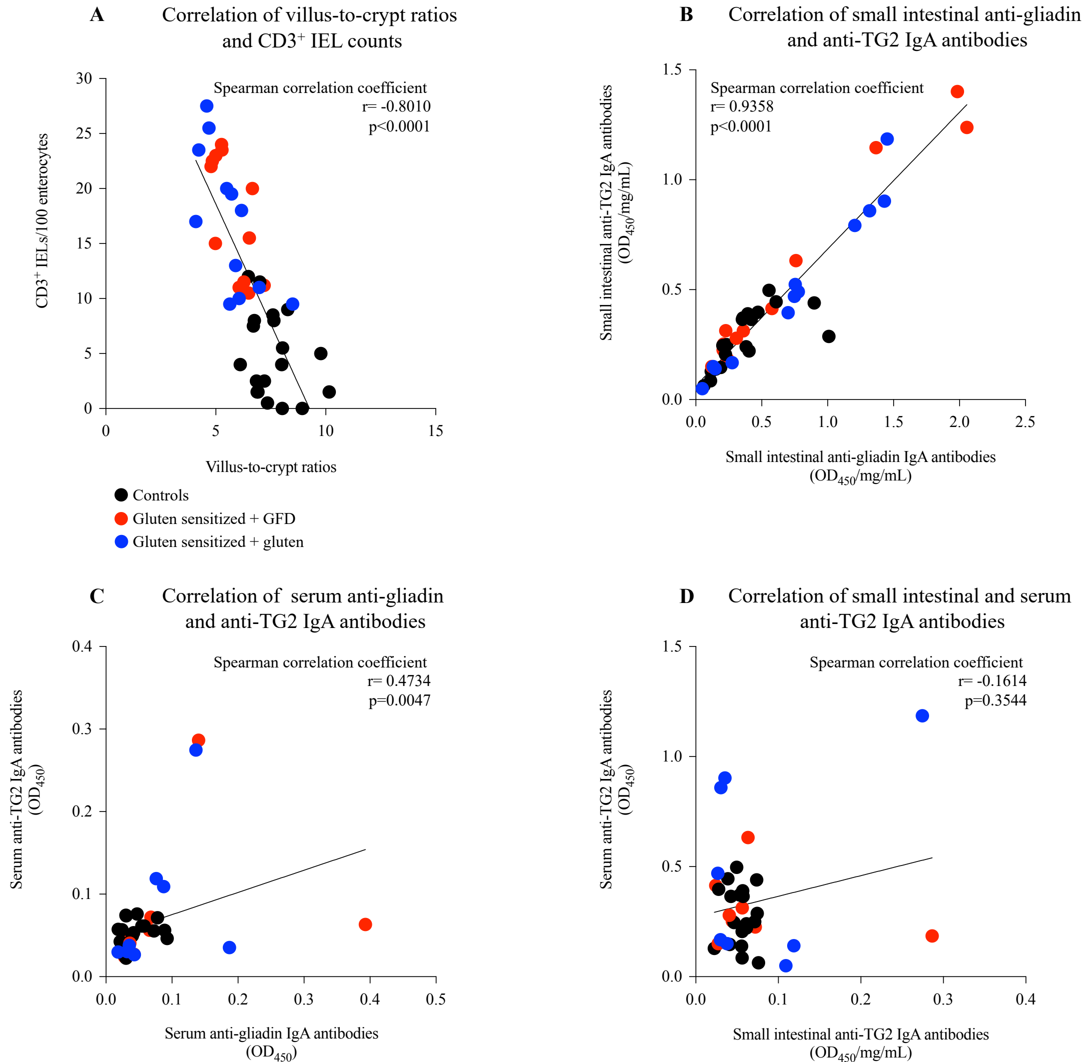

Figure 4.4 Correlations between histological outcome measurements, as well as antibody measurements in DR3-DQ2 mice

Correlations of **(A)** villus-to-crypt ratios against $CD3^+$ IELs, **(B)** small intestinal anti-gliadin against anti-TG2 IgA antibodies, **(C)** serum anti-gliadin against anti-TG2 IgA antibodies, as well as **(D)** small intestinal and serum anti-TG2 IgA antibodies of DR3-DQ2 mice were performed. Each dot represents an individual mouse, with controls in black, gluten sensitized + GFD in red and gluten sensitized + gluten in blue; correlation coefficients and p-values were determined using the non-parametric Spearman's test.

Continued investigation focused on the comparison of antibodies and histological measurements. When comparing small intestinal anti-TG2 IgA antibodies to V/C ratios, there was no remarkable association between the two variables (Figure 4.5, A). However, a statistically significant and moderately positive correlation was found between small intestinal anti-TG2 IgA antibodies and $CD3^+$ IEL counts (Figure 4.5, B). Comparisons with serum anti-TG2 IgA antibodies and histological measures did not yield significant correlations (Figure 4.5, C and D).

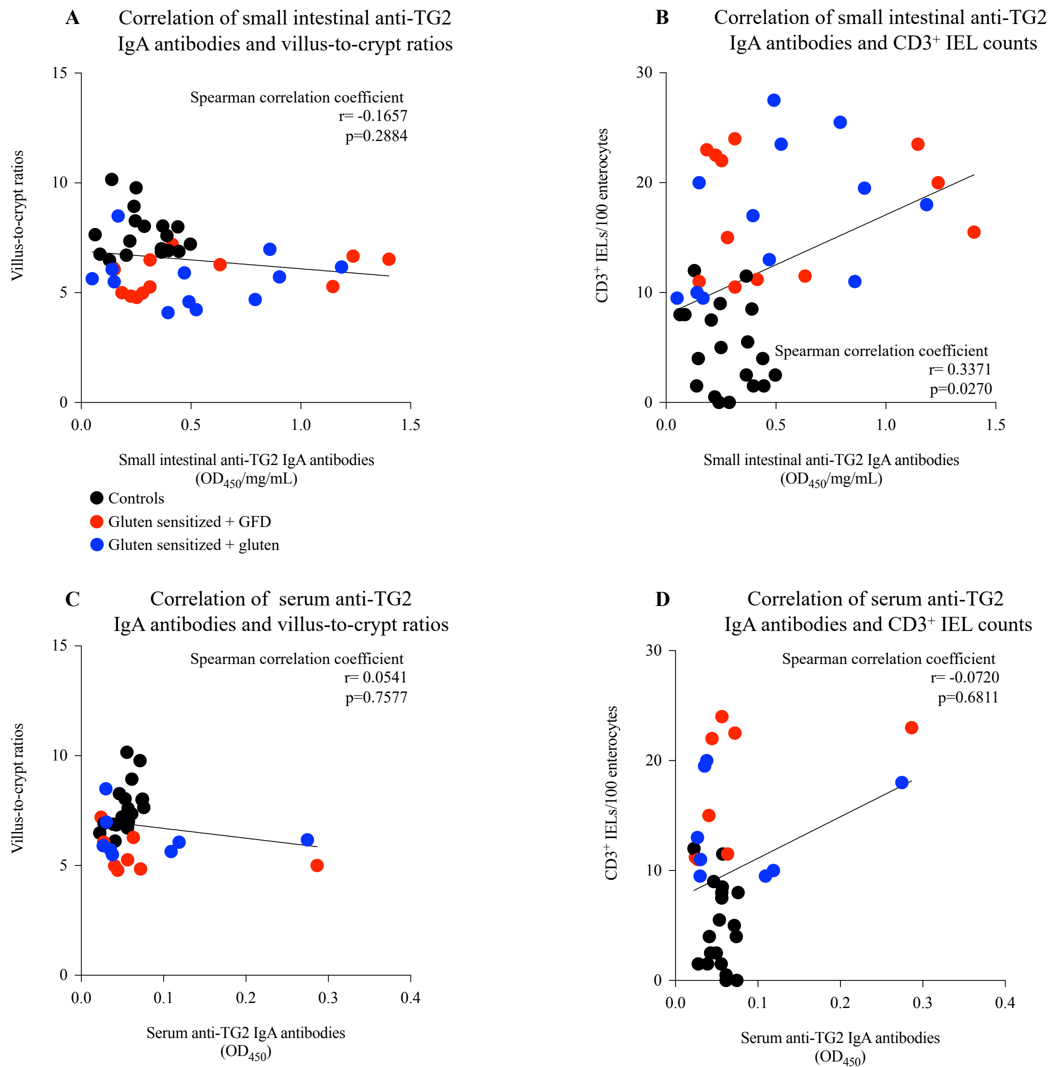

Figure 4.5 Correlations between histological and antibody outcome measurements of DR3-DQ2 mice

Correlations of **(A)** small intestinal anti-TG2 IgA antibodies against villus-to-crypt ratios, **(B)** small intestinal anti-TG2 IgA antibodies against $CD3^+$ IEL counts, **(C)** serum anti-TG2 IgA antibodies against villus-to-crypt ratios, as well as **(D)** serum anti-TG2 IgA antibodies against $CD3^+$ IEL counts of DR3-DQ2 mice were performed. Each dot represents an individual mouse, with controls in black, gluten sensitized + GFD in red and gluten sensitized + gluten in blue; correlation coefficients and p-values were determined using the non-parametric Spearman's test.

4.5 DR3-DQ2 mice sensitized before weaning and kept gluten-free long-term partially recover

Due to the unexpected development of moderate enteropathy in mice sensitized with gluten in early life and kept gluten-free throughout the study, and some of these mice developing IgA antibodies for anti-gliadin and anti-TG2, a long-term recovery model was investigated.

We first looked at the recovery of the histological damage that we saw in the short-term mice from the first aim. Mice sensitized to gliadin before weaning and being kept gluten-free onwards did not fully recover their villus-to-crypts ratios (Figure 4.6, A and B). To further support this, the changes previously identified in villi height were still present, and crypt height differences remained unremarkable (Figure 4.6, C and D).

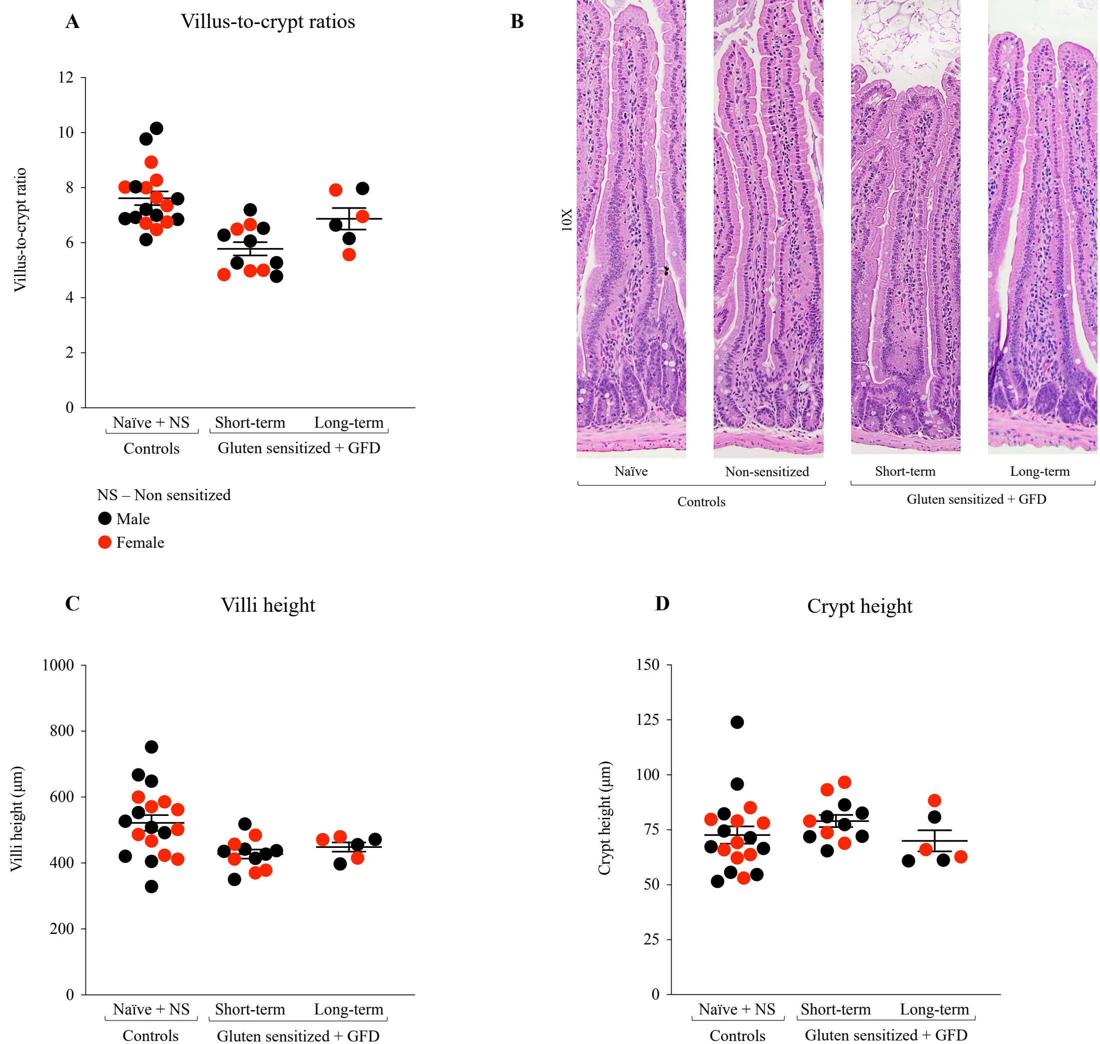

Figure 4.6 DR3-DQ2 mice sensitized to gluten and kept gluten-free long-term do not recover their villus-to-crypt ratios

Villus-to-crypt ratios, villi height and crypt height were quantified in the proximal jejunum of DR3-DQ2 mice. **(A, C, D)** Villus-to-crypt ratios, villi height and crypt height were measured from hematoxylin and eosin-stained slides. Each dot represents an individual mouse, with females in red; data are represented as mean ± standard error mean (SEM); p-values were determined using a linear mixed model with sex and treatment as fixed effects, the mice's litter as a random effect and a normal distribution; controls were evaluated for significant differences, before pairing them to compare to both experimental groups. **(B)** Representative photos were taken at 10× magnification.

In contrast, the measured CD3^{+} IEL counts of long-term gluten-free mice had decreased, normalizing to numbers more like those found in controls (Figure 4.7).

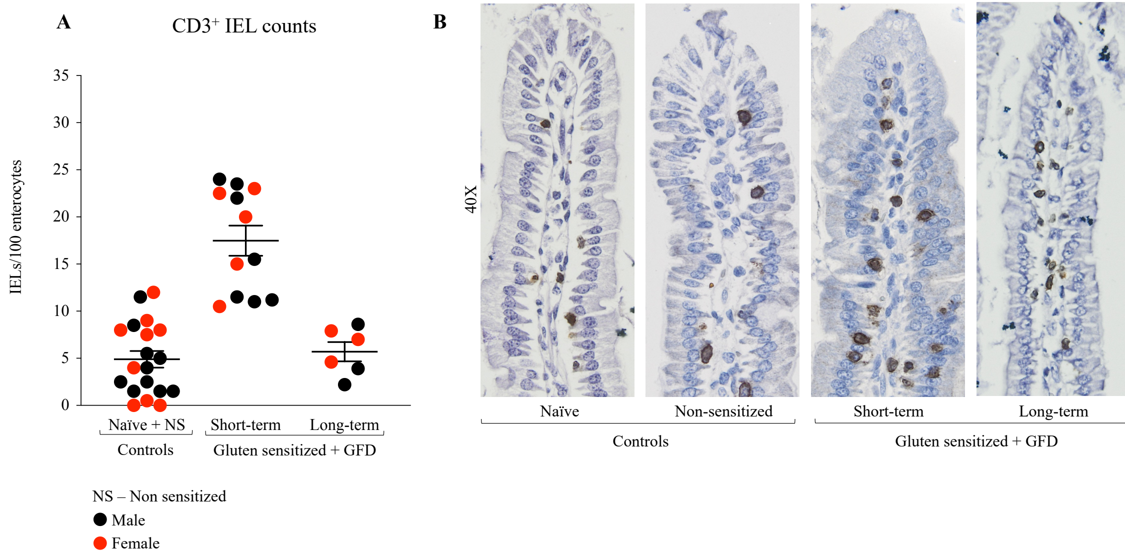

Figure 4.7 DR3-DQ2 mice sensitized to gluten before weaning and kept gluten-free long-term have normalized CD3^{+} IEL counts

CD3^{+} intraepithelial lymphocytes (IELs) were quantified in the proximal jejunum of DR3-DQ2 mice. **(A)** CD3^{+} IELs were quantified using immunohistochemistry. Each dot represents an individual mouse, with females in red; data are represented as mean ± standard error mean (SEM); p-values were determined using a linear mixed model with sex and treatment as fixed effects, the mice's litter as a random effect and a normal distribution; controls were evaluated for significant differences, before pairing them to compare to both experimental groups. **(B)** Representative photos were taken at 40× magnification.

We again measured antibodies responses in mice, as previously described above, to determine if there was recovery for this feature of the model. We found that no long-term gluten-free mice had positive antibody levels in the small intestine (Figure 4.8). However, positive antibody levels were maintained in serum by some mice.

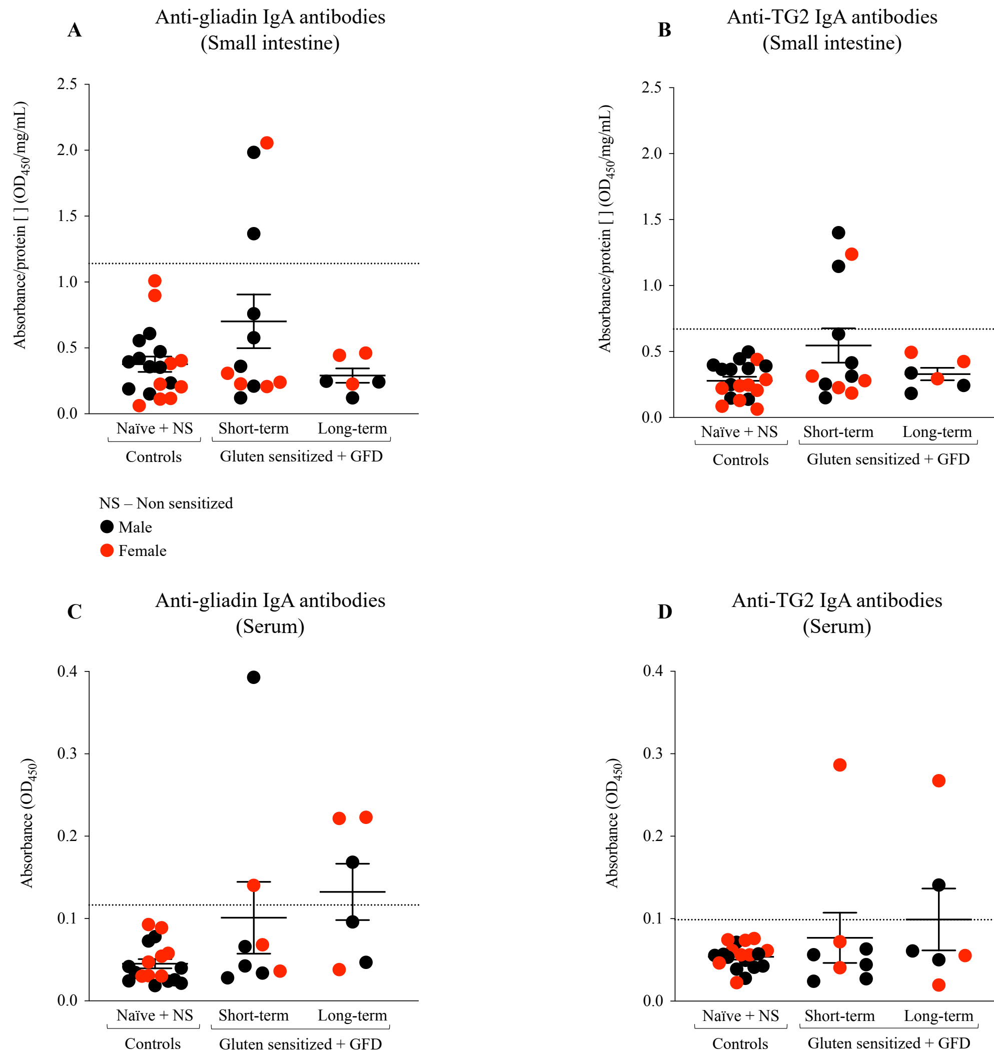

Figure 4.8 Some DR3-DQ2 mice sensitized to gluten before weaning when kept gluten-free long-term continue to produce anti-gliadin and anti-TG2 IgA antibodies reduces

Antibodies in serum and intestinal content of DR3-DQ2 mice were measured. The presence of small intestinal anti-gliadin **(A)** and anti-TG2 **(B)** antibodies (Abs), as well as serum anti-gliadin **(C)** and anti-TG2 **(D)** Abs were determined using ELISAs. Each dot represents an individual mouse, with females in red; positive reactivity was determined using a positive cut-off value of ≥ 3 standard deviations (SD) above the mean of control mice (dotted line); data are represented as mean ± standard error mean (SEM); p-values were determined using a generalized linear mixed model with sex and treatment as fixed effects, the mice's litter as a random effect and a binomial distribution; controls were evaluated for significant differences, before pairing them to compare to both experimental groups.

These results showcase that our animal model is capable of some enteropathy recovery characterized by the normalization of $CD3^{+}$ IEL counts, but not V/C ratios. In addition, intestinal antibody responses normalized, but some mice sustained the production of anti-gliadin and anti-TG2 IgA antibodies in serum. More research is needed to understand the long-lasting effects of this early life gluten sensitization and why it can induce gluten-immunopathology without re-exposure to gluten.

5. CHAPTER 5: THESIS DISCUSSION

THESIS DISCUSSION

5.1 Summary and discussion

Maintaining gut homeostasis is key for maintaining the balance between health and chronic inflammation (König et al., 2016). Therefore, complex systems are in place in the GALT to ensure appropriate immune responses are developed towards threats, while ignorance towards innocuous antigens is developed (König et al., 2016; Mörbe et al., 2021). The intestinal barrier in combination with the immune system and microbiome will interact to protect the host and maintain gut homeostasis (König et al., 2016). Importantly, Foxp3^{+} Treg-mediated active suppression, also known as oral tolerance, will lead to the suppression of immune reactions to innocuous food antigens (Pabst & Mowat, 2012). The breakdown of different intestinal pathway will lead to inflammation and disease (König et al., 2016). For example, oral tolerance breakdown can lead to the development of food allergies and other food sensitivities such as CeD (Pabst & Mowat, 2012).

CeD is a very well-defined food sensitivity where oral tolerance fails (Tye-Din et al., 2018). CeD, a CD4^{+} T-cell mediated immune response, is driven by a group of food proteins (gluten) and aided by cytotoxic transformation of IELs, culminating in the destruction of IECs and the generation of villus atrophy (Tye-Din et al., 2018). We have detailed knowledge of key pathogenic steps in CeD. These include: insufficient digestion of gluten proteins, interaction with TG2 (deamidations and transamidation), APC presentation of gluten in the context of MHC class 2 molecules (HLA-DQ8 or -DQ2), CD4^{+} T cell activation and B cell help, as well as activation and cytotoxic transformation of IELs (Tye-Din et al., 2018). However, many unknowns remain, in particular why some

individuals with genetic susceptibility and exposed to gluten develop CeD and others do not (Tye-Din et al., 2018).

Treatment of CeD is less than ideal, as the only existing treatment is a GFD which is not effective in all patients and the adherence to said diet comes with its own issues (Green & Cellier, 2007; Niland & Cash, 2018; Tack et al., 2010). There is a specific subset of children (HLA-DQ2 carriers) which are highly susceptible to developing CeD in early life, though there are some homozygous children that will never develop the disease (Green & Cellier, 2007). More importantly, whether unknown environmental factors operate in early life to promote oral tolerance breakdown at this age remains unclear. To better study this specific facet of CeD, an early life preclinical model is needed. **Thus, the overall goal of this thesis was to characterize such a model in DR3-DQ2 transgenic mice, which carry the allele most relevant for early life CeD development.** This goal was addressed in both aims of my thesis, where I showed that DR3-DQ2 mice sensitized before weaning and placed on a gluten-containing diet develop key features of both innate and adaptive immune responses in CeD as well as moderate enteropathy, a key histological feature of CeD.

I showed that moderate gluten-immunopathology assessed by reduction in V/C ratios and increased $CD3^+$ IEL cell counts can be induced in mice sensitized before weaning. These results are aligned with enteropathy induction in adult mice, and indicate sensitization in early or adult life leads to similar degrees of enteropathy in the model (Clarizio, 2020). I also showed that a subset of gluten-sensitized DR3-DQ2 mice develop anti-gliadin and anti-TG2 IgA antibodies in serum and in small intestinal content. These are classical antibodies also present in patients with CeD. The development of intestinal

antibodies has also been found in adult HLA-DQ2 and HLA-DQ8 transgenic models and suggests B cells in the intestinal lumen are being activated (Abadie et al., 2020; Clarizio, 2020; Galipeau et al., 2011; Verdu et al., 2008). Importantly, antibodies in serum for both TG2 and gliadin IgA have been reported in other adult HLA-DQ8 transgenic mouse models, **but not the adult HLA-DQ2 transgenic mouse model** (Abadie et al., 2020; Clarizio, 2020; Galipeau et al., 2011; Korneychuk et al., 2015; Verdu et al., 2008). Therefore, the development of IgA antibodies in the early life model is a significant finding, especially anti-TG2 IgA antibodies as they are highly sensitive and specific for CeD and key in CeD diagnosis (Lebwohl & Rubio-Tapia, 2021; Tack et al., 2010). The variability in response exhibited in this model is not surprising as other existing CeD mouse models have described similar results (Clarizio, 2020; Galipeau et al., 2011; Verdu et al., 2008). This is reminiscent of the many clinical phenotypes associated with CeD, including seroconversion without enteropathy (CeD autoimmunity and potential CeD) and active CeD with various degrees of enteropathy (Marsh I-IV) (Catassi et al., 2022). Importantly, I also showed that the histological outcome variables, V/C ratios and $CD3^{+}$ IEL counts, had moderate negative correlation, where low V/C ratios and high $CD3^{+}$ IEL counts were associated. This indicates that these variables concur for intestinal damage in these mice, as has been shown in celiac patients (Biagi et al., 2004). Furthermore, I showed that the antibodies measured in the small intestine, anti-TG2 and anti-gliadin IgA had a strong positive correlation, where high levels of anti-TG2 IgA were linked to high levels of anti-gliadin IgA. The same antibody measurements in serum were also found to correlate to one another, where high levels of anti-TG2 IgA were linked to high levels of anti-gliadin IgA.

This again indicates that these variables concur and are reliable measurements as biomarkers of CeD in these mice, such as seen in humans (Lebwohl & Rubio-Tapia, 2021; Tack et al., 2010). When comparing intestinal antibody levels to their presence in serum, no correlation was found. This is not surprising as antibodies in serum can be sustained for extended periods of time when gluten has been removed and small intestinal antibodies are no longer being produced (Rubio-Tapia et al., 2010). Furthermore, the lack of correlation and the low antibody levels observed in serum throughout this thesis may also be an indicator that seroconversion had only begun in the DR3-DQ2 mice. For this, we would expect to see high levels of intestinal antibodies and low levels of serum antibodies, as we have described. Perhaps a longer exposure to the gluten diet would yield a stronger positive correlation between these two variables. I also showed that small intestinal anti-TG2 IgA antibodies and $CD3^+$ IEL counts had a moderate positive correlation, where high levels of one variable resulted in high levels of the other. No other comparison of intestinal or serum antibodies with histological measures yielded significant correlations. Importantly, the association between the intestinal anti-TG2 IgA antibodies and the $CD3^+$ IEL counts implies that the intestinal damage we see in the DR3-DQ2 mice results from exposure to gluten.

Interestingly, mice sensitized to gluten before weaning and kept gluten-free, an experimental group which has yet to be investigated in other CeD models, developed a trend for lower V/C ratios and higher $CD3^+$ IEL counts, compared to pooled controls. Some also developed both anti-gliadin and anti-TG2 IgA antibodies in serum and intestinal content (Korneychuk et al., 2015). The lack of normalization of IEL counts after only 5

weeks on the GFD aligns with results in humans, which can take 12 months or more to recover (Rubio-Tapia et al., 2010; Zanini et al., 2016). These results raise the intriguing hypothesis that additional environmental factors to gluten may be responsible for the sustained IEL responses observed in these mice. Given that sensitized mice placed on a gluten diet achieved statistically significant differences in their enteropathy, and gluten-sensitized mice on a GFD did not, we can infer that gluten sensitization is key to the development of immunopathology and that the gluten diet allows for a sustained immune response, which lead to statistically significant enteropathy.

The results observed in the sensitized and gluten-free mice led to the development of the second aim of this thesis, where we anticipated that 6 months on the GFD would be sufficient for complete recovery and normalization of the parameters described above. After 6 months of gluten exclusion, V/C ratios had not fully normalized, but $CD3^{+}$ IEL counts had decreased. This has also been seen in humans, where recovery of IELs is not necessarily followed by improvement in villi structure (Rubio-Tapia et al., 2010). This observation is often hypothesized to be caused by gluten contamination (Rubio-Tapia et al., 2010). No gliadin-sensitized mice had **intestinal** anti-TG2 or anti-gliadin IgA antibodies after 6 months of the GFD. However, some continued producing both TG2 and gliadin IgA antibodies in **serum**. The response measured in serum is surprising as serum antibodies in human have a half-life of 30 to 60 days, which is well within the 6 months of treatment for this group (Castillo et al., 2015). This suggests that the serological antibody response is being maintained. Serum antibodies have also been found to be maintained in a subset of patients (Rubio-Tapia et al., 2010). Again, this phenomenon is often

hypothesized to be caused by gluten-contamination (Rubio-Tapia et al., 2010). In humans, antibody titers will become negative before full histological recovery, but the process can take up to 12 months to normalize (Tursi et al., 2003). These results raise the intriguing hypothesis that additional environmental factors to gluten may be responsible for the sustained villus damage and serum responses in this model. Further investigation may elucidate a mechanism relevant to celiac patients reacting similarly, which could prove useful for their treatment. These collective results indicate that early life gluten-sensitization of SPF DR3-DQ2 mice mimic some aspects of the kinetics of serological and histological recovery observed in humans with CeD.

Thus, for the first time, this model reproduces key features of CeD after early life breakdown of oral tolerance to gluten and will be useful to expand on further environmental modulation of these responses in the future.

The most important feature of CeD is the development of villus atrophy. While this model revealed some specific characteristics related to the kinetics of recovery, this model still develops **moderate** enteropathy, which is very comparable to other mouse models of CeD (Korneychuk et al., 2015). It can be argued that rather than a limitation, this represents an advantage for further experiments. With such a model, we can test different environmental triggers to evaluate which may prevent or worsen the model's pathological phenotype. Importantly, it is also possible that 5 weeks on a gluten-containing diet is not sufficient to induce complete atrophy in this model.

This model can also be criticized for its requirement of mucosal adjuvant – in this case, CT – to sensitize the mice to gluten to produce enteropathy. This method is used to

mimic inflammatory events known to trigger CeD (i.e., viral infections) and to shorten the time needed to produce such a response, as it has been previously shown in an adult DR3-DQ2 gluten-sensitized model that these mice are able to spontaneously (with no sensitization phase) develop key features of CeD but will take a minimum of 5 months to do so (Brown et al., 2018; Clarizio, 2020). Considering that the breeding protocol for the early life model already considerably increases the time needed to complete an experiment (minimum 2.5 months) and that inducing an inflammatory response is especially clinically relevant in CeD development, the use of CT to produce key features of CeD is a benefit. The use of such a mucosal adjuvant will also allow for more easily reproducible data.

5.1.1 Limitations and future directions

Although this thesis has led to the development of other experiments in our lab, there are some limitations that must be discussed, as no one animal model is able to model all features of a disease.

Ongoing studies have branched from this thesis as there are many unanswered questions left by both this model, but also in the field of CeD itself. Mice sensitized to gluten that were kept gluten-free short-term did not fully recover their histopathology. Although the GFD used in this study should not contain gluten, it could be argued that small amounts of contamination in the diet occurred as the diet is processed into food pellets in a facility that makes other rodents chows which contain gluten. To confirm that this is not the case, a gluten immunogenic peptide test, which detects the presence of the 33-mer peptide, should be performed on both food pellets samples and mouse fecal samples collected during the study. Another possible explanation is that the phenotype of the IELs

present in villi tips is different, despite numbers remaining increased. Indeed, TCR $\gamma\delta^+$ cells are increased in CeD after treatment with the GFD and have a longer life span than their $\alpha\beta^+$ counterparts (Rubio-Tapia et al., 2010). IHC staining to determine which IELs express TCR$\alpha\beta^+$ or TCR$\gamma\delta^+$ can help understand why IELs remain long-after gluten has been removed. This option, rather than contamination, is further supported by the results in the second aim of this thesis, where an extended recovery time indeed resulted in mucosal recovery.

Another important question to address is why certain gluten-sensitized mice develop antibodies and others do not. I collected tissues and fecal samples to perform NanoString mouse inflammation gene expression panel and 16S ribosomal RNA gene sequencing analysis, which may reveal inflammatory changes associated with sustained microbiome shifts that could underlie the different responses between mice as well as the delayed recovery in the gluten-free mice.

After further reflection, there are several other parameters that should be evaluated to further characterize this early life gluten sensitization model. For example, the second aim of the thesis requires an additional group for comparison, consisting of mice sensitized to gluten before weaning and kept on a gluten-containing diet for 6 months. This group will allow for a better assessment of the recovery of the long-term gluten-free mice. Another additional parameter is the measurement of IgE antibodies in the serum of gluten sensitized mice, to confirm that the pathology exhibited in these mice is not related to allergic responses. Furthermore, given the close relationship between type 1 diabetes and CeD, the insulitis previously described in NOD-DQ8 mice as well as the steady weight gain observed

in the long-term GFD group, the endocrine pancreas of DR3-DQ2 mice merits evaluation for insulitis all while monitoring blood-glucose levels (Galipeau et al., 2011; Pham-Short et al., 2015).

I faced many delays and setbacks during my thesis. Most of my MSc work was done during the SARS-CoV-2 pandemic. This slowed the breeding needed to complete this study as experimental breeding was halted for some months, requiring additional time for the completion of this thesis. Moreover, delays and bottlenecks in the sequencing facility, due to interruptions during COVID-19 pandemic, led to delays in sample processing. It is important to note that there were also significant delays in obtaining the antibody results for this thesis, as the established ELISA protocols for both anti-gliadin and anti-TG2 IgA demonstrated non-specific binding. This issue was finally resolved by increasing the percentage of BSA blocking solution and the blocking time for both antibodies.

Future studies should focus on completing the experiments explained above, before exploring environmental triggers relevant to early life development of CeD, such as using different strains of gluten in the mice's diet (i.e., ancient v. modern) or using opportunistic pathogens linked to CeD (i.e., *Pseudomonas aeruginosa*) during the sensitization phase (Caminero et al., 2016). Importantly, the quantity of gluten introduced during the sensitization in combination with an infection agent (in place of using CT) should be investigated, as high amounts of gluten and high frequency of infections and/or antibiotic use in early life have been linked to an increased risk of celiac autoimmunity (Anti-TG2 antibodies in the presence of normal villi) (Kemppainen et al., 2017).

5.2 Conclusions

The work presented within this thesis has characterized a novel transgenic mouse that will be useful in the study of early life CeD development and the investigation of environmental co-factors involved in CeD development. The animal model described provides a novel preclinical tool for studying early life CeD development in the context of HLA-DQ2 positive patients, the majority of which are likely to develop CeD in early life. The features exhibited within this model will help expand our understanding of CeD, especially factors operating in early life that could be targeted for prevention of CeD in high-risk children.

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