

**NOVEL MECHANISMS AND THERAPIES
FOR CELIAC DISEASE**

**EXPLORING NOVEL MECHANISMS AND THERAPIES FOR
CELIAC DISEASE**

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ABSTRACT

The gastrointestinal tract forms the body's largest interface with the external environment and is exposed to a vast amount of foreign material, including pathogenic and commensal bacteria, as well as food antigens. The gastrointestinal tract has multiple functions that are performed through complex interactions by its different components. It must be able to degrade food, absorb nutrients and eliminate waste, while at the same time maintain a balance between immune tolerance and protection against pathogenic and antigenic material. This concept of mucosally-induced tolerance is a key feature of the gut immune system, whereby a state of local and systemic unresponsiveness to food protein or systemic ignorance of commensal bacteria is maintained under homeostatic conditions through interactions between the host, dietary factors, and the intestinal microbiota. Dysfunctional interactions can lead to a breakdown in tolerance to otherwise innocuous antigens. One of the best characterized food sensitivities is celiac disease (CD). CD is a chronic immune-mediated disease triggered by the ingestion of gluten, the water insoluble protein fraction in wheat, rye and barley, in patients who are HLA/DQ2 or DQ8 positive. Celiac patients can experience a loss of oral tolerance to gluten any time throughout life. The clinical presentation of CD is variable and is often associated with extra-intestinal autoimmune diseases, such as type 1 diabetes (T1D). The increasing incidence of CD and the observation that only a small proportion of genetically susceptible individuals go on to develop active inflammation suggest a role for additional environmental factors in disease pathogenesis. The current treatment for CD is a strict, life-long adherence to a gluten-free diet (GFD), which is very demanding. Frequent gluten contamination can lead

to persistent mucosal damage and symptoms, which have a negative effect on quality of life. Understanding the environmental and host factors that contribute to gluten tolerance is critical for the development of adjuvant therapies to the GFD. Therefore, the overall aim of my thesis is to characterize a humanized mouse model of gluten sensitivity in order to study factors that influence host-responses to gluten and to investigate potential therapeutic strategies.

In **chapter 3** of this thesis I characterized host responses to gluten using transgenic non-obese diabetic (NOD)/DQ8 mice. I found that gluten sensitization in NOD/DQ8 mice induced barrier dysfunction with a moderate degree of enteropathy and the development of anti-gliadin and anti-tissue-transglutaminase antibodies. I also explored the potential role of gluten in the development of T1D and found that gluten-induced barrier dysfunction was not sufficient to induce insulinitis; a partial depletion of regulatory T cells (Tregs) plus gluten sensitization was required. In **chapter 4**, I utilized this model to demonstrate that the microbiota can modulate host responses to gluten. I found that both the presence and absence of a microbiota, as well as the composition of the microbiota influenced host responses to gluten in NOD/DQ8 mice. Finally, **Chapters 5 and 6** of this thesis utilized transgenic DQ8 mice to explore two different adjuvant therapies for CD. In **chapter 5**, I showed that administration of a gluten binding polymer, P(HEMA-*co*-SS, to gluten-sensitive HLA/DQ8 mice reduced short-term and long-term gluten-induced barrier dysfunction and inflammation. In **chapter 6**, I discovered that elafin, a human anti-protease, was decreased in patients with active CD and *in vitro*, it inhibited the deamidation of gliadin peptides, a key step in the pathogenesis of CD. I

showed that administration of elafin prevented gluten-induced barrier dysfunction and intraepithelial lymphocytosis. Together these results, (a) provide an in depth characterization of a humanized animal model for studying gluten-induced intestinal and extra-intestinal immune responses, (b) demonstrate the role of the microbiota as an environmental modulator of gluten-induced immune responses, (c) support the preclinical potential of two novel adjuvant therapies to the GFD. These findings emphasize the translational value of using relevant animal models to study the complex interactions between environmental and host factors that contribute to intestinal health and disease.

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LIST OF ABBREVIATIONS AND SYMBOLS

³ H	Tritium	Da	Dalton
⁵¹ Cr	Chromium	DAPI	4',6-diamidino-2-phenylindole
Ab	Antibody	DC	Dendritic cells
ACD	Active celiac disease	DGGE	Denaturing gradient gel electrophoresis
AGA	Anti-gliadin antibodies	DNA	Deoxyribonucleic acid
Ag	Antigen	DPBS	Dulbecco's phosphate buffered saline
AGU	Axenic gnotobiotic unit	E	Elafin
AJ	Adherens junction	EDTA	Ethylenediaminetetraacetic acid
AMP	Antimicrobial peptides	ELISA	Enzyme linked immunosorbent assay
ANOVA	Analysis of variance	F	Female
ASF	Altered Schaedler flora	FACS	Fluorescence activated cell sorting
ATB	Antibiotic	FBS	Fetal bovine serum
BLQ	Below limit of quantification	FCS	Fetal calf serum
BSA	Bovine serum albumin	FISH	Fluorescent <i>in situ</i> hybridization
C-section	Caesarean section	g	Gram
CBA	Cytometric bead assay	GALT	Gut-associated lymphoid tissue
CD	Celiac disease	GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
CFSE	Carboxyfluorescein succinimidyl ester	GFD	Gluten-free diet
CFU	Colony forming units	GFP	Green fluorescent protein
Ci	Curie	GRD	Gluten-related disorder
CK	Cytokeratin	h	Hour
cm	Centimetre	H&E	Hematoxylin and eosin
Con A	Concanavalin A	HCD4	Human CD4
Cpm	Counts per minute	HD	High dose
CT	Cholera toxin		
Ctrls	Controls		
d	Day		

HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid	LPS	Lipopolysaccharide
HLA	Human leukocyte antigen	M	Male
HRP	Horseradish peroxidase	M	Molarity, Mol/L
IBD	Inflammatory bowel disease	M cells	Microfold cells
IE-CTL	Intraepithelial cytotoxic T lymphocytes	mAb	Monoclonal antibody
IEC	Intestinal epithelial cells	MCP	Monocyte chemoattractant protein
IEL	Intraepithelial lymphocyte	MFI	Mean fluorescence intensity
IFN	Interferon	mg	Milligram
Ig	Immunoglobulin	MHC	Major histocompatibility complex
IgA	Immunoglobulin A	MICA/B	MHC class I-related chain A/B
IL	Interleukin	Min	Minute
ILC	Innate lymphoid cells	ml	Millilitre
ILF	Isolated lymphoid follicle	MLN	Mesenteric lymph nodes
IMDM	Iscove's Modified Dulbecco's Media	mM	Millimolar
iNKT cells	Invariant natural killer T cells	mol	Mole
i.p.	Intraperitoneal	mS	Millisiemens
Isc	Short-circuit current	N	Normality, Equivalent/L
JAM	Junctional adhesion molecules	NC	Non-celiac
kDa	Kilodalton	NCGS	Non-celiac gluten sensitivity
Kg	Kilogram	NK	Natural killer
L	Litre	NLR	NOD-like receptors
LAB	Lactic acid bacteria	nm	Nanometre
LC-MS	Liquid chromatography–mass spectrometry	NOD	Non-obese diabetic
LD	Low dose	NOR	Non-obese resistant
Ll	<i>Lactobacillus lactis</i>	NS	Nonsensitized
LOD	Limit of detection	OD	Optical density
		OTU	Operational taxonomic unit

OVA	Ovalbumin	SPF	Specific pathogen free
P(HEMA- <i>co</i> -SS)	Poly(hydroxyethyl methacrylate- <i>co</i> -styrene sulfonate)	<i>Spp</i>	Species, plural
PAGE	Polyacrylamide gel electrophoresis	T1D	Type 1 diabetes
PBMC	Peripheral blood mononuclear cells	TBS	Tris-buffered saline
PBS	Phosphate buffered saline	TCD	Treated celiac disease
PBS-T	Phosphate buffered saline-Tween 20	TCR	T-cell receptor
PCR	Polymerase chain reaction	TdT	Terminal deoxynucleotidyl transferase
pg	Picogram	TG-2	Tissue transglutaminase
PLN	Pancreatic lymph node	TGF	Transforming growth factor
PRR	Pattern recognition receptors	Th	T helper
PT	Pepsin-trypsin	TJ	Tight junction
QIIME	Quantitative Insights Into Microbial Ecology	TLR	Toll-like receptors
RA	Retinoic acid	TNF	Tumour necrosis factor
RBC	Red blood cell	Tr1	Regulatory type 1
RPMI	Roswell Park Memorial Institute medium	Tregs	Regulatory T cells
rRNA	Ribosomal ribonucleic acid	TSLP	Thymic stromal lymphopoietin
SCFA	Short chain fatty acids	Ttg	Tissue transglutaminase
SCID	Severe combined immunodeficiency	TUNEL	TdT dUTP nick-end labeling
SD	Standard deviation	V/C	Villus-to-crypt
SDS	Sodium dodecyl sulfate	Vol	Volume
SEM	Standard error of the mean	V	Volume
SFB	Segmented filamentous bacteria	wk	Week
SI	Stimulation index	WT	Wild-type
SIgA	Secretory IgA	wt	Weight
		Y	Year
		ZO	Zonula occludens
		°C	Degrees Celsius
		α	Alpha

β	Beta
γ	Gamma
δ	Delta
μ	Micro
μA	Microampere
μg	Microgram
μl	Microlitre
μm	Micrometre

CHAPTER 1

INTRODUCTION

1.1 Regulation of intestinal homeostasis

The gastrointestinal tract forms the body's largest interface with the external environment and is faced with numerous insults and luminal antigens, which include dietary antigens, bacteria, viruses, and fungi. There are a number of physiological and immunological adaptations that enable the gastrointestinal tract to maintain a state of physiological inflammation, in order to fight invading pathogens, but avoid unnecessary inflammation (Hooper and Macpherson, 2010). Maintaining a state of physiological inflammation, or homeostasis, is dependent on complex interactions between the intestinal epithelial barrier, the intestinal immune system, and the luminal antigens it is exposed to, from either dietary or microbial origin. Together, these interactions aid in digestion and the absorption of nutrients, while at the same time protecting the host from the massive intraluminal load of potentially harmful antigens (Menard et al., 2010).

1.1.1 Small intestinal barrier function

The small intestinal barrier is made up of a single layer of polarized intestinal epithelial cells (IECs) and provides a physical and chemical barrier between the external environment and the internal milieu. The epithelial barrier in the small intestine is organized into villi and crypts and is made up of four major cell types: absorptive enterocytes, mucus-producing goblet cells, antimicrobial-producing Paneth cells and hormone-producing enteroendocrine cells. These cells arise from pluripotent intestinal epithelial stem cells that reside in the base of the crypts. The small intestine is highly adapted to aid in digestion and nutrient and water absorption. Brush border enzymes

produced by intestinal epithelial cells, work together with pancreatic enzymes and bile to break down macromolecules, which can be absorbed by enterocytes. Intestinal epithelial cells are interconnected by tight and adherens junctional proteins which regulate paracellular permeability, epithelial cell polarity, and the entry of nutrients, ions and water (Groschwitz and Hogan, 2009; Menard et al., 2010).

The intestinal epithelium acts as a selective barrier which is mediated through two major pathways: the transcellular and paracellular pathways (Figure 1.1). The transcellular pathway is involved in the transport and absorption of nutrients including amino acids, small peptides, fatty acids, vitamins, short-chain fatty acids (SCFA) and sugars through epithelial cells (Suzuki, 2013). This pathway is generally regulated by selective transporters and channels found on the apical and basolateral surface of epithelial cells. Larger antigens (>600Da), such as dietary peptides, may also be transferred through enterocytes by endocytosis and released at the basolateral membrane where these antigens can interact with immune cells (Menard et al., 2010). This process is thought to be critical for the generation of oral tolerance (Pabst and Mowat, 2012). Larger antigenic molecules, such as microbial antigens, may also cross the epithelial barrier through specialized enterocytes called microfold (M) cells. M cells are located in the epithelium of Peyer's patches or isolated lymphoid follicles (ILF) and can transport antigens, including bacterial or dietary antigens to underlying immune cells (Pabst and Mowat, 2012). The uptake of luminal antigens through M cells and absorptive enterocytes therefore plays a key role in maintaining homeostasis in the gut.

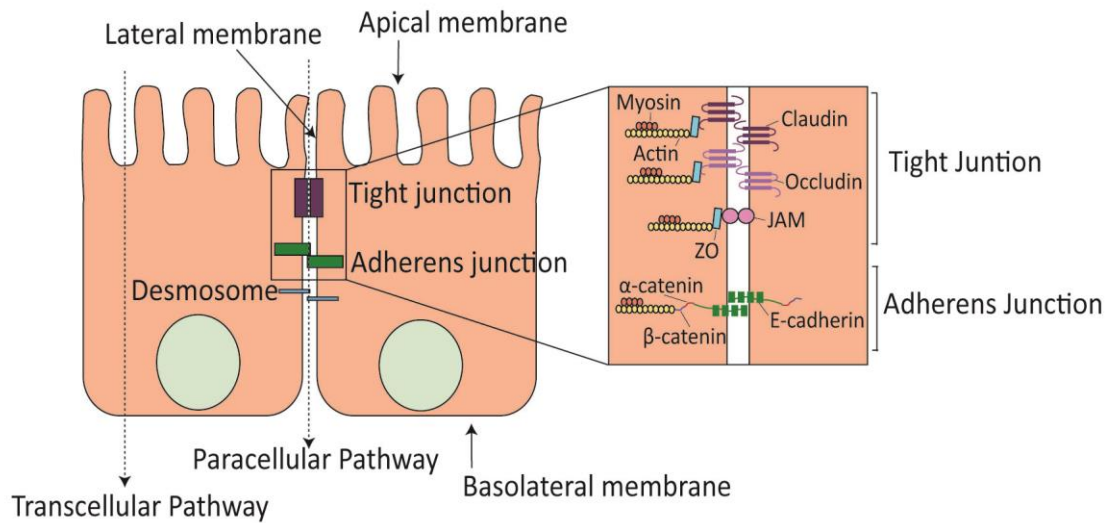


Figure 1.1 Pathways of intestinal permeability and overview of tight junction proteins. Intestinal permeability is controlled through two major pathways: paracellular and transcellular transport. Paracellular permeability is controlled by tight and apical junctional proteins.

The paracellular pathway is involved in the transport of small molecules, ions, and solutes between epithelial cells. Epithelial cells are connected and held together by apical junction complexes, which are composed of tight junctions, adherens junctions, and desmosomes. Tight junctions are found at the most apical surface of the lateral membrane and tightly regulate paracellular permeability by facilitating the movement of ions and solutes between epithelial cells, while blocking the translocation of larger antigens, toxins, and microbes. Tight junctions are dynamic structures made up of several protein complexes that include transmembrane proteins (occludin, claudins, junctional adhesion molecules or JAM) and scaffolding proteins (zonula occludens, ZO), which anchor the transmembrane proteins to the actin cytoskeleton (Figure 1.1). Tight junction structure and stability can be regulated by a number of intrinsic and extrinsic factors including

cytokines and growth factors, cellular stress, pathogens, probiotics, and peptides (Groschwitz and Hogan, 2009; Suzuki, 2013). Increased paracellular permeability and epithelial damage can lead to the excessive entry of dietary or microbial antigens, which may be a contributing or driving factor for inflammatory diseases.

Intestinal permeability can be measured by *in vivo* and *in vitro* techniques. *In vivo* methods are used in both humans and in experimental animals and usually involves measuring the urinary excretion of orally ingested inert probes such as sugars or ⁵¹Chromium (Cr)-EDTA. The lactulose: mannitol ratio is commonly used in clinical settings as an indication of small intestinal paracellular permeability. However, many factors such as intestinal transit time, intestinal surface area, renal function, and the probe itself can influence *in vivo* permeability measurements (Arrieta et al., 2006; Odenwald and Turner, 2013). *In vitro* techniques often involve the use of Ussing chambers to measure ion flux or the permeability to probes. Intestinal biopsies or segments of the intestine can be mounted in Ussing chambers and the electrical resistance (which reflects the passage of ions) and the movement of specific probes across the intestine can be measured. ⁵¹Cr-EDTA flux and electrical resistance are often used as an indicator of paracellular permeability *in vitro*, but may not reflect changes in permeability to larger dietary antigens (Menard et al., 2010; Perrier and Corthesy, 2011).

In addition to acting as a physical barrier, intestinal epithelial cells also participate in immune defense through the production of antimicrobial peptides (AMP) and mucins. Mucin glycoproteins, or mucus, are produced by specialized epithelial cells called goblet cells. In the large intestine, a dense inner mucus layer prevents luminal antigen contact

with epithelial cells (Johansson et al., 2008) and an outer mucus layer provides nutrients for mucosal-associated bacteria (Johansson et al., 2011b). On the other hand, the mucus in the small intestine is organized as a single layer, is loosely associated with the epithelium, and is permeable to bacteria (Johansson et al., 2011a). AMPs also function to limit contact between luminal antigens and the epithelial barrier or underlying immune cells. Although Paneth cells, located in small intestinal crypts, are the main producers of AMPs, including defensins, C-type lectins, cathelicidins and lysozyme, IECs and other innate cells are also capable of producing AMPs (Bevins and Salzman, 2011). Finally, dimeric immunoglobulin A (IgA) produced by plasma cells in the lamina propria, is transcytosed across the epithelial barrier by epithelial cells and released into the intestinal lumen as secretory IgA (sIgA) where it contributes to the separation of luminal antigens from the internal mucosa (Brandtzaeg and Prydz, 1984).

1.1.2 Intestinal immune system

In addition to the physical and chemical properties of the intestinal barrier, the underlying intestinal immune system, or the gut-associated lymphoid tissue (GALT), is critical for maintaining intestinal homeostasis. The intestinal immune system must be able to mount an appropriate immune response against invading pathogens, while maintaining tolerance to innocuous luminal antigens, including dietary antigens and commensal bacteria.

The GALT can be divided into sites of induction and effector sites (Figure 1.2). Antigen-specific immune responses are generated at sites of induction, which include

mesenteric lymph nodes (MLNs), Peyer's patches, and ILF. Peyer's patches are found scattered in the submucosa of the small intestine and ILF are found in the lamina propria of the small and large intestine. The lamina propria and epithelium make up the effector sites and harbour large populations of lymphoid cells (Pabst and Mowat, 2012).

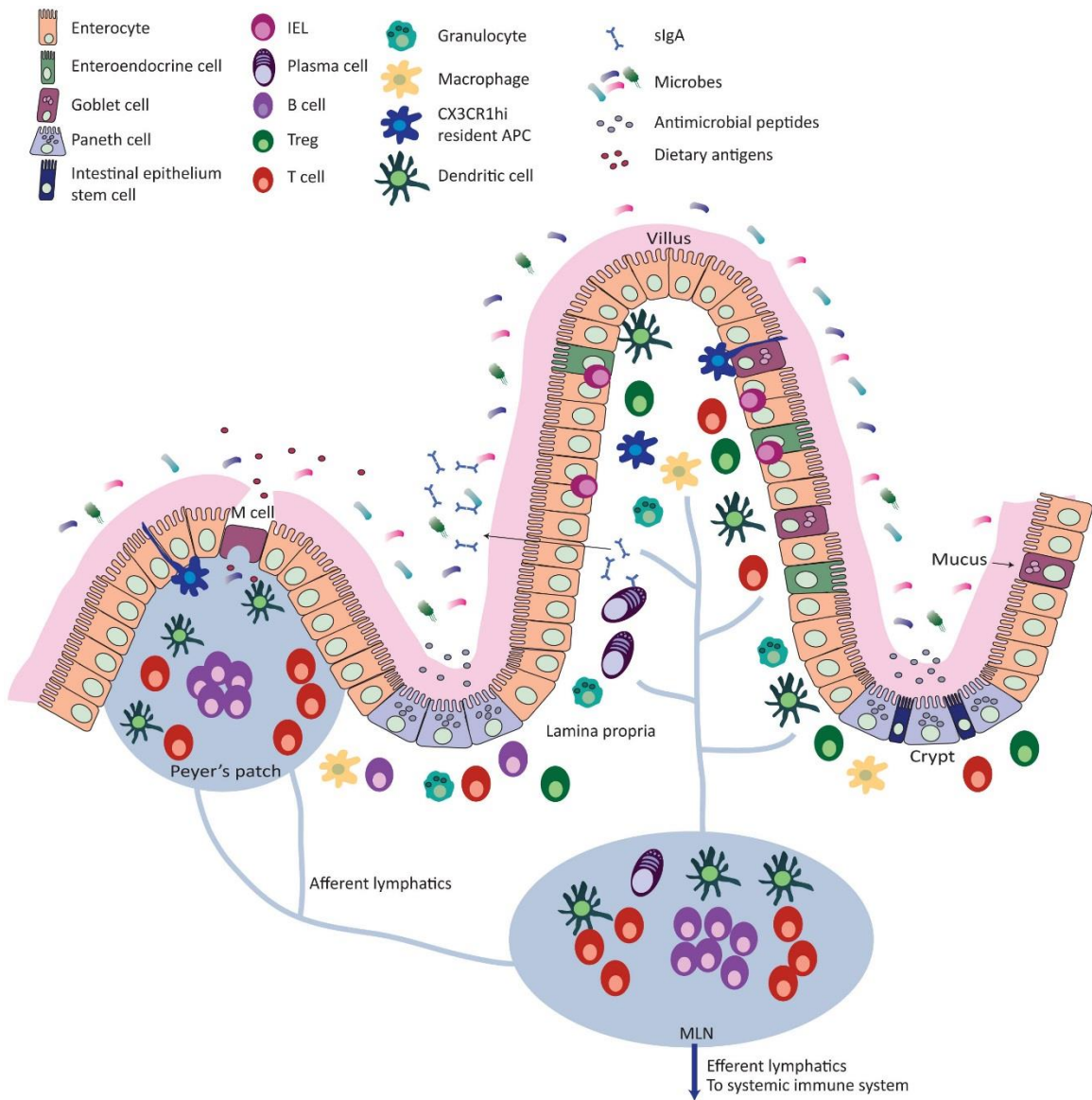


Figure 1.2 Overview of the mucosal immune system. The intestinal immune system is comprised of inductive sites (Peyer's patches, mesenteric lymph nodes (MLN)) where antigen-specific immune responses are induced, and effector sites (lamina propria, epithelium) that harbour diverse populations of innate and adaptive immune cells. The Peyer's patches and lamina propria are drained by afferent lymphatics to the MLN, which connects to the systemic immune system via efferent lymphatics.

The epithelium overlaying Peyer's patches and ILF contain specialized M cells, which allows for the uptake of antigens and continuous sampling of luminal contents (Mabbott et al., 2013). Antigens that have gained access through M cells are taken up by underlying antigen presenting cells (APCs), such as dendritic cells (DCs), which mature, migrate to T cell zones of the PP and present antigens to naive CD4⁺ T cells. This results in the activation of antigen-specific T helper (Th) cells. Antigens can also be taken up by lamina propria DC-like cells that extend dendrites between epithelial cells to sample luminal antigens, or by epithelial cells (Niess et al., 2005). Migratory DCs then travel to draining MLNs where antigens are presented to CD4⁺ T cells. Interactions between CD4⁺ T cells and B cells in the Peyer's patches and MLN also results in the activation and expansion of B cells to become antibody-producing plasma cells. The local cytokine environment plays a key role in determining the nature of immune responses. Following activation, CD4⁺ T cells and B cells home back to the lamina propria as effector cells or antibody-producing plasma cells.

In addition to effector T cells and antibody-producing plasma cells, the intestinal lamina propria and epithelium contain a number of innate immune cells, granulocytes and various subsets of phagocytes. Tissue resident and migratory DCs and macrophages are

found throughout the lamina propria and play important roles in priming the adaptive immune response, but also function as innate effector cells.

Intraepithelial lymphocytes (IELs) are a heterogeneous population of specialized effector T cells that reside within the epithelial barrier. IELs provide one of the first lines of defence for invading pathogens but are also involved in maintaining barrier function due to their direct contact with IECs and close proximity to luminal antigens (Cheroutre et al., 2011). In contrast to conventional peripheral T cells, IELs have an antigen-experienced phenotype and a more limited oligoclonal T-cell receptor (TCR) and exist in a state of heightened activation. This allows them to respond quickly to stress signals, thus representing an innate-like population of T cells (Abadie et al., 2012). IELs express either the TCR $\alpha\beta$ or TCR $\gamma\delta$ and can be further categorized into type a (conventional) or type b (unconventional) subtypes in mice (Table 1.1). Type a IELs are absent at birth and arise from conventional thymus-derived TCR $\alpha\beta$ ⁺ CD4⁺ or CD8 $\alpha\beta$ ⁺ T cells. They are activated in the periphery in response to non-self antigens and migrate to the intestinal epithelium (Umesaki et al., 1993). In the small intestine, the majority of type a IELs bear the CD8 $\alpha\beta$ co-receptor (Abadie et al., 2012). Type b IELs express either the TCR $\alpha\beta$ or TCR $\gamma\delta$, but lack CD4 or CD8 $\alpha\beta$ co-receptors. The majority of type b IELs also express the CD8 $\alpha\alpha$ homodimer. Unlike type a IELs, type b IELs are present at birth and are thought to acquire their activated phenotype in the thymus in response to self-antigens (Cheroutre et al., 2011; Pérez-Cano et al., 2005), after which they migrate directly to the intestinal epithelium. In mice, the majority of type b IELs express the TCR $\gamma\delta$ (Goodman and Lefrançois, 1988; Guy-Grand et al., 1991).

Table 1.1 Small intestinal IEL subsets in mice

<i>IEL subset</i>	<i>TCR phenotype</i>	<i>Frequency</i>	<i>Development</i>	<i>Function</i>
<i>Type a (Conventional, induced)</i>	TCR $\alpha\beta$ +CD4+ CD8 $\alpha\alpha$ + CD8 $\alpha\alpha$ -	< 15%	Absent at birth, arise from conventional CD4+ or CD8 $\alpha\beta$ + TCR $\alpha\beta$ + in the thymus, acquire activated phenotype in response to non-self antigens in the periphery, migrate to the intestinal intestine	Protection against infection, protect mucosal barrier function, regulatory, maintaining oral tolerance, conversion to cytotoxic cells,
	TCR $\alpha\beta$ +CD8 $\alpha\beta$ + CD8 $\alpha\alpha$ + CD8 $\alpha\alpha$ -	20-30%		Cytolytic activity, effector pro-inflammatory T cells, protection against infection
<i>Type b (Unconventional, natural)</i>	TCR $\alpha\beta$ + CD8 $\alpha\alpha$ + CD4-CD8-	20-50%	Present at birth, arise from CD4-CD8- T cells in the thymus, acquire activated phenotype in response to self-antigens in the thymus, migrate directly to the intestinal epithelium	Cytolytic activity, immune regulation, prevent T-cell induced inflammation
	TCR $\gamma\delta$ + CD8 $\alpha\alpha$ + CD4-CD8-	40-70%		Anti-inflammatory, immune regulation, anti-microbial, epithelial growth and repair, IgA class switching, maintaining oral tolerance, cytolytic activity

IEL, intraepithelial lymphocytes; TCR, T cell receptor

Both type a and type b IELs have cytolytic functions and are capable of producing pro-inflammatory cytokines in response to invading pathogens. However, IELs also have regulatory functions that promote barrier function and epithelial repair after injury. TCR $\gamma\delta$ + IELs play a primary role in maintaining epithelial barrier integrity following intestinal injury through the secretion of growth factors and anti-microbial peptides

(Boismenu and Havran, 1994; Chen et al., 2002; Ismail et al., 2009). In response to signals from IECs, TCR $\gamma\delta$ ⁺ IELs also produce anti-inflammatory cytokines interleukin (IL)-10 and transforming growth factor (TGF)- β and may control cytotoxic IEL responses (Bhagat et al., 2008). Finally, TCR $\gamma\delta$ ⁺ IELs also have cytotoxic and pro-inflammatory properties that provide protection against infections (Dalton et al., 2006; Lefrancois and Goodman, 1989; Simpson et al., 1997). Although type b TCR $\alpha\beta$ ⁺ IELs express self-reactive TCRs, they are not self-destructive under normal conditions and are thought to play an immunomodulatory role by inhibiting unnecessary inflammation (Abadie et al., 2012). Type a TCR $\alpha\beta$ ⁺CD8 $\alpha\beta$ ⁺ IELs, or intraepithelial cytotoxic T cells (IE-CTLs), are critical for protection against invading pathogens through cytolytic activity against infected cells and the production of pro-inflammatory cytokines (Chardes et al., 1994; Roberts et al., 1996). However, in steady-state conditions, these IELs display low cytolytic activity, express natural killer (NK) inhibitory receptors (NKG2A), and are quiescent in order to prevent unnecessary inflammation. On the other hand, inflammatory signals such as IL-15, can activate potent cytotoxic responses in IE-CTLs which can result in excessive inflammation (Meresse et al., 2004).

Several important differences in IEL composition, phenotype, and frequency occur between mice and humans, as well as between the colon and the small intestine. Compared to the small intestine, IELs are present in smaller numbers in the colon and are dominated by TCR $\alpha\beta$ ⁺CD4⁺ IELs (Beagley et al., 1995). Although similar numbers of IELs can be found in mice (10 IELs per 100 IECs) and humans (10-20 IELs per 100 IECs), there are several differences in the phenotype and frequency of IELs that reside in

the small intestine under homeostatic conditions (Abadie et al., 2012) (Table 1.2). The population of TCR $\gamma\delta$ + IELs in humans is much smaller (~10%) compared to what is observed in mice (~60%). Furthermore, mice harbour a large population of TCR $\alpha\beta$ +CD8 $\alpha\alpha$ + IELs that have not yet been identified in humans, and humans harbour a small population of CD3⁻IELs that have not been identified in mice. Although there are differences in IEL phenotypes between mice and humans, it is believed that human and murine IELs express oligoclonal TCRs that are distinct from peripheral T cells (Abadie et al., 2012).

Table 1.2 Small intestinal subsets in humans

<i>TCR phenotype</i>	<i>Frequency</i>
<i>TCRα+CD4+</i>	10-15%
<i>TCR$\alpha\beta$+CD8$\alpha\beta$+</i>	>70%
<i>TCR$\gamma\delta$+ CD8+ CD4-CD8-</i>	5-20%
<i>CD3-CD103+</i>	<10%

TCR, T cell receptor

1.1.3. Protease and anti-protease balance

Proteases control cellular processes through the cleavage of protein substrates and are produced and secreted by bacteria in the intestinal lumen, by IECs at the brush border membrane, and by immune cells in the intestinal lamina propria. These proteases play a critical role in maintaining barrier function and intestinal homeostasis by controlling tissue remodeling and regulating intestinal permeability (Giuffrida et al., 2014; Heutinck et al., 2010). Proteases can be classified into four classes: metalloproteinases, serine

proteases, cysteine proteases, and aspartic proteases (Biancheri et al., 2013). Serine proteases are the biggest family of proteases and include trypsin and chymotrypsin, as well the neutrophil produced cathepsin-G, neutrophil elastase, and proteinase-3.

Under steady-state conditions, certain proteases are constitutively expressed at low levels, helping to maintain barrier function. On the other hand, proteolytic imbalances are a feature of inflammation and can exacerbate inflammation (Giuffrida et al., 2014). Maintaining a proteolytic balance in the gut is therefore critical for homeostasis and the production of protease inhibitors plays an important role in maintaining this proteolytic balance. Elafin is a serine protease inhibitor that has potent inhibitory capacity against neutrophil elastase and proteinase-3. Elafin is constitutively produced by epithelial cells at mucosal surfaces throughout the human body and is rapidly induced to high levels during inflammation (Shaw and Wiedow, 2011). In addition to its function as a protease inhibitor, elafin has been shown to have anti-microbial properties (Verrier et al., 2012). More details regarding the function of elafin will be described in **chapter 6** of this thesis.

1.1.4 Intestinal microbiota and dietary antigens

1.1.4.1 The intestinal microbiota

At birth, we are colonized with a complex community of microbes that reaches up to a density of 10^{12} bacterial cells per gram of content in the adult colon. These microbes live in a symbiotic relationship with the host and play a major role in health and disease by influencing nutrient absorption, epithelial barrier function, and immune development (Collado et al., 2012). The composition and density of the microbiota varies with age as

well as along the length of the intestine (Brown et al., 2013; Clemente et al., 2012) (Figure 1.3). The intestinal microbiota fluctuates over the first two to three years of life, with high interindividual variability, but becomes more stable and converges to resemble that of an adult microbiota by two to three years of age (Koenig et al., 2011; Yatsunenko et al., 2012). Even still, general trends can be observed in early colonization of the human gastrointestinal tract. The neonatal microbiota has low diversity, but gradually increases with time, particularly with the introduction of solid food (Koenig et al., 2011; Palmer et al., 2007). Immediately following birth, the neonatal intestine is colonized by facultative anaerobes and is dominated by enterobacteria, *Staphylococcus*, and *Streptococcus*. The depletion of oxygen then allows colonization by strict anaerobes that include *Bifidobacterium*, *Clostridium*, and *Bacteroides* (Matamoros et al., 2013). Following weaning, the microbiota continues to mature, and the diversification of *Bacteroides* and *Clostridium* rapidly increases, while the proportion of bifidobacteria stabilizes (Collado et al., 2012). By two years of age, the microbiota is dominated by members belonging to the Firmicutes and Bacteroidetes phyla and resembles that of an adult-like microbiota (Clemente et al., 2012).

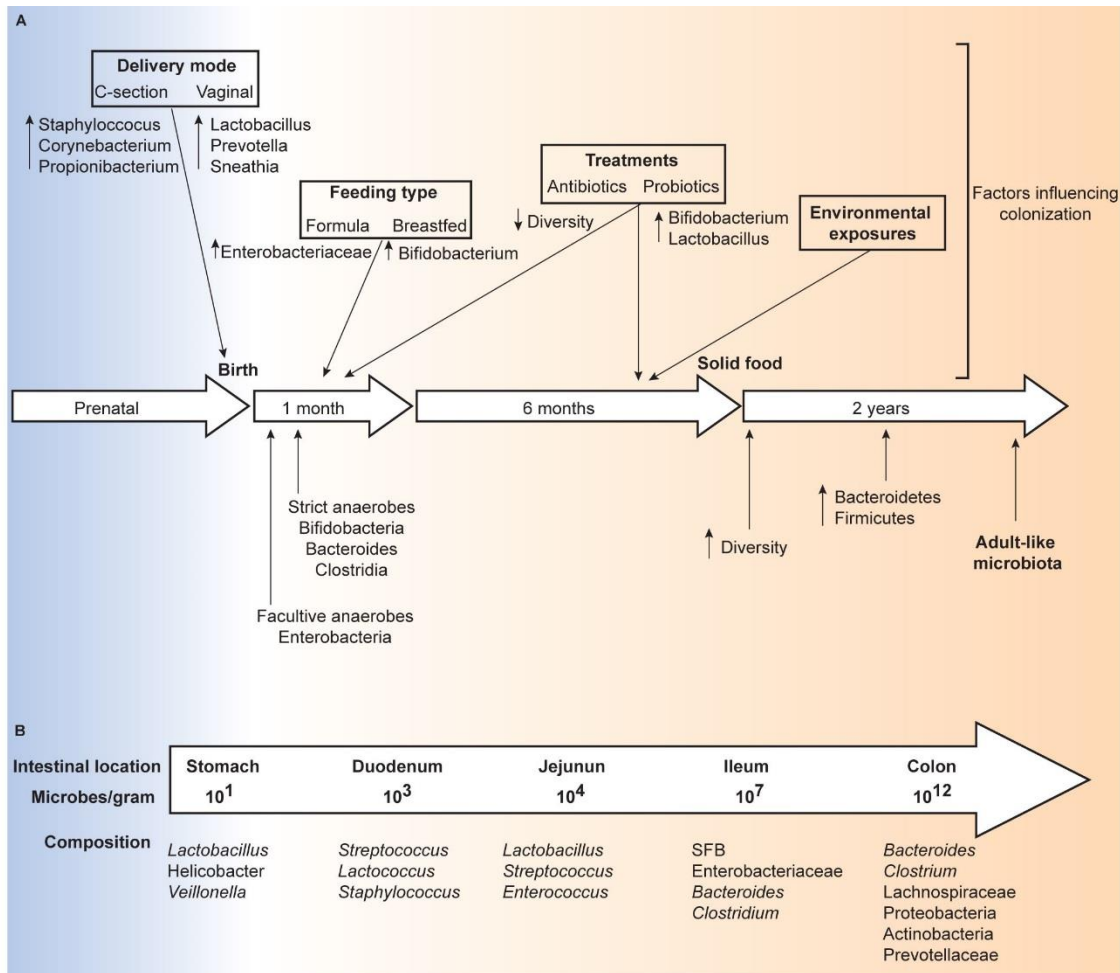


Figure 1.3 Development of the intestinal microbiota. A) Factors influencing microbial colonization over time. B) Composition and density of the microbiota along the gastrointestinal tract.

The adult microbiota contains more than 10^{14} microorganisms, about 10 times as many cells as the human body. Although the adult microbiota has high interindividual variability at the species level, it is highly conserved at higher-taxa levels (Cho and Blaser, 2012). The adult microbiota is dominated by members of the Firmicutes and Bacteroidetes phyla, with members from Proteobacteria, Actinobacteria, Fusobacteria and Verrucomicrobia accounting for less than 10% of the total population (Hooper and

Macpherson, 2010). Differences in microbial composition and density are observed along the gastrointestinal tract (Figure 1.3B), with much lower densities in the proximal intestine due to harsher conditions. The densities in the proximal small intestine reach 10^3 cells per gram of content and densities in the distal colon reach 10^{12} per gram of content (Brown et al., 2013; Sommer and Bäckhed, 2013). While composition of the large intestine has been well studied, less is known about the small intestinal microbial composition due to more restricted and difficult sampling. However, studies have demonstrated that the microbiota in the small intestine is less stable than that of the colon and is dominated by facultative anaerobes including *Streptococcus* (Hollister et al., 2014; Zoetendal et al., 2012). A number of factors can influence microbial colonization early in life, which include birth delivery mode, neonatal antibiotic or probiotic use, and feeding patterns or diet (Collado et al., 2012) (Figure 1.3A). While changes in the intestinal microbiota (dysbiosis) and factors that influence early microbial colonization have been associated with inflammatory diseases, the long-term effects of these factors on the microbial composition have not been well characterized.

1.1.4.2 Dietary antigens

Following ingestion, food is systematically broken down by mechanical and enzymatic mechanisms throughout the gastrointestinal tract which allows the absorption of nutrients. Partially digested food from the stomach is emptied into the small intestine where the chemical breakdown of lipids, starches, and proteins continues. The majority of food proteins are broken down to amino acids, dipeptides, or tripeptides by digestive enzymes including trypsin, chymotrypsin, elastase, and carboxypeptidase that can be

efficiently absorbed by enterocytes (Bornhorst and Paul Singh, 2014). However, some food components and proteins, such as cellulose, β -lactoglobulin (Reddy et al., 1988), and gliadin (Hausch et al., 2002), are resistant to enzymatic hydrolysis in the stomach and small intestine. Non-digestible carbohydrates pass through the small intestine until they reach the colon where they are fermented by bacteria to produce end-products such as SCFA, which promote gut health (Tremaroli and Bäckhed, 2012). Incomplete protein digestion results in large, potentially immunogenic peptides or proteins that can reach the underlying subepithelial tissues (Menard et al., 2010). Normally, antigens in the diet induce a systemic non-responsiveness called oral tolerance. In addition to providing hosts with essential vitamins and minerals, the diet can influence microbial composition and host immunity and these interactions play an important role in health and disease.

1.1.4.3 Microbial and dietary factors influence host physiology and immunity.

Studies using germ-free and gnotobiotic mice have demonstrated the importance of the microbiota on the development of host physiology and a mature immune system (Figure 1.4). Germ-free mice have an enlarged cecum, longer and thinner villi in the small intestine, and shallow small intestinal crypts with few proliferating stem cells (Sommer and Bäckhed, 2013). Colonization results in widening and shortening of the villi and increases enterocyte proliferation. A number of innate and adaptive immune adaptations also occur following colonization (Kabat et al., 2014). The development of the GALT is impaired in germ-free mice, resulting in underdeveloped and immature MLNs and Peyer's patches in germ-free mice (Sommer and Bäckhed, 2013). Germ-free animals also have a thinner mucus layer and fewer goblet cells (Sommer and Bäckhed, 2013). The

production of certain AMP, including RegIII γ , is also dependent upon the microbiota (El Aidy et al., 2012; Natividad et al., 2013). The microbiota also modulates the differentiation of immune cell subsets. Germ-free mice have reduced intestinal CD4⁺ T cells, reduced small intestinal Th17 cells (Ivanov et al., 2008), reduced colonic regulatory T cells (Tregs) (Geuking et al., 2011), and reduced TCR $\alpha\beta$ +IELs (Helgeland et al., 1996; Imaoka et al., 1996). Importantly, these differences are restored following colonization. The differentiation of B cells to IgA-producing plasma is also dependent on the microbiota (Hapfelmeier et al., 2010; Uematsu et al., 2008).

Accumulating evidence suggests that particular members of the microbiota can differentially modulate the development or function of intestinal immune cells (Figure 1.4). Segmented filamentous bacteria (SFB) are potent inducers of Th17 cells (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009). Other microbes, such as the altered Schaedler flora (ASF), a cocktail of eight commensals, were also shown to induce Th17 cells, but to a lesser extent than SFB (Geuking et al., 2011). On the other hand, *Clostridia*, *Bacteroides fragilis* (*B. fragilis*) and ASF were shown to induce colonic Tregs (Atarashi et al., 2013; Atarashi et al., 2011; Geuking et al., 2011; Round and Mazmanian, 2010). Microbial products, such as lipopolysaccharide (LPS) or SCFA, may also stimulate colonic Tregs (Arpaia et al., 2013; Hrnčir et al., 2008). Induction of immune responses may therefore be influenced by not only the presence or absence of live bacteria, but also on the relative abundance of particular members of the microbiota and their by-products.

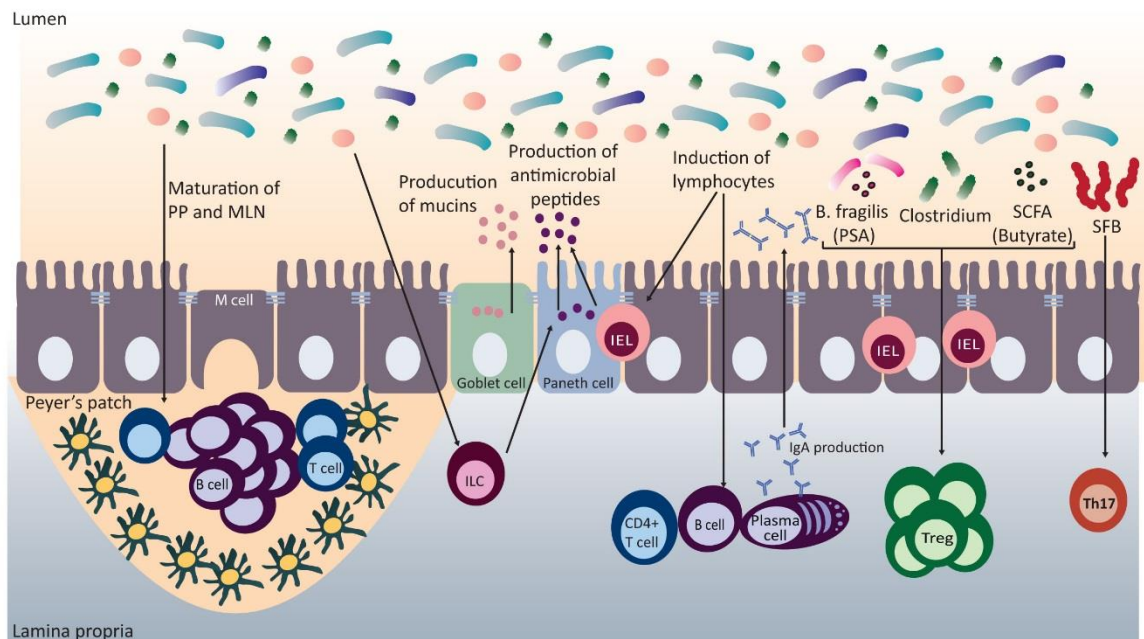


Figure 1.4 Influence of microbiota on immune responses. The microbiota induces maturation of the gastrointestinal lymphoid tissue (Peyer’s patches, MLN). Signals from the microbiota induce production of the antimicrobial peptide RegIII γ from paneth cells and intraepithelial lymphocytes (IELs), and the production of mucin from goblet cells. Microbes also influence the development of T cell subsets and are critical for the induction of IgA-producing plasma cells.

Dietary factors also directly or indirectly contribute to the development of host immunity and physiology. For example, vitamin A, which is contained in the diet, is converted in the body to retinoic acid (RA) by enzymes produced by intestinal DCs. RA promotes the upregulation of gut homing markers on T and B cells (Iwata et al., 2004), promotes the induction of Tregs (Coombes et al., 2007; Sun et al., 2007), and influences Th17 cell differentiation (Mucida et al., 2007). Vitamin B9, or folic acid is an essential nutrient that can be obtained through the diet, or generated by members of the intestinal microbiota (Pompei et al., 2007) and has been shown to promote Treg maintenance

(Kinoshita et al., 2012). Vitamin D and its metabolites can also modulate CD4+ T cell differentiation, DC maturation, and monocyte function (Borges et al., 2011). Macronutrients also contribute to the maintenance of T cell responses; glucose and amino acids provide energy for effector T cells (Kau et al., 2011). In addition to direct effects on immune function, dietary factors may indirectly influence host immune responses and physiology through effects on the microbiota. Diet can affect the microbial composition, with significant microbial differences observed between those consuming a ‘Western’ diet high in fat and sugar compared to those consuming a plant-based diet rich in fibre (De Filippo et al., 2010; Turnbaugh et al., 2009). SCFAs are the end-products of fibre metabolism by bacteria in the gastrointestinal tract, and can influence immune and barrier function. The concentration of SCFA in the lumen can be modified by the amount of fibre in the diet, which in turn can effect host immune function and barrier function (Kau et al., 2011).

1.1.5 Mechanisms to maintain homeostasis: Mucosally-induced tolerance

Homeostasis is the tendency of a physiological system to maintain a stable equilibrium by adjusting its physiological processes. The gastrointestinal tract of mammals is exposed to a vast amount of foreign material, including microbes and dietary antigens, which normally do not elicit inflammatory immune responses. The intestinal microbiota and the host have co-evolved for millions of years and exist in a mutualistic relationship. In addition to remaining tolerant to the huge microbial and dietary load in the intestinal lumen, the host must mount an appropriate response to invading pathogens. Complex interactions between the microbiota, dietary factors and the host help to

maintain a state of equilibrium, or homeostasis in the gut. This state of physiological inflammation is critical for determining health and disease. A number of innate and adaptive immune adaptations exist to maintain homeostasis within the gut (Figure 1.5).

The mucus layer, AMPs, sIgA, and the layer of epithelial cells all function to confine the luminal antigens to the intestinal lumen and limit contact with ECs. Maintaining intestinal barrier function is critical for maintaining intestinal homeostasis. Additional mechanisms ensure that inappropriate pro-inflammatory immune responses are not generated against commensals but pathogens that penetrate the epithelial barrier are rapidly killed and are not exposed to the systemic immune system (Hooper and Macpherson, 2010). Phagocytes in the lamina propria can rapidly take up and kill invading microbes, but can do so without inducing an inflammatory response (Kamada and Núñez, 2014). Antigen-specific T cells and B cells generated in the GALT also contribute to maintaining homeostasis in the gut. In steady-state conditions, the production of RA, thymic stromal lymphopoietin (TSLP), TGF- β , and IL-10 from IEC and tolerogenic DCs promotes the generation of Tregs and IgA-producing plasma cells (Coombes et al., 2007; Gutzeit et al., 2014; Sun et al., 2007). CD4⁺CD25⁺FOXP3⁺ Tregs and CD4⁺FOXP3⁻ regulatory type 1 T (Tr1) cells produce IL-10 under steady-state conditions, which is critical for maintaining homeostasis through the suppression of inflammatory responses (Kamada and Núñez, 2013). Tregs also play a critical role in the induction of IgA-producing plasma cells (Gutzeit et al., 2014). Non-specific sIgA and antigen-specific sIgA function to trap luminal antigens in the mucus, and prevent adhesion of bacteria to IECs without activating an inflammatory response (Brown et al.,

2013). The production of RA from DCs in the Peyer's patches or MLN results in the upregulation of gut-homing markers on activated T and B cells, allowing them to home back to the intestinal lamina propria (Iwata et al., 2004; Jaensson et al., 2008). Finally, the MLNs act as a firewall to prevent systemic exposure to microbes, confining the induction of any immune response to the mucosal immune system and maintaining systemic ignorance of commensal bacteria (Macpherson and Uhr, 2004).

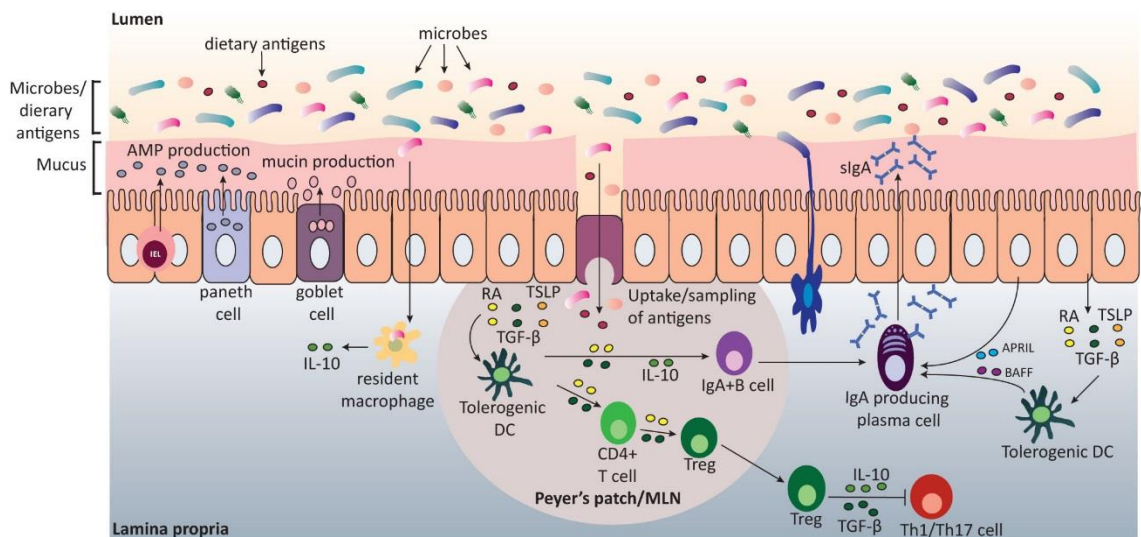


Figure 1.5 Mechanisms to maintain intestinal homeostasis. The production of antimicrobial peptides (AMPs), mucin, and sIgA promote bacterial exclusion from the intestinal epithelium. Resident macrophages are able to take up and kill invading microbes and produce anti-inflammatory cytokines. Antigens are sampled or taken up by M cells overlying Peyer's patches or dendritic cells (DCs) in the lamina propria, which migrate to Peyer's patches or mesenteric lymph nodes (MLNs). The local cytokine environment in lymphoid tissues and lamina propria (retinoid acid; RA), TGF- β , IL-10, TSLP) promote tolerogenic DCs, IgA producing plasma cells, and the generation and maintenance of regulatory T cells (Treg).

Mechanisms also exist to maintain tolerance to ingested food proteins, in a process referred to as oral tolerance. Oral tolerance is the mucosal and systemic unresponsiveness that is induced following the oral administration of an innocuous antigen, such as food protein. Unlike tolerance to commensal bacteria, tolerance to food protein generated in the small intestine involves both local and systemic immune responses (Pabst and Mowat, 2012). Food antigens are sampled by DCs in the small intestine, which then migrate to the MLNs; a process critical for the generation of oral tolerance to food proteins (Worbs et al., 2006). The local environment in the gut promotes tolerogenic responses and the induction of Tregs in the MLNs. After homing back to the small intestinal lamina propria, Tregs suppress inappropriate inflammatory responses to dietary antigens (Hadis et al., 2011). Less is understood regarding how these local mucosal immune responses suppress responses to systemic antigen exposure. These highly adapted physiological and immune responses are critical for maintaining tolerance to commensals and food antigens; disruptions in any of these pathways may have detrimental effects on the host.

1.2 Celiac disease as a model to understand the breakdown of intestinal homeostasis

Maintaining intestinal homeostasis requires interactions between the microbiota, dietary factors, and the host; a breakdown in any of these pathways may disrupt the balance between homeostasis and disease, and promote chronic inflammation (Figure 1.6). Host factors (genetics) and environmental factors (drug use, infections, or diet), which influence immune responses, barrier function, and the intestinal microbiota, may contribute to a breakdown in intestinal homeostasis. However, the overall contribution of

these factors to the disruption of homeostasis and disease development is not understood. For example, alterations in microbial composition, referred to as dysbiosis, may influence the balance between homeostasis and inflammation, and increase susceptibility to inflammatory diseases including inflammatory bowel disease (IBD), obesity, and celiac disease (CD) (Maloy and Powrie, 2011). As such, dysbiosis has been described in many chronic inflammatory diseases. However, the causative role of dysbiosis has been difficult to prove.

A disruption in intestinal homeostasis may also lead to a breakdown in tolerance to normally innocuous luminal antigens (Figure 1.6). A breakdown in immune tolerance to food antigens is referred to as a food sensitivity, which differs from non-immune mediated food intolerances (i.e. lactose intolerance). Sensitivities to food antigens include gluten sensitivity and peanut allergy. One of the best characterized examples of a food sensitivity is CD which is an immune mediated condition triggered by the ingestion of dietary gluten. Unlike many other chronic inflammatory diseases, the trigger (gluten) and the genetics of CD are well-defined. This makes it an ideal disease to the mechanisms leading to the breakdown in tolerance towards innocuous antigens, which is critical for developing preventative and therapeutic strategies for these diseases.

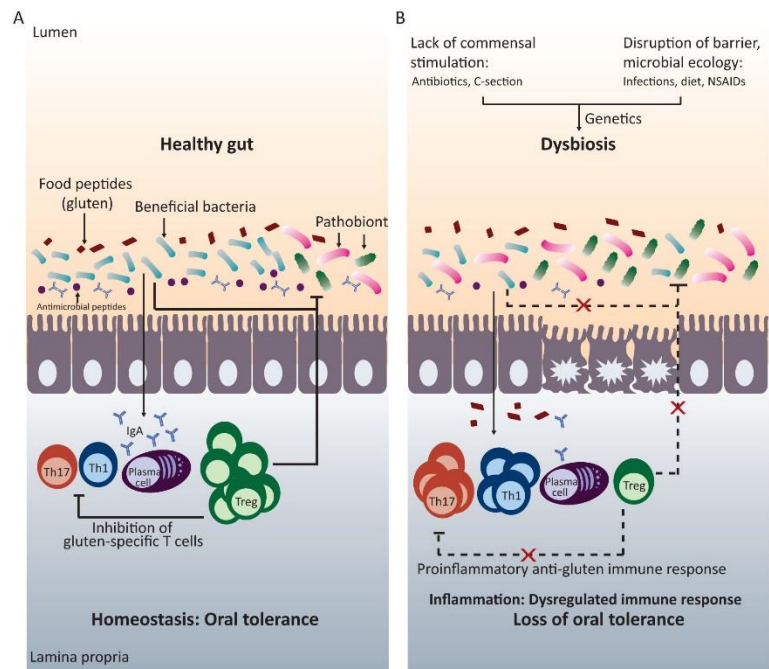


Figure 1.6 Microbiota and immune homeostasis in oral tolerance. A) In the healthy gut there is a balance between beneficial and potentially harmful bacteria (pathobionts) resulting in a balance between the pro-inflammatory Th1 and Th17 cells and regulatory T cells, allowing the generation of oral tolerance to food proteins such as gluten. B) A disruption of early microbial colonization (C-section, neonatal antibiotic use) or a disruption of barrier function or microbial ecology (changes in diet, infections, or drugs) can result in intestinal dysbiosis. This can lead to imbalances between pro-inflammatory and regulatory immune cells. In genetically susceptible individuals, immune imbalances may promote loss of tolerance to food proteins, such as gluten. Figure adapted from (Galipeau and Verdu, 2014)

1.2.1 Celiac disease: An overview

Celiac disease (CD) is an autoimmune enteropathy triggered by the ingestion of gluten in genetically susceptible individuals. The prevalence of CD has risen rapidly over the past 50 years, and currently affects about 1% of the European and North American population (Fasano et al., 2003; Green and Cellier, 2007; Mustalahti et al., 2010; Rubio-

Tapia et al., 2009). CD belongs to a spectrum of gluten-related disorders (GRD), which also includes non-celiac gluten sensitivity (NCGS), dermatitis herpetiformis, and gluten ataxia. There is also a spectrum of clinical presentation within CD. Classical, or typical CD symptoms include small intestinal villus atrophy presenting with malabsorption, diarrhea, and weight loss (Ludvigsson et al., 2012). However, non-classical, or atypical symptoms are being increasingly recognized (Green, 2005). These include chronic fatigue, joint pain, migraines, infertility, neurological symptoms, and anemia. The onset of symptoms can occur at any time throughout life; however, some present with no symptoms and are classified as asymptomatic or silent. Finally, potential CD is characterized by normal small intestinal mucosa, but positive serology, resulting in an increased risk of developing CD. NCGS is characterized by gluten-dependent intestinal or extra-intestinal symptoms in the absence of CD (positive serology and villus atrophy). Although an attempt to define all the terms related to CD and GRD was recently made (Ludvigsson et al., 2012), inconsistencies within the literature remain, causing confusion and making it difficult to distinguish between disorders and clinical presentations of the diseases. The use of gluten sensitivity in this thesis denotes a loss of oral tolerance to gluten, and should not be confused with NCGS. In addition to the variable clinical presentation, CD is associated with a number of autoimmune diseases, including type 1 diabetes (T1D), autoimmune thyroiditis, and autoimmune hepatitis (Cohn et al., 2014; Di Sabatino et al., 2013).

1.2.2 Celiac disease pathogenesis

The pathogenesis of CD involves an interplay between environmental, genetic, and immune factors (Figure 1.7). The major susceptibility genes in CD are the major histocompatibility complex (MHC) class II molecules, human leukocyte antigen (HLA)-DQ2 and HLA-DQ8, which are necessary, but not sufficient to cause disease. The DQ genes account for approximately 40% of the genetic risk. Other genetic factors, including non-HLA genes that are involved in barrier and immune function, also contribute to disease susceptibility but account for a small proportion of the overall risk (Gujral et al., 2012; Kupfer and Jabri, 2012)

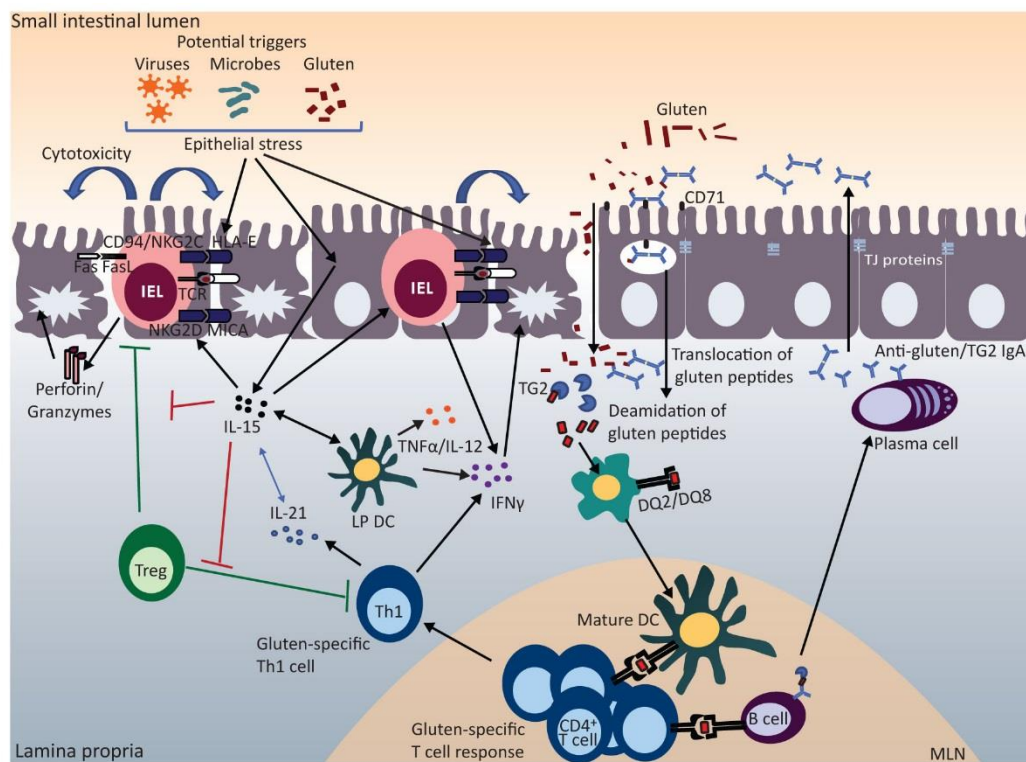


Figure 1.7 Overview of celiac disease (CD) pathogenesis. Gluten peptides in the small intestinal lumen translocate the epithelial barrier, and induce a pro-inflammatory immune response in a genetically predisposed host. Activation of the innate immune response is a key initial step in CD. Increased epithelial cell stress may be triggered by gluten peptides, bacteria, or viruses, which can upregulate stress molecules on epithelial cells (HLA-E, MICA/B) and induce IL-15 production from epithelial cells. IL-15 can induce IEL proliferation and activation and cytotoxic killing of ECs leading to tissue damage. IL-15 can also inhibit the regulatory effects of Tregs. Figure adapted from (Galipeau and Verdu, 2014)

CD is triggered by the ingestion of gluten from wheat, and related prolamins found in barley (hordeins) and rye (secalins). Gluten is made up of gliadins and glutenins, both of which are capable of triggering CD. Several properties of gluten may explain their toxicity for patients with CD. Gluten proteins have a high concentration of proline and glutamine residues, which renders them resistant to enzymatic degradation by digestive enzymes, leaving large, potentially immunogenic peptides that may reach the underlying mucosa (Hausch et al., 2002; Shan et al., 2002). One of these peptides is the well-studied 33-mer, which is highly immunogenic in a large proportion of CD patients (Camarca et al., 2009). Once gluten peptides have crossed the epithelial barrier by paracellular or transcellular mechanisms, the high proline and glutamine content renders them excellent substrates for tissue transglutaminase (TG-2) (Vader et al., 2002). TG-2 is usually contained intracellularly in an inactive form. However, during inflammation, TG-2 is released and becomes active through mechanisms that are not clearly understood (Siegel et al., 2008). Once activated, TG-2 deamidates gluten peptides which converts glutamine to negatively charged glutamic acid residues. The negatively charged residues on

deamidated gluten peptides and the presence of proline residues increase the ability of the peptides to bind to HLA-DQ2/8 molecules on APCs (Bergseng et al., 2005; Molberg et al., 1998). Gluten-peptides bound to HLA-DQ2/8 on APCs are presented to gluten-specific CD4⁺T cells in MLNs, which results in their activation and migration to the small intestinal lamina propria. Gluten-specific Th1 cells from CD patients preferentially recognize deamidated gluten-peptides (Molberg et al., 2001; Molberg et al., 1998) and produce the Th1 cytokines IFN- γ and IL-21 (Bodd et al., 2010; Nilsen et al., 1998). Gluten-specific Th1 cells also participate in the activation of B cells into anti-gluten and anti-TG-2 antibody producing plasma cells (Sollid et al., 1997). The presence of anti-TG-2 IgA antibodies are a valuable tool for diagnosing CD; however, it is not clear whether anti-gliadin or anti-TG-2 antibodies contribute to CD pathogenesis.

In addition to the gluten-specific adaptive immune response, innate immune activation is critical in CD pathogenesis (Figure 1.7). Central to this pathway are the IELs. Although not CD-specific, increased IELs are a hallmark of CD, and a value of 25 IELs/100 enterocytes has been suggested as the normal cut-off value (Walker et al., 2010). The migration of IELs towards the villi tip is also a morphological feature of CD (Biagi et al., 2004). Both TCR $\alpha\beta$ ⁺ and TCR $\gamma\delta$ ⁺ IEL subsets are increased in patients with untreated CD, with TCR $\alpha\beta$ ⁺ making up the majority of the IEL infiltrate (Abadie et al., 2012). Treatment with a gluten-free diet (GFD) results in a decrease in both IEL subsets, however, only the TCR $\alpha\beta$ ⁺ IELs return to basal levels. The density of TCR $\alpha\beta$ ⁺ IELs (IE-CTLs) correlates with intestinal damage, while TCR $\gamma\delta$ ⁺ IELs remain elevated in treated

patients, raising the hypothesis that they play a protective role in CD (Bhagat et al., 2008; Kutlu et al., 1993).

Increased epithelial expression of stress markers and IL-15 have also been proposed to play a key role in CD pathogenesis. Microbes, infections, or gluten peptides themselves, have been suggested to modulate the expression of epithelial stress molecules and the increased epithelial expression of IL-15 in CD (Sollid and Jabri, 2013). These stress molecules include the MHC class I-related chain A and B (MICA/B) and HLA-E in humans and RAE-1 and H60 in mice and are ligands for the NK activating receptor NKG2D. Increased IL-15 has been shown to upregulate of NKG2D on TCR $\alpha\beta$ +CD8 $\alpha\beta$ + IELs (IE-CTLs) (Meresse et al., 2004). Binding of NKG2D to its ligands (stress-induced molecules) on IECs activates a cytotoxic pathway in these IE-CTLs, resulting in epithelial destruction in a TCR-independent manner (Hüe et al., 2004; Meresse et al., 2004; Meresse et al., 2006).

1.2.3 Environmental factors in celiac disease

Approximately 30% of the general population carry the HLA-DQ2/8 CD susceptibility genes; however, only 2-5% of these individuals will go on to develop CD (Rossi and Schwartz, 2010). It is unclear why only a proportion of susceptible individuals go on to develop disease. This, along with the increasing incidence of CD suggests a role for additional environmental factors in disease development. Furthermore, why some individuals develop intestinal symptoms versus extra-intestinal manifestations, or extra-intestinal autoimmune co-morbidities is unclear. The additional factors that influence CD

development and its extra-intestinal autoimmune co-morbidities are unknown, but may include alterations in the intestinal microbiota or altered barrier or immune function.

1.2.3.1 Dysbiosis and celiac disease

Many studies have demonstrated a link between intestinal dysbiosis and CD. A number of studies have explored overall differences in microbial composition and diversity between patients with active CD, treated CD and healthy controls (see Appendix I, Table S1). Increases in potentially harmful gram-negative bacteria (*Bacteroides*) and decreases in protective, anti-inflammatory bacteria (*Bifidobacterium*) have been detected in active CD patients, and are not completely normalized after treatment with a GFD (Collado et al., 2008; Collado et al., 2009; Di Cagno et al., 2011). Others have detected changes in *Staphylococcus*, *Streptococcus*, *Lactobacillus*, and *Clostridium* in patients with active CD, some of which are reversed after treatment with a GFD (Collado et al., 2009; De Palma et al., 2010b; Nadal et al., 2007; Sánchez et al., 2010; Sánchez et al., 2013; Sánchez et al., 2012). While it is clear that differences in microbial composition exist between CD patients and healthy controls, the literature is inconsistent (see Appendix I, Table S1). Differences in the age of the study population (children vs. adults), methodology (FISH-PCR, DGGE, 16s rRNA sequencing), and the sample (biopsy vs. fecal) may contribute to inconsistent findings in the literature. Furthermore, differences in microbial composition may depend on the clinical presentation of the disease (Wacklin et al., 2013). These differences also make it difficult to compare results across different studies and determine whether the microbiota contributes to CD pathogenesis. Thus, whether changes in the intestinal microbiota play a causative role in

CD development or are a consequence of the disease are unclear from these studies. More recently, the link between factors that alter microbial composition early in life and subsequent CD risk has been studied.

1.2.3.2 Factors influencing intestinal colonization

Studies exploring how perturbation of the microbiota early in life can influence disease susceptibility have enhanced our understanding of the role of the microbiota in CD pathogenesis. A number of factors, which include mode of delivery, antibiotic use, and feeding patterns and diet can influence early intestinal colonization (Figure 1.4A). Given the importance of microbial exposure on host physiology and educating the immune system, the initial microbiota that colonizes the gastrointestinal tract may have important consequences for health and disease. Altered microbial colonization early in life may impair the establishment of host-microbe homeostasis and impair generation of tolerance to gluten.

Birth delivery mode: The mode of delivery influences the initial colonizers of the neonatal intestine, and these changes may persist beyond the first days following birth (Grönlund et al., 1999; Jakobsson et al., 2013). Vaginal birth results in the transmission of bacteria from the mother's vaginal flora, which is dominated by *Lactobacilli* and *Prevotella*. On the other hand, infants born by caesarean section (C-section) acquire a microbiota that resembles the mother's skin, and is dominated by *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* (Dominguez-Bello et al., 2010). Differences in intestinal microbial composition could also be detected in vaginal versus caesarean

delivered infants at 6 months of age, suggesting mode of delivery may have longer-lasting effects (Grönlund et al., 1999). Caesarean delivered infants also have a delayed colonization of *Bacteroides* and *Bifidobacterium* and a much lower overall diversity during the first two years of life (Grönlund et al., 1999; Jakobsson et al., 2013). Interestingly, increases in clostridia could also be detected in 7-year olds born vaginally compared to children born by C-section (Salminen et al., 2004). Although more long-term studies are needed to fully understand the impact of birth delivery mode on long-lasting microbial changes, an association between cesarean delivery and increased risk of CD has been demonstrated in several studies (Decker et al., 2010; Mårild et al., 2012).

Antibiotics: Antibiotics are designed to target pathogenic bacteria; however, they often also affect related commensals, which can lead to long-lasting shifts in the microbial community (Jakobsson et al., 2010; Jernberg et al., 2007). Although many variables, including the spectrum, dosage, and route of administration affect the extent of antibiotic-induced changes in the microbiota (Jernberg et al., 2010), antibiotic use leads to a reduction in microbial diversity and an increase in antibiotic-resistant strains (Willing et al., 2011). Most members of the microbiota will return to pre-treatment levels within days or weeks (De La Cochetiere et al., 2005; Dethlefsen et al., 2008). However, some members are lost from the community, resulting in long-term microbial changes (Clemente et al., 2012; Willing et al., 2011). Antibiotic use during the first month of life resulted in decreases in *Bifidobacterium* and *B. fragilis* (Penders et al., 2007; Penders et al., 2006), and changes following clindamycin treatment were reported up to two years after treatment (Jernberg et al., 2007). Antibiotic use by pregnant mothers or

breastfeeding mothers may also result in changes in the neonatal intestinal microbiota (Fallani et al., 2010). Antibiotic use has been linked to increased risk of CD (Mårild et al., 2013), but the design of this study made it difficult to determine whether antibiotic use truly preceded CD onset. However, a more recent study found that antibiotic use during the first year of life increased risk of developing CD later in life, and this risk increased with increasing courses of antibiotics (Canova et al., 2014).

1.2.3.3 Microbial modulation of gluten-induced immune responses

Given the association between dysbiosis and CD, the pathogenic potential of CD-associated bacteria and the microbial modulation of gluten-induced immune responses have been explored. *Escherichia coli* (*E. coli*), *B. fragilis*, and *Staphylococcus epidermidis* clones isolated from CD patients were shown to have increased virulence genes (Sanchez et al., 2012; Sánchez et al., 2008; Sánchez et al., 2012). The effect of CD-associated bacteria on gluten-induced immune responses has been tested in a number of *in vitro* and *in vivo* studies. *E. coli* and *Shigella* were shown to enhance gluten-induced pro-inflammatory immune responses and intestinal barrier dysfunction in epithelial and peripheral blood mononuclear cell (PBMC) cultures (Cinova et al., 2011; De Palma et al., 2010a; De Palma et al., 2012b). Conversely, *Bifidobacterium* reversed gluten-induced barrier dysfunction and immune responses *in vitro* and *in vivo* (Cinova et al., 2011; Laparra et al., 2012; Laparra and Sanz, 2010; Lindfors et al., 2008). Finally, in germ-free rats long-term gliadin but not albumin feeding resulted in moderated enteropathy and crypt hyperplasia (Štěpánková et al., 1996). Together, these studies suggest that microbes can modulate immune responses to gluten (Figure 1.8), but how these responses

contribute to CD pathogenesis or to the loss or induction of gluten tolerance in genetically susceptible hosts is unknown. Microbial modulation of gluten-induced immune responses in an animal model of CD is further explored in **chapter 4** of this thesis.

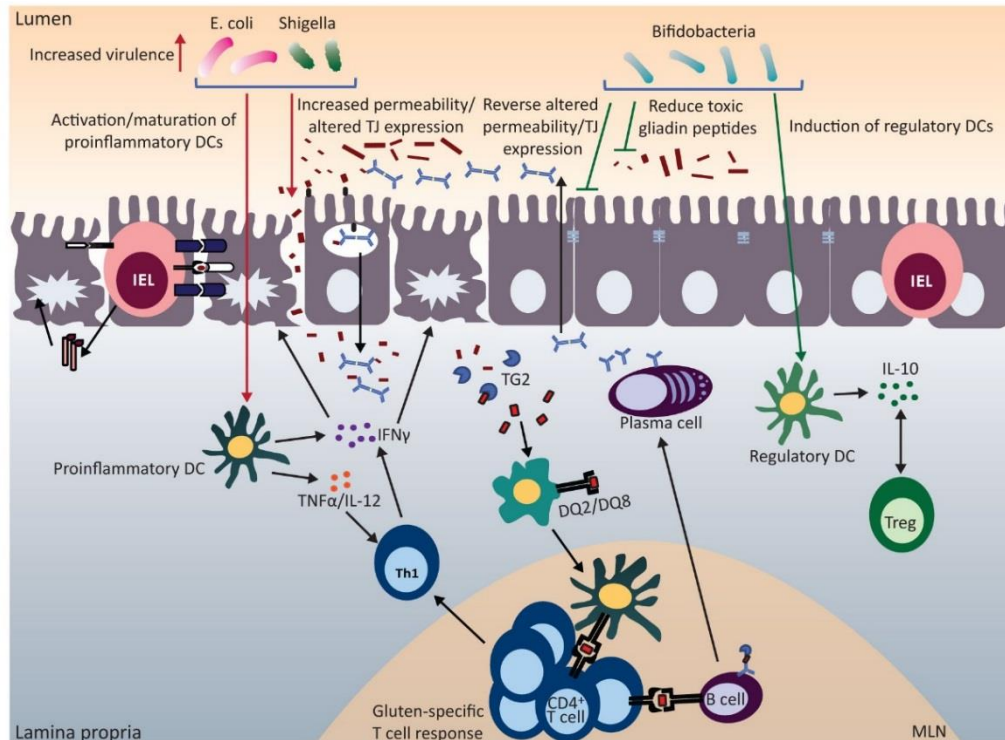


Figure 1.8 Potential microbial role as a disease modulator in celiac disease (CD). Pathogenic and protective role of microbiota in CD. Potential pathobionts, including *E. coli* and *Shigella*, may promote pro-inflammatory anti-gluten immune responses. First, *E. coli* strains isolated from CD patients were shown to have increased virulence. Second, *E. coli* and *Shigella* can induce the maturation of dendritic cells (DCs) and the production of pro-inflammatory cytokines (IL-12, TNF α) after gliadin stimulation *in vitro*. Finally, *Shigella* and *E. coli* can increase intestinal permeability and alter tight junction (TJ) protein expression. On the other hand, potentially beneficial bacteria, such as bifidobacteria, may reverse pathogenic, gluten-induced responses. Bifidobacteria can reverse gluten-induced increased permeability and altered TJ expression. *Bifidobacterium* species may also reduce the number of toxic, immunogenic gliadin peptides generated during digestion. Finally, bifidobacteria can promote the production of IL-10 from DCs *in vitro*. Figure adapted from (Galipeau and Verdu, 2014).

1.2.3.4 Feeding patterns and diet

The timing and quantity of initial gluten introduction have also been suggested to influence CD risk in genetically susceptible children. The early introduction (before four months of age) and the introduction of large amounts of gluten have been shown to increase CD risk (Ivarsson et al., 2002; Norris et al., 2005). However, other studies have failed to confirm these findings and have found no association between early gluten introduction and increased CD risk (Hummel et al., 2007; Ivarsson et al., 2002). Increased CD risk has also been associated with late (after six months of age) gluten introduction whereas the introduction of small amounts of gluten between four and six months has been suggested to be protective (Ivarsson et al., 2013). Based on these studies, the European Society of Paediatric Gastroenterology, Hepatology and Nutrition recommends the introduction of small amounts of gluten between four and six months of age, while maintaining breastfeeding. However, the heterogeneity of studies on this topic and the lack of randomized controlled trials is a major limitation, hindering the ability to draw conclusions. Several more recent clinical studies have found no association between early gluten introduction or duration of breastfeeding and subsequent CD risk (Jansen et al., 2014; Lionetti et al., 2014; Vriezinga et al., 2014).

Diet is one of the key factors that modulates the intestinal microbiota (Turnbaugh et al., 2009; Wu et al., 2011). Changes in diet can rapidly induce microbial changes and (David et al., 2013) and early life feeding patterns can influence the developing microbiota. In particular, breastfeeding versus formula feeding can influence the colonization of the infant gastrointestinal tract (Albenberg and Wu, 2014). Breastfeeding

is an important source of probiotics and prebiotics for the infant microbiota (Martín et al., 2012; Matamoros et al., 2013). Human milk oligosaccharides are a major component of breast milk and promote the growth of beneficial bacteria, including bifidobacteria (Albenberg and Wu, 2014). Breast-fed infants have higher counts of *Lactobacillus* and *Bifidobacterium* whereas formula fed infants have higher counts of potentially pathogenic species of *Bacteroides*, *Clostridium*, *Staphylococcus*, and *Enterobacteriaceae* (Matamoros et al., 2013; Saavedra and Dattilo, 2012). Breastfeeding may also have a protective effect against CD (Akobeng et al., 2006) by reducing microbial differences that are associated with HLA-DQ genotype (De Palma et al., 2012a).

The interaction between feeding practices and microbiota was further hypothesized to influence CD risk after rod-shaped bacteria were detected in CD children from the so-called “Swedish epidemic”. In Sweden, the annual incidence rate of CD in children increased dramatically from 1985-1995 (Ivarsson et al., 2000), which coincided with changes in feeding practice guidelines. National feeding guidelines recommended postponing the introduction of gluten from four to six months. As a result, a larger proportion of children received gluten after breastfeeding had ended. At the same time, the average consumption of gluten from commercial milk products increased in children under 2 years of age. Rod-shaped bacteria constituted a large fraction of the small intestinal microbiota of children born during this epidemic and this interaction between changes in feeding practices and the presence of rod-shaped bacteria may be an important risk factor that contributed to the increase in disease incidence (Ou et al., 2009).

1.2.4 Celiac disease treatments

1.2.4.1 The gluten-free diet

Currently the only treatment for CD is a strict adherence to a gluten-free diet (GFD), which leads to improvement of symptoms and mucosal damage. However, the complete removal of gluten from the diet is very difficult as it is a common substance in many foods. Furthermore, many foods that are believed to be safe or are naturally gluten-free are contaminated with small amounts of gluten (Collin et al., 2004). Studies have shown that 50mg of gluten per day, and in some patients as little as 10mg of gluten per day, is sufficient to induce villus-to-crypt alterations in CD patients (Catassi et al., 2007). Without proper supervision, following a GFD can also lead to nutritional deficiencies, which can affect quality of life (Theethira et al., 2014). The GFD is very demanding, expensive and may have a negative impact on lifestyle (Black and Orfila, 2011; Stevens and Rashid, 2008), which leads to high non-adherence rates, often voluntarily (Hall et al., 2009). Finally, even CD patients that are compliant with the diet to the best of their abilities are not satisfied with the GFD and report having a very high burden treatment, similar to what was reported for patients with end-stage renal disease (Shah et al., 2014). Incomplete clinical and mucosal recovery has also been reported in CD patients following a GFD for up to 5 years (Midhagen and Hallert, 2003; Rubio-Tapia et al., 2010). Although the limit for labelling foods as “gluten-free” is set to 20ppm in Canada and the United States, very small amounts of gluten can induce mucosal changes in some patients (Catassi et al., 2007). Failure to adhere to a strict GFD can increase the risk of complications, including decreased bone density, malnutrition and malignancy (Rubio-

Tapia et al., 2013). This highlights the need for development of adjuvant therapies to the gluten-free diet.

1.2.4.2 Alternatives or adjuvant therapies to the gluten-free diet

A number of different dietary and non-dietary therapeutic approaches, which target different areas of CD pathogenesis, are currently in development (Table 1.3). Emerging therapies include the modification of wheat prior to ingestion or during digestion to reduce the immunogenicity of gluten. Of these treatments, ALV003, a microbial-derived protease therapy, is in advanced clinical trials. Also in advanced phase II clinical trials is Larazotide acetate, a modulator of intestinal permeability. Other treatments in development include the inhibition of TG-2 or HLA-DQ2, which aim to block the activation of gluten-specific T cells. Finally, the restoration of gluten tolerance through a mucosal vaccine would be a curative approach. **Chapter 5 and 6** of this thesis will explore the therapeutic potential of two different novel adjuvant therapies for CD.

Table 1.3 Alternative therapeutic strategies for celiac disease

Therapy	Mode of action	Treatment effect	Compound	Stage	References
Gluten modification					
Genetically modified wheat	Selective breeding RNA interference	Reduced immunogenicity of gliadin epitopes		Pre-clinical	(Gil-Humanes et al., 2014a; Gil-Humanes et al., 2014b; Gil-Humanes et al., 2010; Spaenij–Dekking et al., 2005)
Pre-treatment of wheat flour	Treatment of wheat with lactobacilli/fungal derived proteases	Hydrolysis of immunogenic gluten peptides		Phase I clinical	(Di Cagno et al., 2010; Di Cagno et al., 2004; Di Cagno et al., 2002)

	Treatment of wheat flour with microbial transglutaminase	Transamidation of gluten peptides, reduces immunogenicity		Pre-clinical	(Gianfrani et al., 2007)
	Treatment of wheat flour with the probiotic preparation VLS#3	Hydrolysis of gluten peptides, reduced toxicity	VSL#3	Pre-clinical	(Angelis et al., 2006)
Luminal Therapies					
Oral proteases	Microbial derived prolyl endopeptidases (PEP)	Hydrolysis of gliadin peptides	<i>Flavobacterium meningosepticum</i> (FM)-PEP	Phase I	(Pyle et al., 2005a; Pyle et al., 2005b)
			<i>A. niger</i> (AN)-PEP	Phase IIa	(Mitea et al., 2008; Stepniak et al., 2006; Tack et al., 2013; Tack et al., 2010)
	PEP + glutamine endoprotease (EP)	Hydrolysis of gliadin peptides	<i>ALV003</i> (<i>Sphingomonas capsulata</i> (SC)-PEP + EP)	Phase IIb ongoing	(Lähdeaho et al., 2014; Lahdeaho et al., 2011; Siegel et al., 2012)
	Food grade protease		<i>Stan1</i>	Phase I-II ongoing	(Ehren et al., 2009)
Gluten-binding polymer	Specific sequestration of gliadin intraluminally	Reduces production of immunogenic peptides, minimizes gluten absorption	<i>P(HEMA-co-SS)/BL-7010</i>	Phase I ongoing	(Pinier et al., 2011; Pinier et al., 2009)
Modulation of intestinal permeability					
Larazotide acetate	Modulates permeability by opening intestinal epithelial tight junctions	Promote tight-junction assembly and improves paracellular permeability	<i>Larazotide acetate</i>	Phase IIb	(Kelly et al., 2013; Leffler et al., 2012; Paterson et al., 2007; Wang et al., 2014)
Modulation of T cell activation and migration					
TG2 inhibitors	Blocks TG2 activity	Reduces affinity of gliadin peptides for HLA-DQ2/8, reduces T cell activation	<i>Cystamine</i>	Pre-clinical	(Molberg et al., 2001)
			<i>KCC009</i> (di-hydroisoxazole)	Pre-clinical	(Choi et al., 2005)
			<i>R283</i>	Pre-clinical	(Maiuri et al., 2005)
HLA-DQ2 inhibitors	Blocks HLA-DQ2 binding site	Suppresses gliadin peptide presentation by APCs, inhibiting	<i>Azidoproline gluten analogue</i>	Pre-clinical	(Kapoerchan et al., 2008)
			<i>Dimeric gluten analogue</i>	Pre-clinical	(Xia et al., 2007)

		T cell activation			
CCR9 antagonist	Blocks CCR9, a chemokine that controls T cell trafficking to the small intestine	Blocks the homing and recruitment of T cells to the small intestine	<i>CCX282-B (Traficet-EN)</i>	Phase II ongoing	(Keshav et al., 2013; Kurppa et al., 2014)
Restoring gluten tolerance/ Immune modulation					
Vaccine	Intradermal immunization with DQ2 gluten peptides	Inhibit pathogenic gluten-specific T cell responses	<i>Nexvax2</i>	Phase I	(Anderson and Jabri, 2013; Brown et al., 2011; Kurppa et al., 2014; Tye-Din et al., 2010)
Genetically modified bacteria	Oral administration of <i>Lactococcus lactis (L. lactis)</i> modified to produce a DQ8-specific gluten peptide	Suppresses systemic gluten-specific, restores gluten tolerance	<i>Gluten-peptide producing L. lactis</i>	Pre-clinical	(Huibregtse et al., 2009)
Helminth infection	Modulation of the immune system by upregulation of Th2 immune responses	Reduced Th1 and Th17 responses and increased IL-10	<i>Necator americanus</i>	Phase IIa	(Daveson et al., 2011; McSorley et al., 2011)
Probiotics	Administration of live organisms that provide health benefits to the host	Modulation of barrier function and gluten-induced immune responses	<i>Bifidobacterium longum CECT7347</i>	Phase II	(Laparra et al., 2012; Olivares et al., 2014)
			<i>Bifidobacterium infantis</i>	Phase II	(Smecuol et al., 2013)

1.2.5 Animal models to study gluten-related disorders

Several animal models have been developed to study CD and GRD including dermatitis herpetiformis and milder conditions of gluten sensitivity (Table 1.4). Although the use of animal models has greatly improved our understanding of the pathogenesis of GRD, no single model encompasses all elements of CD. These include non-spontaneous and spontaneous models, only some of which are MHC Class II dependent (Marietta and

Murray, 2012). The development of an MHC Class II dependent model that spontaneously develops small intestinal atrophy following gluten exposure has been an area of intense research in the past few years. The use of spontaneous models, which currently center on the overexpression of pro-inflammatory cytokines, provide information on the interaction between particular cytokines and genetic factors. However, they do not allow for the investigation of unknown environmental factors that likely contribute to disease development. Recently developed mouse models include transgenic mice that lack all mouse MHC Class II molecules, but express the human DQ8 gene (HLA/DQ8 mice) (Black et al., 2002). The HLA/DQ8 mouse has been further characterized by our lab to study gluten-induced gut dysfunction following gliadin-sensitization (Verdu et al., 2008). These mice develop neuromotor and permeability abnormalities in the absence of enteropathy following gliadin-sensitization, which is reminiscent of NCGS. Thus, it constitutes a model of mild gluten sensitivity where environmental and host factors that contribute to gluten-induced enteropathy and extra-intestinal co-morbidities can be investigated. This thesis will utilize the HLA/DQ8 mouse (**chapter 5**) as well as transgenic DQ8 mice in the autoimmune predisposing non-obese diabetic (NOD) background (NOD/DQ8 mice; **chapter 3, 4, 6**). Details and characterization of NOD/DQ8 mice will be provided in **chapter 3** of this thesis. These humanized transgenic mice develop varying degrees of gluten-induced enteropathy and immune responses and can be used to further investigate environmental factors involved in disease development and phenotype (**chapter 4**) and potential therapeutic strategies (**chapter 5 and 6**).

Table 1.4 Animal models to study gluten-related disorders

<i>Model</i>	<i>Genetic background/ association</i>	<i>Spontaneous/ Sensitization</i>	<i>Gluten-dependent enteropathy</i>	<i>AGA anti-TG-2</i>	<i>Reference</i>
<i>Dog (Irish Setter)</i>	No MHC Class II association	Spontaneous	Partial villus atrophy	AGA	(Batt et al., 1984; Hall and Batt, 1992; Polvi et al., 1998)
<i>Rhesus macaque</i>	MHC Class II association unknown	Spontaneous	Partial villus atrophy	AGA anti-TG-2	(Bethune et al., 2008; Sestak et al., 2011)
<i>Horse</i>	MHC Class II association unknown	Spontaneous	Yes, partial villus atrophy	AGA anti-TG-2	(van der Kolk et al., 2012)
<i>Germ-free rats</i>	Wistar-AVN, no MHC Class II association	Gliadin-feeding following birth	Mild, IEL increase	No	(Štepánková et al., 1996)
<i>Mice</i>	Balb/c	Gluten-feeding for 30 days	Partial villus atrophy, IEL increase	AGA	(Papista et al., 2012)
	Rag-/-	Transfer of T cells from sensitized C56BL/6 mice to Rag-/- mice	Partial villus atrophy	AGA	(Freitag et al., 2009)
	HLA/DQ8 transgenic, MHC Class II dependent	Sensitization	IEL increase	AGA	(Black et al., 2002; Verdu et al., 2008)
	HLA/DQ2 transgenic, MHC Class II dependent	Sensitization	No	AGA anti-TG-2	(de Kauwe et al., 2009)
	NOD	Gluten-feeding	Mild, IEL increase	Not detected	(Maurano et al., 2005)
	IL-15-DQ8 transgenic, MHC Class II dependent	Gliadin-feeding for 10 days	IEL increase	AGA anti-TG-2	(DePaolo et al., 2011)

AGA, anti-gliadin antibodies; TG-2, tissue transglutaminase; MHC, major histocompatibility complex; IEL, intraepithelial lymphocyte; NOD, non-obese diabetic;

1.2.6 Gaps in knowledge of celiac disease pathogenesis: Past, present and future

The earliest descriptions of CD date to the second century by the Greek physician Aretaeus, but the symptoms and characteristics were further defined by physicians in the 19th century. However, it wasn't until the 1940's that Willem Karel Dicke demonstrated that the exclusion of wheat, rye and oats from the diet led to a dramatic improvement in symptoms, and gluten was identified as the toxic component that triggered symptoms (van Berge-Henegouwen and Mulder, 1993). In the 1980's and 1990's, the genetic susceptibility genes (HLA-DQ2/DQ8) were identified and the autoantigen (tissue transglutaminase) was identified by Detlef Schuppan and colleagues (Dieterich et al., 1997). Up until this time, CD was predominantly thought of as a childhood disease characterized by total villus atrophy in the small intestine that presented with gastrointestinal symptoms and failure to thrive (Fasano and Catassi, 2001). However, it became increasingly recognized in the 1980's that CD often presented with other autoimmune diseases, and presented with a spectrum of intestinal and extra-intestinal symptoms (Fasano and Catassi, 2001; Mäki et al., 1988; Pare et al., 1988). The reasons for this are still not understood. Today, CD is considered a clinical chameleon presenting at all ages, and case finding in high-risk groups is becoming widely accepted. Importantly, patient monitoring after diagnosis is suboptimal. Dietary compliance is a problem, as well as complications associated with its failure. Although many of the mechanisms involved in CD pathogenesis have been identified, many others remain unclear, including factors influencing disease onset, the spectrum of clinical presentation, and disease co-morbidities and complications. In addition to clinical research in CD, the

development and use of animal models will be critical to investigate these unknown factors and how they relate to CD risk and disease progression.

CHAPTER 2

THESIS OBJECTIVES

2.1 Thesis scope

The awareness and understanding of CD has come a long way in the past few decades; however we are still far from fully understanding disease pathogenesis, the spectrum of disease manifestations, and the factors involved in disease onset and progression. However, CD is a unique autoimmune disease in that the disease trigger (gluten) and the major susceptibility genes have been identified. This makes it an ideal disease to study the environmental and host factors that influence disease risk and how we might manipulate these factors as a preventative or therapeutic strategy. Moreover, animal models that recapitulate some pathophysiological alterations present in CD will be of aid in the preclinical development of pharmacological therapies for CD. **The overall goal of my thesis was to characterize a humanized animal model of gluten sensitivity in order to study factors that influence host-responses to gluten and potential therapeutic strategies.** The major findings of this thesis are organized into three aims and four chapters, each chapter a published or submitted manuscript. In **chapter 3** of this thesis I characterized a new model for studying GRD using humanized transgenic NOD/DQ8 mice. I investigated the potential role of gluten in the development of T1D. In **chapter 4** of this thesis I utilized NOD/DQ8 mice to investigate the influence of the microbiota on gluten-induced responses in the host. Finally, in **chapters 5 and 6** of this thesis I explored two different adjuvant therapies that could be applicable to CD or other GRD using HLA/DQ8 or NOD/DQ8 mice. Both gluten and gliadin are used for sensitizations and/or challenges throughout the thesis. For simplicity, the term gluten will

be used to denote all reactions to gluten and its proteins, which includes gliadin. The specific aims for each chapter are outlined below.

2.2 Thesis aims

Aim 1: To characterize gluten-induced enteropathy and barrier dysfunction as well as the potential role of gluten in the development of insulinitis in humanized, transgenic NOD/DQ8 mice.

CHAPTER 3: The effects of gluten sensitization on innate and adaptive intestinal and extra-intestinal host responses to gluten were tested in transgenic NOD/DQ8 mice. These findings were published in the following manuscript:

Galipeau HJ, Rulli NE, Jury J, Huang X, Araya R, Murray JA, David CS, Chirido FG, McCoy KD, Verdu EF. Sensitization to gliadin induces moderate enteropathy and insulinitis in nonobese diabetic-DQ8 mice. *J Immunol* 2011; Oct 15;187(8):4338-46. doi: 10.4049/jimmunol.1100854

Aim 2: To investigate whether microbial colonization modulates host responses to gluten using transgenic NOD/DQ8 mice.

CHAPTER 4: The effects of microbial colonization on host responses to gluten was tested using transgenic NOD/DQ8 mice 1) colonized with a limited, benign microbiota free of any pathogens and opportunistic bacteria 2) maintained germ-

free, and 3) colonized with a complex conventional microbiota harbouring opportunistic bacteria. As a secondary approach, the influence of perinatal antibiotic use on gluten-induced responses in NOD/DQ8 mice was investigated. These findings have been prepared in a manuscript that has been submitted to *Mucosal Immunology*.

Galipeau HJ, McCarville JL, Huebener S, Litwin O, Meisel M, Jabri B, Murray JA, Jordana M, Alaedini A, Chirido FG, Verdu EF. Microbiota modulates the response to dietary gluten in gnotobiotic mice. In submission at *Muc Immunol*.

Aim 3: To investigate the therapeutic potential of two novel therapies for GRD, the gluten binder P(HEMA-co-SS) and the anti-proteolytic molecule elafin, using gluten-sensitive transgenic DQ8 mice.

CHAPTER 5: The therapeutic potential of P(HEMA-co-SS), a gluten binding polymer, was tested *in vitro* and in gluten-sensitive transgenic HLA/DQ8 mice. This thesis will focus on the results from HLA/DQ8 mice in the manuscript. These findings were published in the following manuscript:

Pinier M, Fuhrmann G, Galipeau HJ, Rivard N, Murray JA, David CS, Drasarova H, Tuckova L, Leroux JC, Verdu EF. The copolymer P(HEMA-co-SS) binds gluten and reduces immune response in

gluten-sensitized mice and human tissues. *Gastroenterology* 2012; Feb;142(2):316-25.e1-12. doi: 10.1053/j.gastro.2011.10.038.

CHAPTER 6: The therapeutic potential of elafin, a protease inhibitor, was tested using small intestinal biopsies from treated and untreated CD patients, *in vitro* assays, and gluten-sensitive transgenic NOD/DQ8 mice. These findings were published in the following manuscript:

Galipeau HJ, Wiepjes W, Motta JP, Schulz JD, Jury J, Natividad JM, Pinto-Sanchez I, Sinclair D, Rousset P, Martin-Rosique R, Bermudez-Humaran L, Leroux JC, Murray JA, Smecuol E, Bai JC, Vergnolle N, Langella P, Verdu EF. Novel role of the serine protease inhibitor elafin in gluten-related disorders. *Am J Gastroenterol* 2014; May;109(5):748-56. doi: 10.1038/ajg.2014.48.

Details regarding author contribution to each individual manuscript can be found in the preface preceding each chapter.

CHAPTER 3

**SENSITIZATION TO GLIADIN INDUCES MODERATE ENTEROPATHY AND
INSULITIS IN NOD-DQ8 MICE**

Sensitization to gliadin induces moderate enteropathy and insulinitis in NOD-DQ8 mice

Gliadin, enteropathy, and insulinitis

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Preface: The research conducted in this paper was conducted from Sept 2008 to July 2011. I am the primary author of the paper. Experiments were designed by my supervisor, Dr. Elena Verdu, and myself. I conducted the majority of the experiments, analyzed the data, wrote the manuscript, and addressed concerns from the reviewer with my supervisor. Experiments were conducted with technical assistance by N.E. Rulli (T cell proliferation assays), J. Jury (Ussing chamber experiments), X. Huang (T cell proliferation assays, immunohistochemistry, ELISA), and R. Araya (T cell proliferation assays). NOD/DQ8 mice were provided by J.A. Murray and C.S David. K.D. McCoy provided experimental advice on flow cytometry experiments and provided NOD and NOR mice. N.E Rulli, J.A. Murray, F. Chirido and K.D McCoy contributed scientific input and critically appraised the manuscript.

Summary and significance: This article utilized transgenic NOD/DQ8 mice to investigate the effects of gluten sensitization on intestinal and extra-intestinal inflammation. In particular, the study explored the factors that contribute to the observed clinical association between CD and T1D. Gluten sensitization led to intestinal barrier dysfunction, moderate enteropathy and the development of anti-gliadin and anti-tissue transglutaminase antibodies, but mice were protected from insulinitis, a precursor to T1D. However, a prior immune dysregulation plus gluten-induced barrier dysfunction resulted in the development of severe insulinitis and gluten-specific immune responses in the pancreatic lymph nodes. These findings link mucosal intolerance to a dietary protein and insulinitis development, and provide evidence to support the clinical association between CD and T1D. Moreover, the moderate degree of enteropathy and development of gluten-specific immune responses observed in gluten-sensitive NOD/DQ8 makes it a valuable model for studying environmental disease modulators as well as the effectiveness of alternative therapies.

Title: Sensitization to gliadin induces moderate enteropathy and insulinitis in NOD-DQ8 mice

Short title: Gliadin, enteropathy, and insulinitis

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Abbreviations used in this article: CD, celiac disease; IEL, intraepithelial lymphocyte; MLN, mesenteric lymph node; NOR, nonobese resistant; PBS-T, PBS-0.05% Tween-20; PLN, pancreatic lymph node; PT-gliadin, peptic-tryptic digest of gliadin; T1D, type 1 diabetes; Treg, regulatory T cell; tTG, tissue transglutaminase.

Abstract

Celiac disease (CD) is frequently diagnosed in patients with type 1 diabetes (T1D), and T1D patients can exhibit Abs against tissue transglutaminase, the auto-antigen in CD. Thus, gliadin, the trigger in CD, has been suggested to have a role in T1D pathogenesis. The objective of this study was to investigate whether gliadin contributes to enteropathy and insulinitis in NOD-DQ8 mice, an animal model that does not spontaneously develop T1D. Gliadin-sensitized NOD-DQ8 mice developed moderate enteropathy, intraepithelial lymphocytosis, and barrier dysfunction, but not insulinitis. Administration of anti-CD25 mAbs before gliadin-sensitization induced partial depletion of CD25⁺Foxp3⁺ T cells and led to severe insulinitis, but did not exacerbate mucosal dysfunction. CD4⁺ T cells isolated from pancreatic lymph nodes of mice that developed insulinitis showed increased proliferation and proinflammatory cytokines after incubation with gliadin but not with BSA. CD4⁺ T cells isolated from nonsensitized controls did not respond to gliadin or BSA. In conclusion, gliadin sensitization induced moderate enteropathy in NOD-DQ8 mice. However, insulinitis development required gliadin-sensitization and partial systemic depletion of CD25⁺Foxp3⁺ T cells. This humanized murine model provides a mechanistic link to explain how the mucosal intolerance to a dietary protein can lead to insulinitis in the presence of partial regulatory T cell deficiency.

Introduction

The environmental factors that trigger most autoimmune diseases remain unknown. One exception is celiac disease (CD), which is one of the most common autoimmune disorders (1). CD is caused by the ingestion of gluten, a mixture of wheat proteins composed of gliadin and glutenin (1–4). Genetic factors also have a crucial role in CD pathogenesis (4). Almost all patients with CD carry HLA-DQ2 or HLA-DQ8, and these alleles contribute 40% of disease susceptibility (5). HLA-DQ8 and -DQ2 heterodimers confer susceptibility to CD by presenting a set of toxic gliadins and glutenins to specific CD4⁺ T lymphocytes. Adult CD diagnoses have dramatically increased in North America because of increased diagnostic efficiency and increased prevalence. CD is now a public health concern with a prevalence of ~1% among North Americans (2, 6).

Type 1 diabetes (T1D) is an autoimmune disease that results from a T cell-mediated attack of β cells in pancreatic islets. As with CD, there is a strong genetic component to T1D, of which the HLA class II genes *DQ* and *DR* play a major role (7). These susceptibility genes are thought to be important regulators of immune responses (8). However, the declining proportion of newly diagnosed disease in children with high-risk genotypes suggests that environmental factors have an important pathogenic role in T1D, but the exact triggers remain largely unclear (9).

CD and T1D are comorbid conditions (10–12). CD is diagnosed in 3–11% of T1D patients, and ~10% of children and 2% of adults with T1D exhibit Abs against tissue

transglutaminase (tTG), the autoantigen in CD (3, 10, 13–15). The association between CD and T1D has been attributed to their similar genetic basis (16–18). HLA haplotypes, such as DR3-DQ2, have been associated with defective oral tolerance and enhanced immune responses to dietary proteins (19, 20). Intriguingly, parallel birth cohort studies showed that infants at genetic risk for CD and T1D are at greater risk of either disease when exposed early to dietary cereals (21, 22). A genotyping study that enrolled 8064 T1D patients and 9339 control subjects showed that patients with T1D and CD express seven common alleles that regulate autoimmune responses (23, 24). Thus, genetic predisposition may confer susceptibility through DQ2/DQ8 HLA and non-HLA mechanisms that involve dysregulated immune responses to gut-encountered Ags (25). Studies in clinical and T1D animal models have proposed that exposure to gliadin in the diet contributes to diabetes in genetically susceptible hosts (22, 23, 26–29).

In this study, we used mice that lack all mouse endogenous class II molecules, but express the human *DQ8* gene, in the autoimmune predisposing background of NOD mice (30) to investigate the role of gliadin-sensitization in the development of enteropathy and insulinitis. In contrast to NOD mice, a validated animal model of diabetes (31, 32), NOD-DQ8 mice do not spontaneously develop diabetes (33, 34). Our findings demonstrate that gliadin-sensitization resulted in barrier dysfunction and moderate enteropathy, but no insulinitis. However, a partial depletion of regulatory T cells before gliadin sensitization induced severe insulinitis. The presence of gliadin-responsive T cells in the pancreatic lymph nodes (PLNs) of mice that develop insulinitis suggests that gliadin-specific T cell reactivity has a role in insulinitis development in this model.

Materials and Methods

Mice

Transgenic male mice that express HLA-DQ8 in an endogenous MHC class II-deficient background were backcrossed to NOD mice for 10 generations and intercrossed to produce congenic NOD AB^o DQ8 mice (33). Eight- to 10-wk-old male mice were used for experiments. Mice were weaned and maintained on a low-fat (4.4%), gluten-free diet, purchased from Harlan Laboratories and bred in a conventional, specific pathogen-free colony at McMaster University. Fourteen-week-old male NOD mice, 6-wk-old NOD mice, and 14-wk-old nonobese resistant (NOR) mice (obtained from Dr. J. Danska, Hospital for Sick Children, Toronto, ON, Canada) were used in additional experiments as positive and negative controls, respectively, for the development and evaluation of insulinitis. Glycemic status was monitored weekly using a glucometer (Abbott Diabetes Care) and was determined at the time of sacrifice using a Roche modular instrument. All experiments were conducted with approval from the McMaster University Animal Care Committee.

Anti-CD25 Ab treatment

Prior to gliadin sensitization, mice received two i.p. injections of monoclonal anti-CD25 Abs (PC61, 250 µg; Leinco Technologies, St. Louis, MO), 1 wk apart. This procedure induces a partial depletion of CD4⁺CD25⁺Foxp3⁺ T cells and induces an

immune dysregulation. Control groups were pretreated with two i.p. injections of PBS (250 μ l) 1 wk apart.

Gliadin sensitization

One week after anti-CD25 Ab treatment, mice were sensitized with a peptic-tryptic digest of gliadin (PT-gliadin). PT-gliadin was prepared as described previously (35). Gliadin (Sigma-Aldrich) was dissolved in endotoxin-free 0.2 N HCl for 2 h in a 37°C water bath with 1 g pepsin (Sigma-Aldrich). After 2 h of digestion, the pH was adjusted to 7.4 using endotoxin-free 2 M NaOH. Trypsin (Sigma-Aldrich) was added, the solution was boiled vigorously for 30 min, and the PT-gliadin was stored at -20°C. To sensitize the mice, they were gavaged with 500 μ g PT-gliadin plus 25 μ g cholera toxin (Sigma-Aldrich) once per week for 3 wk. Gliadin-sensitized mice were switched to a gluten-containing diet at the time of sensitization. Another set of mice was sensitized with 500 μ g BSA (Sigma-Aldrich) plus 25 μ g cholera toxin once per week for 3 wk. Control groups were gavaged with 25 μ g cholera toxin only. Control groups were maintained on a gluten-free diet throughout the experiments.

Glucose tolerance test

To determine impaired glucose tolerance, mice were fasted for 6 h. Mice were then injected with glucose (Sigma-Aldrich) i.p. at a dose of 1 g/kg. Venous plasma glucose was checked prior to glucose injection, and at 20, 40, 60, 90, and 120 min after glucose injection (36). Blood glucose levels were tested using a glucometer (Abbott

Diabetes Care). Glucose tolerance tests were conducted after the third gliadin-sensitization.

FACS analysis

One week after the second anti-CD25 Ab injections, cell suspensions of spleen, mesenteric lymph nodes (MLNs) and PLNs were prepared in RPMI 1640 (1% Penstrep, 10% FCS, 2 mM l-glutamine) by passing organs through a 100- μ m nylon mesh screen to dissociate the cells. Suspensions were depleted of RBCs by lysis and resuspended in FACS buffer (PBS containing 0.1% azide and 2% BSA). Cells were stained with fluorochrome-labeled cell-surface Abs including CD4-allophycocyanin (RM4-5), CD8a-PerCP (53-6.7), and CD25-PE (7D4) for 30 min at 4°C (BD Biosciences-PharMingen). For intracellular staining, cells were permeabilized using the Foxp3 staining buffer set (eBioscience) and incubated with FITC-conjugated Abs toward Foxp3 (FJK-16s; eBioscience) for 90 min at 4°C. Stained cells were acquired using the LSR II (BD Biosciences) and analyzed with FlowJo software (TreeStar).

Histologic evaluation

Cross-sections of the proximal small intestine were fixed in 10% formalin, embedded in paraffin, and stained with H&E for histologic evaluation by light microscopy. Two sections of the proximal small intestine were evaluated for evidence of inflammation, and villus-to-crypt ratios were determined. Twenty villus-to-crypt ratios were measured for each mouse in a blinded fashion. CD3⁺ intraepithelial lymphocytes

(IELs) per 20 enterocytes in five randomly chosen villus tips were counted according to the previously described methods and expressed as IEL/100 enterocytes (37).

The pancreas was removed, fixed in 10% formalin, and embedded in paraffin. Sections of the pancreas were stained with H&E for histologic evaluation and assessment of insulinitis. As described previously, insulinitis was determined by evaluating islet infiltration from grade 0–4: 0, no infiltration; 1, perivascular or periductal infiltrates; 2, leukocyte penetration of up to 25% islet mass; 3, leukocytes penetration of up to 75% of islet mass; 4, end-stage insulinitis with <20% of islet mass remaining (38–40). An average insulinitis score was determined for each group of mice. The liver and lungs were also removed and fixed in formalin. Sections were embedded in paraffin and stained with H&E and evaluated for signs of inflammation. Slides were viewed by light microscopy (Olympus), and images were acquired and analyzed using Image Pro Plus.

Immunohistochemistry for CD3⁺ cells

Immunostaining for CD3⁺ cells was performed on paraffin sections to detect the presence of IELs in sections of the proximal small intestine as described previously (41). Paraffin sections were incubated overnight at 4°C with rabbit anti-mouse primary Abs to CD3 (1:2000; Dako); this was followed by incubation with HRP conjugated anti-rabbit Abs. The Abs were visualized using 3-amino-9-ethylcarbazole and counterstained with Mayer hematoxylin. Negative controls were performed in the absence of primary Ab. Immunostaining for CD3⁺ cells was performed on paraffin sections of the pancreas as

described above. Slides were viewed by light microscopy (Olympus) under 40× magnification. Images were acquired and analyzed using Image Pro Plus.

Anti-tTg ELISA

tTg was derived from guinea pig liver (Sigma-Aldrich). Transglutaminase was diluted in PBS and 0.1 µg was added to each well of a 96-well Nunc-Immuno plate (Nunc). Plates were incubated overnight at 4°C. Plates were washed five times with PBS and blocked with 1% BSA in PBS for 1 h at room temperature. Serum was added to the plates at 50 µl per well and incubated for 1 h at room temperature. Plates were washed five times, and secondary anti-mouse IgA (Sigma-Aldrich) Abs were added and incubated an additional 2 h. Secondary Abs were HRP conjugated, and tetramethylbenzidine was the substrate (SurModics). Positive reactivity was determined using a positive cutoff value of ≥ 3 SD above the mean of the control group (42).

Anti-gliadin ELISA

Gliadin (Sigma-Aldrich) was dissolved in 70% ethanol and then diluted in PBS. Ninety-six-well Nunc-Immuno plates (Nunc) were coated with gliadin by adding 5 µg to each well. The plate was incubated overnight at 4°C. Plates were washed with PBS-0.05% Tween-20 (PBS-T) and blocked using 1% BSA in PBS for 1 h at room temperature. Serum was diluted into 1% BSA in PBS either 1:10 for detection of IgA or 1:50 for detection of IgG and added to plates for 2 h at room temperature. Plates were washed with PBS-T, and HRP conjugated secondary anti-mouse IgA (Sigma-Aldrich) or

anti-mouse IgG (Sigma-Aldrich) Abs were added to the wells for 1 h at room temperature. Plates were washed with PBS-T, and tetramethylbenzidine (SurModics) was used as the substrate.

Ussing chambers

Two sections of jejunum from each mouse were used for Ussing chamber experiments as described previously (41, 43). A 3–4-cm piece of jejunum was collected, cut into two sections, and placed in Krebs buffer aerated with 95% O₂ and 5% CO₂ (pH 7.3–7.4). Each segment of intestine was cut open along the mesenteric border to form a flat mucosal sheet and mounted into an Ussing chamber. The chamber exposed 0.6 cm² of tissue surface area to 8 ml of circulating oxygenated Krebs buffer containing 10 mM glucose (serosal side) and 10 mM mannitol (mucosal side) maintained at 37°C. Net active transport across the epithelium was measured via a short circuit current response (I_{sc}, μA) injected through the tissue under voltage-clamp conditions. Tissue conductance (the passive permeability to ions) was calculated using Ohm's law. Baseline I_{sc} (μA/cm²) and conductance (mS/cm²) were recorded at equilibrium, 20 min after mounting jejunum sections.

In vitro proliferation assay and cytokine analysis

Cell suspensions of PLNs were prepared in RPMI 1640 (1% penicillin/streptomycin, 10% FCS, 2 mM l-glutamine) by passing organs through a 100-μm nylon mesh screen to dissociate cells. CD4⁺ T cells were isolated from PLNs through

negative selection (EasySep Mouse CD4⁺ T Cell Enrichment Kit; Stemcell). Cell yield was assessed by FACS analysis, with an enriched population of 89–96% CD4⁺ T cells. Isolated CD4⁺ T cells were labeled with CFSE as previously described and washed three times with RPMI 1640 (44). Splenocytes were treated with mitomycin C (Sigma-Aldrich), and dendritic cells were isolated through positive selection (Easysep Mouse CD11c Selection Kit, Stemcell). Dendritic cells (5×10^4 cells/well) were cocultured with isolated CD4⁺ T cells (2×10^5 cells/well) in the presence of PT-gliadin (500 µg/ml), BSA (500 µg/ml), media alone in a round-bottom 96-well plate. For positive controls, cells were cultured with Con A. Cells were cultured for 4 d at 37°C, 5% CO₂. Cells were harvested on day 4 to assess proliferation by FACS analysis. Cells were resuspended in FACS buffer and stained with anti-CD4-allophycocyanin (BD Biosciences-Pharmingen) and 7-AAD (Sigma-Aldrich). CFSE-labeled cells were acquired using the LSR II (BD Biosciences). Viable cells, as determined by 7AAD exclusion, were gated on CD4⁺ T cells. CFSE intensity for this population was determined using FlowJo software (TreeStar).

Supernatants from cell cultures were collected at 48 and 96 h. The presence of proinflammatory cytokines in the supernatant was determined using a cytometric bead array inflammation kit (BD Biosciences) and analyzed using BD FACSAarray Bioanalyzer System (BD Biosciences). The presence of TGF-β was determined by ELISA (R&D Systems).

Statistical analysis

Statistical analysis was performed with GraphPad Prism software. For more than two treatment groups, an ANOVA with a Bonferroni post hoc test for multiple comparisons was used. When two groups were compared, an unpaired *t* test was used; $p < 0.05$ was considered significant. Data are displayed as mean \pm SEM.

Results

Gliadin-sensitized NOD-DQ8 mice developed barrier dysfunction and altered villus/crypt ratios

We have shown previously that HLA-DQ8 mice develop activation of the innate and adaptive arms of the immune system, and increased tissue conductance after gliadin sensitization (41, 43, 45). Despite this finding, gliadin-sensitized HLA-DQ8 mice develop only a mild enteropathy, characterized by increased IELs, similar to a Marsh I lesion in CD (46). To determine whether NOD-DQ8 mice develop gliadin-induced barrier dysfunction and enteropathy, mice were sensitized with a peptic-tryptic digested form of gliadin once per week for 3 wk. Cholera toxin was used as a mucosal adjuvant. A group of mice was pretreated with anti-CD25 mAbs to determine whether a preexisting immune dysregulation could exacerbate gliadin-induced mucosal dysfunction and enteropathy. Regulatory T cells (Tregs) are marked by the expression of CD4, CD25, and the transcription factor Foxp3. Therefore, we define a Treg as a CD4⁺CD25⁺Foxp3⁺ T cell, and will refer to CD4⁺CD25⁺Foxp3⁺ T cells as Tregs or CD4⁺CD25⁺Foxp3⁺ Tregs

hereafter. Furthermore, CD25 (IL-2R α subunit) is essential for the generation, peripheral expansion, and maintenance of Tregs (47–52). Monoclonal anti-CD25 Abs have been used to deplete the Treg population (47, 49, 53). Therefore, NOD-DQ8 mice were treated with two i.p. doses of anti-CD25 mAbs, 1 wk apart, prior to gliadin sensitization. We used Ussing chambers to measure tissue conductance in sections of the small intestine. Enteropathy was evaluated by villus-to-crypt ratios and by immunostaining for CD3⁺ IELs (37, 41).

Gliadin-sensitized NOD-DQ8 mice had increased tissue conductance compared with untreated controls and to anti-CD25 treated mice (Fig. 1). No difference in conductance was seen between untreated controls and anti-CD25 mAb-treated plus BSA-sensitized mice. There was also no difference in tissue conductance between gliadin-sensitized mice and sensitized mice that were pretreated with anti-CD25 mAbs, suggesting that barrier dysfunction was gliadin-dependent and was not induced by anti-CD25 mAb treatment. Gliadin-sensitized mice developed lower villus-to-crypt ratios (Fig. 2) and increased IEL counts (Fig. 3), reminiscent of a Marsh II lesion, compared with untreated control mice, anti-CD25 mAb treated mice, and anti-CD25 mAb-treated plus BSA-sensitized mice. Previous Treg depletion did not further increase the severity of gliadin-induced enteropathy in NOD-DQ8 mice. These results suggest that, compared with HLA-DQ8 mice, DQ8 mice on the NOD background are more sensitive to gliadin-induced enteropathy, and partial depletion of Tregs has no evident role in the induction of enteropathy in the model.

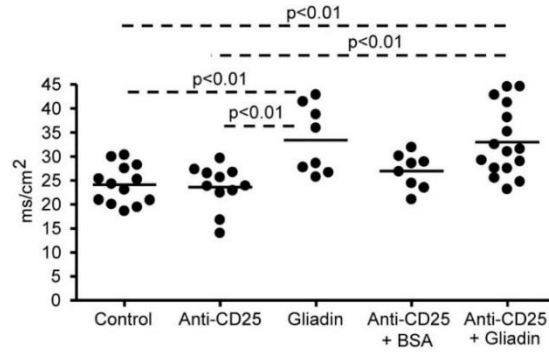


Figure 1. Impaired intestinal barrier function in gliadin-sensitized NOD-DQ8 mice. Sections of small intestine were mounted in Ussing chambers and tissue conductance (mS/cm²) was measured 24 h after the final gavage. Each dot represents an individual mouse; *p* values were computed using an ANOVA with a post hoc test for multiple comparisons.

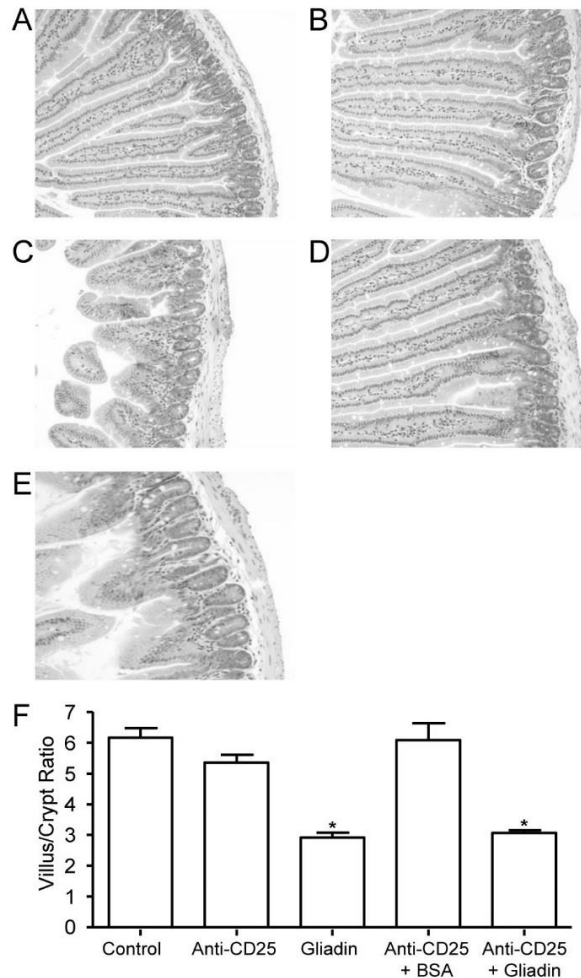


Figure 2. Histopathology of the small intestine showing decreased villus-to-crypt ratio in gliadin-sensitized NOD-DQ8 mice. H&E-stained sections of the proximal small intestine in untreated controls (A) and anti-CD25 mAb-treated (B), gliadin-sensitized (C), anti-CD25 mAb treated plus BSA-sensitized (D), and anti-CD25 mAb treated plus gliadin-sensitized mice (E). Original magnification $\times 10$. F, Quantification of villus/crypt ratios ($n = 8$ for each group). Data represented as mean \pm SEM; p values were computed using an ANOVA with a post hoc test for multiple comparisons. $*p < 0.05$ versus control mice, anti-CD25 mAb treated mice, and anti-CD25 + BSA-treated mice.

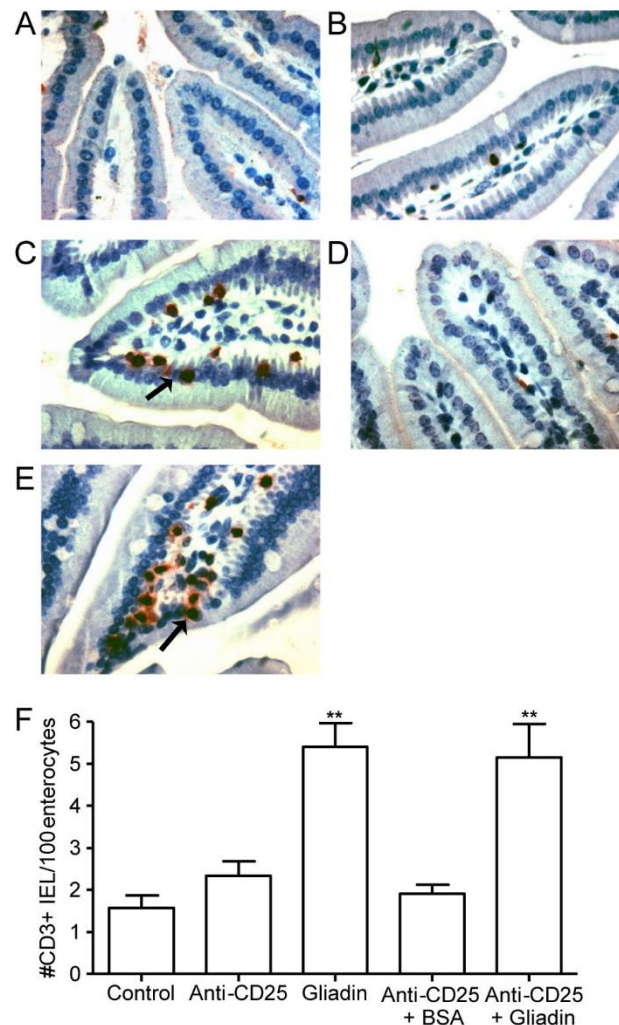


Figure 3. Immunohistochemistry showing increased number of CD3⁺ IELs in gliadin-sensitized mice. CD3⁺-stained sections of the proximal small intestine in untreated

controls (A), anti-CD25 mAb-treated (B), gliadin-sensitized (C), anti-CD25 mAb-treated plus BSA-sensitized (D), and anti-CD25 mAb-treated plus gliadin-sensitized mice (E). Original magnification $\times 40$. Black arrows indicate IELs. F, Quantification of CD3⁺ cells in villi tips, expressed as IEL per 100 enterocytes ($n = 8$ for each group). Data are represented as mean \pm SEM; p values were computed using an ANOVA with a post hoc test for multiple comparisons. ** $p < 0.01$ versus control mice, anti-CD25 mAb treated mice, and anti-CD25 + BSA-treated mice.

Gliadin-sensitized NOD-DQ8 mice develop anti-gliadin and anti-tTG Abs

In contrast to anti-gliadin IgA and IgG Abs, IgA autoantibodies toward tTG are highly specific for CD (54). HLA-DQ8 mice develop anti-gliadin IgG Abs after gliadin-sensitization (41, 45). We tested whether gliadin sensitization in NOD-DQ8 mice led to the production of Abs toward gliadin and tTG. Four of 11 gliadin-sensitized mice developed anti-gliadin IgG Abs in the serum and three of nine anti-CD25 plus gliadin-sensitized mice developed anti-gliadin IgG Abs. Anti-gliadin IgG Abs were not detected in the serum of nonsensitized mice (Fig. 4A). Anti-tTG IgA Abs were found in the serum of three gliadin-sensitized mice and two anti-CD25 mAb-treated plus gliadin-sensitized mice. No control, anti-CD25 mAb treated only, or BSA-sensitized mouse tested positive for anti-tTG Abs (Fig. 4B). The presence of anti-tTG Abs in a proportion of gliadin-sensitized mice is in accordance with the more moderate enteropathy observed in these mice compared with HLA-DQ8 mice (41).

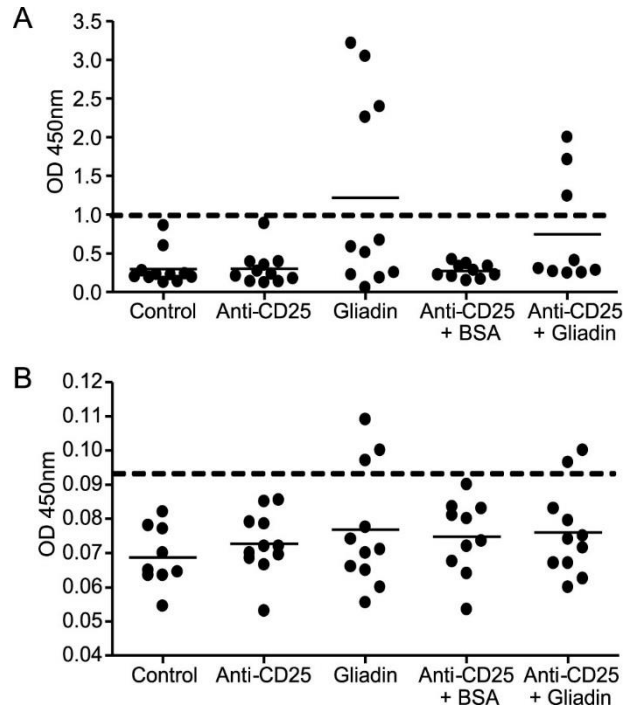


Figure 4. Anti-gliadin and anti-tTG ELISAs. Serum was collected from NOD-DQ8 mice, and the presence of anti-gliadin IgG Abs (A) and anti-tTG IgA Abs (B) was determined by ELISA. Anti-gliadin and anti-tTG positive reactivity was determined using a positive cutoff value of ≥ 3 SD above the mean of control mice (dotted line) (40). Each dot represents an individual mouse.

Anti-CD25 Ab treatment partially depleted CD4⁺CD25⁺Foxp3⁺ T cells

To confirm that anti-CD25 mAb treatment depleted the regulatory population, NOD-DQ8 mice were treated with two i.p. doses of anti-CD25 mAbs, each 1 wk apart. Cells were collected 1 wk following the second Ab treatment, and the percentage of Tregs was determined. Similar to previous findings, we showed a partial depletion of CD4⁺CD25⁺Foxp3⁺ Tregs (Supplemental Fig. 1) (47, 49). Compared with PBS-treated controls, the CD25⁺Foxp3⁺ cells decreased from 10.57 to 2.32% of the total CD4⁺ lymphocyte population in the spleen of anti-CD25 mAb-treated mice (Supplemental Fig.

1A, *left panels*). The remaining Foxp3⁺ cells expressed low levels of CD25 or no CD25. In addition, anti-CD25 mAb treatment resulted in the partial depletion of Tregs in the MLN and PLN. In the MLN, the CD25⁺Foxp3⁺ cells decreased from 11.54 to 3.27% of the CD4⁺ T cell population (Supplemental Fig. 1A, *center panels*). In the PLN, the CD25⁺Foxp3⁺ cells decreased from 11.94 to 3.0% of the CD4⁺ T cell population (Supplemental Fig. 1A, *right panels*). Thus, anti-CD25 mAb treatment before gliadin sensitization led to a partial, but significant depletion of Tregs in the spleen, MLN, and PLN (Supplemental Fig. 1B).

Partial Treg depletion and gliadin sensitization was associated with insulinitis in NOD-DQ8 mice

NOR and NOD mice were used as negative and positive controls, respectively, to validate the insulinitis grading in NOD-DQ8 mice. At 6 wk of age, NOD mice had developed mild periinsulinitis (Supplemental Fig. 2A, 2D), while severe insulinitis developed at 14 wk of age (Supplemental Fig. 2B, 2D). NOR mice developed mild periinsulinitis at 14 wk of age (Supplemental Fig. 2C, 2D), as previously reported (55). Intestinal tissue conductance was significantly higher in 14-wk-old NOD mice, but not in 6-wk-old NOD mice, compared with NOR controls. NOR mice displayed normal barrier function (Supplemental Fig. 3). Thus, although NOD mice displayed higher tissue conductance values at the preinsulinitis stage, only at 14 wk of age (insulinitis stage) did this difference become statistically significant.

We investigated whether gliadin-induced mucosal changes were linked to insulinitis in NOD-DQ8 mice. Previous studies have shown that NOD-DQ8 mice on a gluten-containing diet develop a gliadin-dependent blistering of the skin, but do not develop diabetes (33). In addition, gliadin-sensitized HLA-DQ8 mice have shown increased IL-10 production and increased recruitment of regulatory Foxp3⁺ cells within the lamina propria. It has been hypothesized that this regulatory immune response to gliadin may protect mice from overt autoimmunity (41, 45). Thus, we used anti-CD25 mAbs to partially deplete the Tregs, which we defined as CD4⁺CD25⁺Foxp3⁺ T cells (47, 56) prior to gliadin sensitization. Pancreatic islets were analyzed for infiltration of immune cells 24 h after the final gavage. Untreated control mice had normal islets (Fig. 5A). Anti-CD25 treated mice also had normal islets with no significant infiltration (Fig. 5B). Similar to the findings of Marietta et al. (33), gliadin-sensitized mice did not develop severe insulinitis (Fig. 5C). However, mice that were pretreated with anti-CD25 mAbs and subsequently sensitized with gliadin developed severe insulinitis (Fig. 5E). The insulinitis scores of anti-CD25 plus gliadin-sensitized mice were similar to those of 14-wk-old NOD mice (Supplemental Fig. 2). Treatment of anti-CD25 mAbs plus BSA sensitization did not lead to insulinitis, suggesting that the response was not induced by an unrelated Ag and was gliadin-dependent (Fig. 5D). The insulinitis scores of anti-CD25 mAb treated plus gliadin-sensitized mice were greater than those in untreated controls, anti-CD25 mAb treated mice, gliadin-sensitized, and to anti-CD25 mAb treated plus BSA-sensitized mice (Fig. 5F). The infiltrates were composed of CD3⁺ lymphocytes (Fig. 6). No differences were seen in blood glucose levels (data not shown) or glucose tolerance between groups (data

not shown). These results suggest that NOD-DQ8 mice are susceptible to developing an inflammatory response in β -cell islets when systemic partial depletion of $CD4^+CD25^+Foxp3^+$ cells is induced prior to gliadin sensitization.

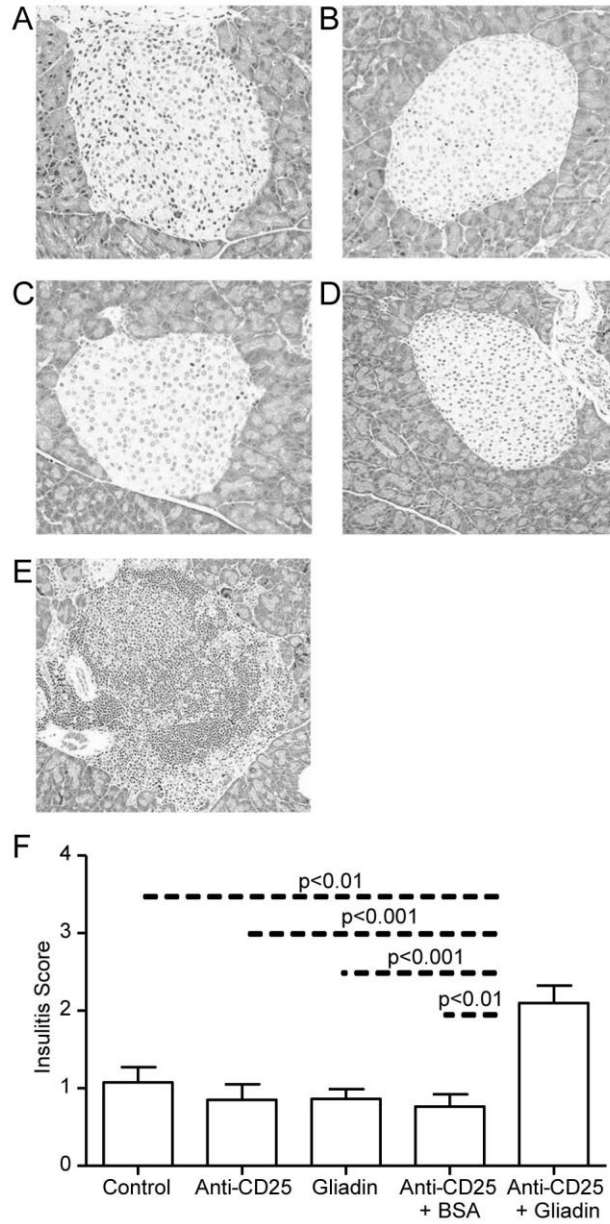


Figure 5. Gliadin-sensitized NOD-DQ8 mice develop severe insulitis in the presence of an immune dysregulation. Insulitis was determined by evaluating H&E-stained sections of the pancreas for islet infiltration, scoring each islet from grade 0 (no infiltration) to grade 4 (end-stage insulitis with <20% of islet mass remaining) (38–40). An average insulitis score was determined for each group of mice: untreated controls (A), anti-CD25 mAb treated mice (B), gliadin-sensitized mice (C), anti-CD25 mAb-treated plus BSA-sensitized mice (D), and anti-CD25 mAb treated plus gliadin-sensitized mice (E). Original magnification $\times 10$. F, Quantification of insulitis scores for multiple mice ($n = 9$ per group). Data are represented as mean \pm SEM; p values were computed using an ANOVA with a post hoc test for multiple comparisons.

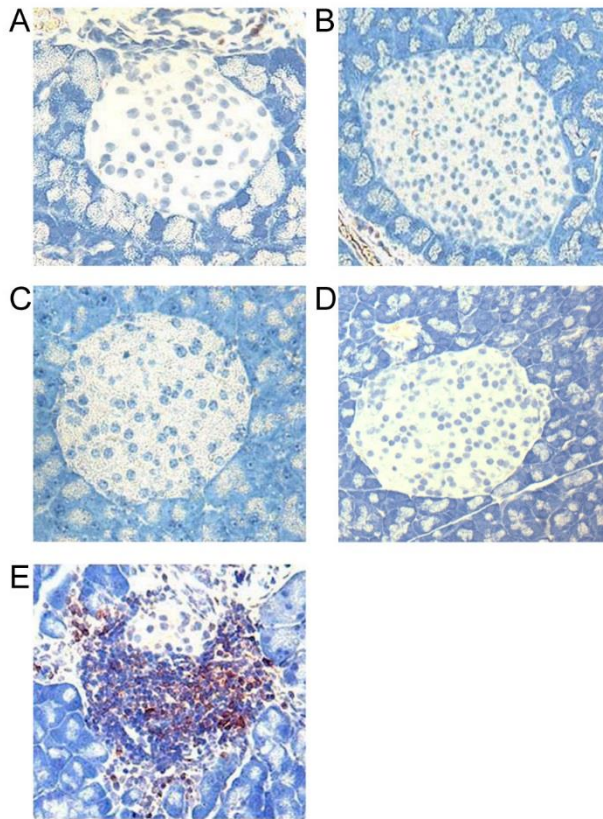


Figure 6. Immunohistochemistry showing the presence of CD3⁺ lymphocytes in pancreatic islets in NOD-DQ8 mice. Representative CD3⁺-stained sections of the pancreas in untreated controls (A), anti-CD25 mAb-treated (B), gliadin-sensitized mice (C), anti-CD25 mAb plus BSA-sensitized (D), and anti-CD25 mAb-treated plus gliadin-sensitized mice (E). Original magnification $\times 20$.

Partial CD4⁺CD25⁺Foxp3⁺ cell depletion and gliadin sensitization did not lead to widespread autoimmunity

To determine whether inflammation was present in other organs, H&E-stained sections of the liver and lungs were examined for signs of infiltration and inflammation. There was no significant infiltration observed in the liver of nonsensitized controls, anti-CD25 mAb treated mice, gliadin-sensitized mice, anti-CD25 mAb treated plus BSA-sensitized mice, or in anti-CD25 mAb plus gliadin-sensitized mice (data not shown). Similarly, none of the groups exhibited inflammation within the lungs (data not shown). Thus, the infiltration observed in anti-CD25 mAb-treated plus gliadin-sensitized mice was restricted to the pancreas, indicating an absence of generalized autoimmunity.

T cells from PLNs of NOD-DQ8 mice that developed insulinitis exhibit increased proliferation and proinflammatory cytokine production when incubated with gliadin

HLA-DQ8 mice have shown increased proliferative responses toward gliadin peptides in both the spleen and MLN (45, 57). Evidence suggests that the gut and the pancreas are immunologically linked. In NOD mice, islet-infiltrating lymphocytes express the $\alpha 4\beta 7$ integrin, a gut-homing receptor (58). The ligand for $\alpha 4\beta 7$, MAdCAM-1, is expressed within the pancreas and is upregulated during insulinitis (59). These findings suggest that lymphocytes displaying gut homing markers are able to circulate between the gut and the pancreas, and that immune responses to dietary Ags may modulate insulinitis. We therefore determined whether cells in the PLNs of gliadin-sensitized NOD-DQ8 mice responded to gliadin by measuring proliferation and cytokine production. CD4⁺ T cells

were isolated from the PLNs of control mice and anti-CD25 mAb plus gliadin-sensitized mice and incubated with gliadin, BSA, or media alone. In anti-CD25 mAb plus gliadin-sensitized mice, gliadin stimulation led to increased proliferation compared with BSA and media alone. In control mice, no increase in proliferation was observed in gliadin-stimulated cultures (Fig. 7A, 7B). Compared with untreated controls, gliadin stimulation increased proliferation in anti-CD25 plus gliadin-sensitized mice. Cytokine production was measured in the PLN cell culture supernatants. There was a significant increase in production of the proinflammatory cytokine TNF- α in the cell cultures from mice that developed insulinitis and were incubated with gliadin compared with BSA (Fig. 7C). In cultures from mice that developed insulinitis and were cultured with media or BSA, the production of MCP-1 and IL-6 was below the limit of detection, whereas incubation with gliadin resulted in detectable levels of both MCP-1 and IL-6 in some of the mice (Fig. 7D, 7E). No differences were found in IL-10 or TGF- β production (data not shown). In PLN cell cultures from control mice, all cytokine levels were below the limit of detection (data not shown). These results suggest that gliadin-specific T-cells are found within the PLNs of mice that develop insulinitis and that these cells produce a Th1 cytokine response.

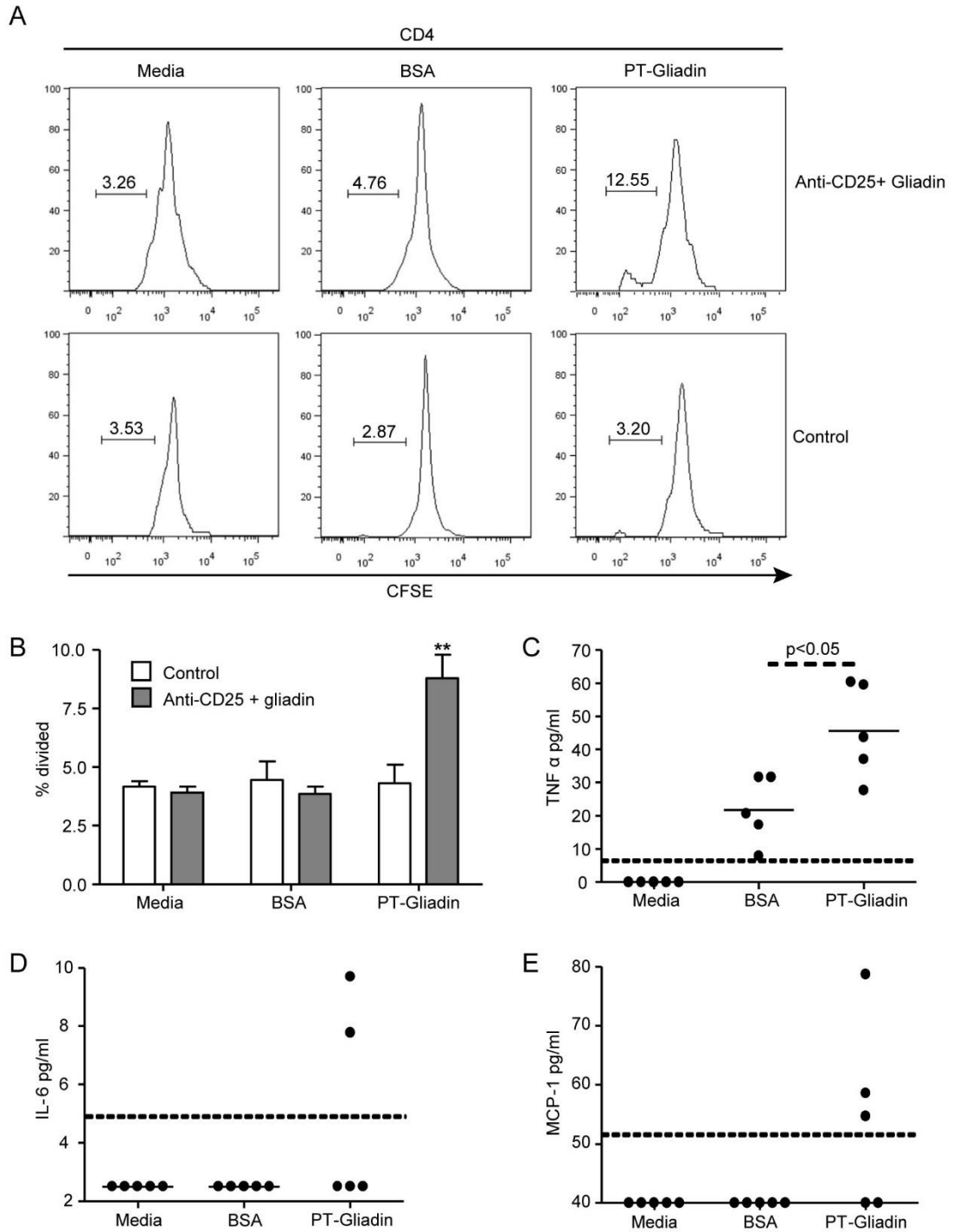


Figure 7. T cells isolated from the PLNs of mice that develop insulinitis respond to gliadin stimulation. A, CD4⁺ T cells were isolated from the PLNs of control and anti-CD25 mAb-

treated plus gliadin-sensitized mice and labeled with CFSE. CD4⁺ T cells were incubated with APCs with PT gliadin (*right panels*), BSA (*center panels*), or media alone (*left panels*) for 4 d. Proliferation was assessed with FACS analysis. Cells were gated on live CD4⁺ lymphocytes. Gated population represents the percentage of proliferated cells. *B*, Quantification of proliferation in *A* for control mice ($n = 5$) and anti-CD25⁺ gliadin mice ($n = 5$). Data are represented as mean \pm SEM; p values were computed using an ANOVA for multiple comparisons. ** $p < 0.01$ versus other groups. TNF- α (*C*), IL-6 (*D*), and MCP-1 (*E*) production was assessed in cell culture supernatants from mice that developed insulinitis, using a cytometric bead array inflammation kit. The dotted line represents the limit of detection; p values were computed using an unpaired t test.

Discussion

The main objective of this study was to investigate the role of gliadin in the development of enteropathy and insulinitis in NOD-DQ8 mice. T cells in NOD-DQ8 mice are selected in the thymus in a DQ8-restricted manner. The presence of the *DQ8* gene ensures efficient gliadin Ag presentation by APCs to CD4⁺ T cells ([45](#), [57](#), [60](#)). We found that gliadin sensitization in NOD-DQ8 mice induced moderate enteropathy, characterized by decreased villus-to-crypt ratios and increased IEL counts in the proximal small intestine. Similar to other DQ8 transgenic mouse models, NOD-DQ8 mice developed gliadin-induced intestinal barrier dysfunction. These changes were induced by gliadin, as the immune and functional changes were not observed after sensitization with an unrelated Ag—BSA. Development of severe insulinitis, however, required partial depletion of CD25⁺Foxp3⁺ T cells prior to gliadin sensitization. T cells isolated from the PLNs of mice that developed insulinitis exhibited enhanced proinflammatory cytokine production and proliferation when cultured with gliadin, compared with culture with BSA or media alone. CD4⁺ T cells isolated from the PLNs of control mice did not respond to gliadin or

BSA. These data suggest that a combination of mucosal damage and breakdown in tolerance to gliadin led to insulinitis in NOD-DQ8 mice.

There is mounting evidence that a defect in the intestinal barrier can promote T1D (61, 62). Several animal models of T1D have demonstrated that increased intestinal permeability can be detected prior to the clinical onset of diabetes (63–65). Barrier dysfunction has also been observed in patients with T1D and in their relatives (66, 67). A recent study in NOD mice has shown that infection with *Citrobacter rodentium* resulted in increased intestinal permeability and accelerated onset of insulinitis and diabetes (68). The results suggest an adjuvant role of intestinal barrier dysfunction in diabetes development. In our study, 14-wk-old NOD mice used as positive controls for insulinitis exhibited barrier dysfunction and severe insulinitis. Because gliadin is known to affect intestinal permeability in animal models of gluten sensitivity and in humans with CD (41, 65, 69–71), we investigated whether NOD-DQ8 mice developed gliadin-induced barrier dysfunction and insulinitis. It has been suggested that the patients with CD and the greatest risk of developing extraintestinal autoimmunity are those who have had a longer duration of gluten exposure (72). Studies in animal models and humans have shown a reduction in T1D risk following implementation of a gluten-free diet (29, 73, 74). We found that gliadin sensitization led to increased tissue conductance and an enteropathy, characterized by decreased villus-to-crypt ratios and increased IELs, that is reminiscent of moderate gluten sensitive enteropathy (75). In addition, a proportion of gliadin-sensitized mice developed both anti-gliadin IgG and anti-tTG IgA Abs. Similarly, a recent article using DQ8 transgenic mice that overexpress IL-15 in the lamina propria found that mice fed

gliadin developed anti-gliadin and anti-tTG Abs, intraepithelial lymphocytosis, and IFN- γ -producing T cells, but lacked overt villous atrophy (76). Innate immune responses toward gliadin can directly cause mucosal damage, a process that involves production of IL-15. tTg also plays a key role in modifying the anti-gliadin immune response in CD. Whether these key innate immune responses have critical roles in the development of mucosal damage in NOD-DQ8 still needs to be elucidated. Despite these mucosal abnormalities in gliadin-sensitized NOD-DQ8 mice, insulinitis did not develop. These findings suggest that loss of barrier function and moderate enteropathy induced by gliadin are not sufficient, on their own, to induce insulinitis.

Recent studies have shown that inducible Tregs have a crucial role in maintaining oral tolerance (77). In the MLNs, IL-2 and TGF- β are required to induce production of CD4⁺CD25⁺Foxp3⁺ inducible Tregs from naive CD4⁺CD25⁻ T cells (78). Migration to the gut and expansion of these cells in the lamina propria by intestinal dendritic cells is crucial for the generation of oral tolerance (77, 78). In accordance with previous results using this technique, we showed that treatment with anti-CD25 mAbs led to partial depletion of Foxp3⁺ Tregs (47, 49, 52). Importantly, this includes the inducible Treg population, which is critical for controlling the immune response in the intestinal mucosa against oral Ags (52, 77). The partial depletion was likely sufficient to disrupt the immunologic balance between proinflammatory and regulatory mediators in NOD-DQ8 mice because insulinitis developed after sensitization with the relevant Ag in this model—gliadin. However, anti-CD25 mAb-treated plus gliadin-sensitized mice did not develop hyperglycemia or impaired glucose tolerance and were not, therefore, clinically diabetic

at the time they were analyzed. Similarly, a study using anti-CD25 mAbs demonstrated severe autoimmune gastritis only after administration of autoimmune gastritis target Ag plus anti-CD25 mAb treatment (53). Although insulinitis was evident in anti-CD25 mAb plus gliadin-sensitized mice, a longer follow-up time after gliadin sensitization may be needed for development of hyperglycemia and progression to diabetes. However, the degree of insulinitis in anti-CD25 plus gliadin-sensitized mice was similar to the insulinitis seen in 14-wk-old NOD mice in our study and reported elsewhere (79). Importantly, several studies suggest that a proinflammatory environment within the pancreatic islets can promote the development of diabetes. NOD-DQ8 mice have been shown to develop diabetes if TNF- α is overexpressed in β islets (34). Other studies have shown that the expression of the costimulatory molecule, B7-1, or IFN- α within the islets can promote diabetes development, even in a diabetes-resistant background (80, 81). Our results indicate that partial CD25⁺Foxp3⁺ T cell depletion prior to gliadin sensitization was associated with islet infiltration in NOD-DQ8 mice. Extraintestinal inflammation in gliadin-sensitized NOD-DQ8 mice with prior CD25⁺Foxp3⁺ cell depletion was localized to the pancreas, as liver and lung tissues were normal.

CD4⁺ T cells isolated from the PLNs of mice that developed insulinitis responded to gliadin, and not to BSA. In NOD mice and patients with T1D, diabetogenic T cells within the islets display the gut homing marker α 4 β 7 (82). Furthermore, the ligand for the α 4 β 7 integrin, MadCam-1, is expressed on the endothelium in the pancreas and is upregulated during insulinitis (83). It has been shown that T cells isolated from the MLNs of 3-wk-old NOD mice are able to induce the development of diabetes in NOD.SCID mice more

efficiently than lymphocytes isolated from peripheral lymph nodes, pancreatic lymph nodes, and the spleen (84). This finding suggests the initial priming of diabetogenic T cells may take place in the gut and that activated T cells can home to the pancreas to cause insulinitis (84). In our model, oral sensitization to gliadin in mice with partially depleted Tregs led to the production of gliadin-responding T cells in the PLN. This gliadin-associated insulinitis could be permissive of T1D development in a genetically predisposed host. Gliadin predominantly activates innate mechanisms in animal models, but the adaptive immune system has also been shown to be activated in transgenic HLA DQ8 mice (41, 45). We demonstrate the presence of gliadin-responsive T cells in the PLNs of NOD-DQ8 mice, providing an experimental setting where both innate and adaptive immune mechanisms can be studied within and outside the gut.

The current prevalence of T1D is estimated to double by 2020 in children younger than 5 y, and ~10% of children with T1D have documented CD (10, 85). CD is common worldwide, with a dramatic increase in prevalence from just 0.2–0.9% during the last five decades (6). With 95% of CD cases estimated to be currently undiagnosed in North America, and the increasing incidence of both diseases, the potential effects of gliadin intolerance on T1D are alarming. Diabetes imposes a substantial cost burden on society, in part because of its complications and comorbidities (86, 87). Our results provide a model to elucidate the mechanisms through which a common dietary intolerance may increase T1D risk and test innovative dietary approaches to prevent and improve metabolic control in T1D (88).

Disclosures

The authors have no financial conflicts of interest.

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Footnotes

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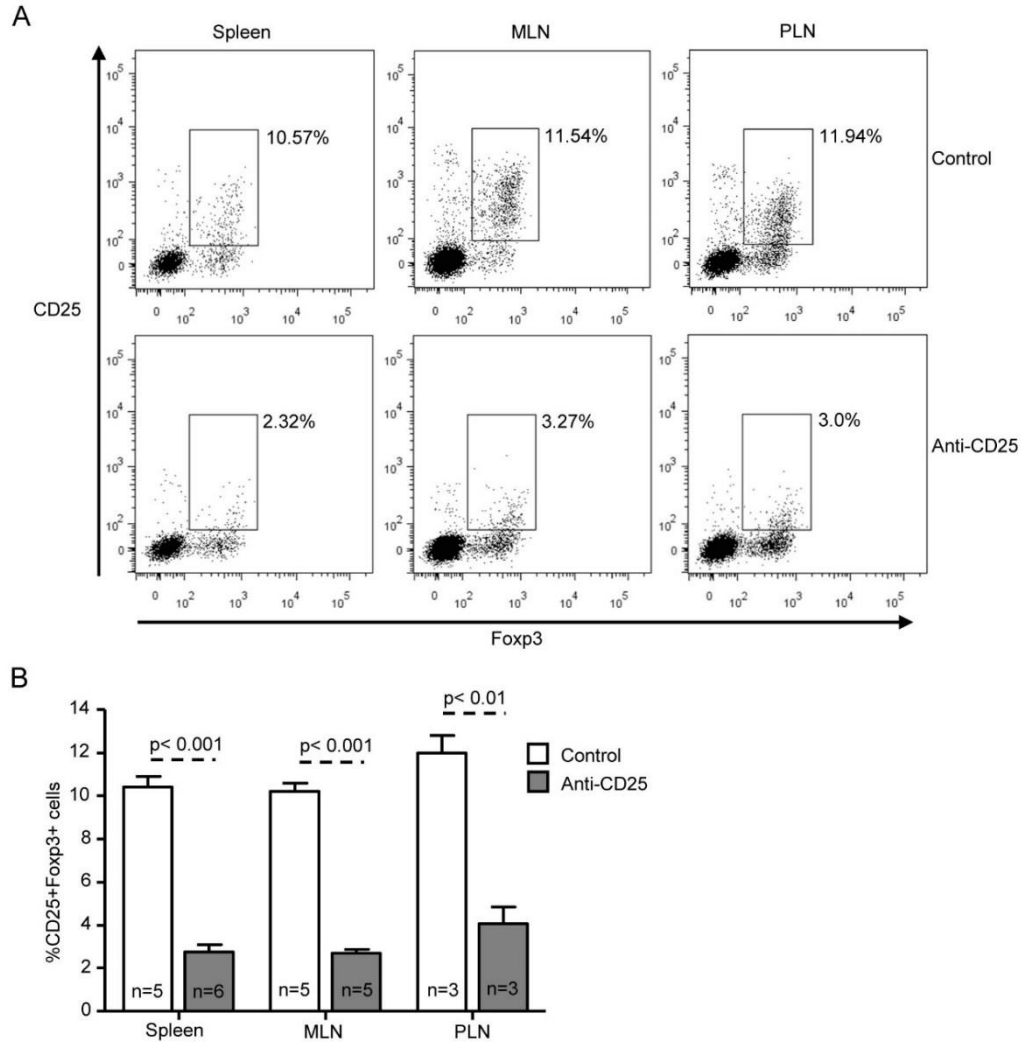
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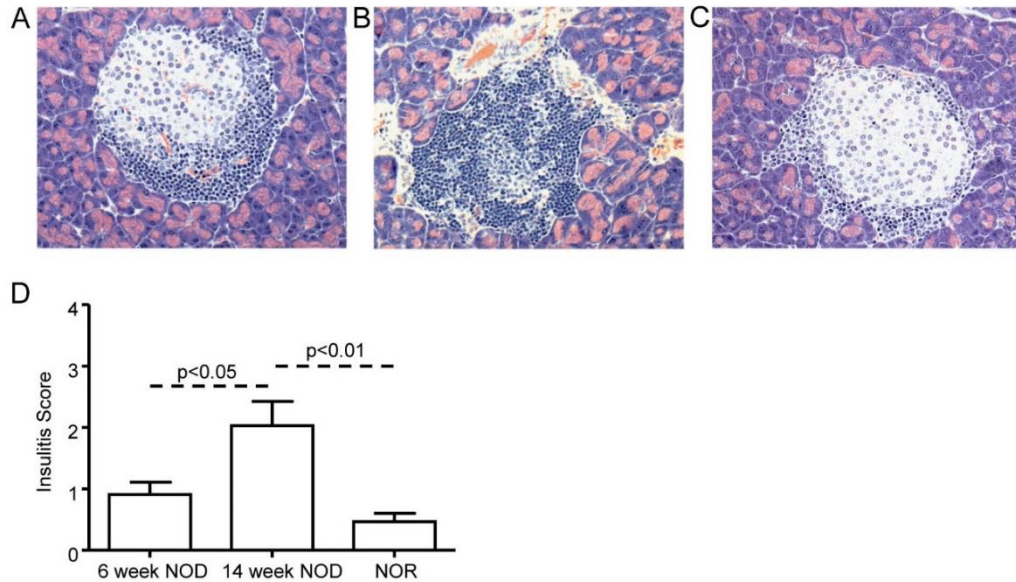
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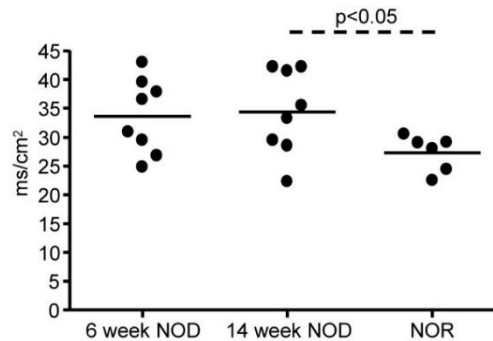
Supplemental Figures



Supplemental figure 1. Anti-CD25 antibody treatment partially depletes Tregs. (A) NOD-DQ8 mice were treated with two i.p. injections of anti-CD25 antibodies or PBS, one week apart. Splenocytes, MLN cells, and PLN cells were collected from PBS treated control mice (top panels) and anti-CD25 treated mice (bottom panels) one week following the second injection and stained with mAbs for CD4, CD25, and Foxp3. Lymphocytes are gated on CD4+ cells. Numbers represent the percentage of CD25+Foxp3+ lymphocytes falling within the gate. Representative plots from 3 independent experiments are shown. (B) Quantification of data in A for multiple control and anti-CD25 mAb treated mice (n=3-6 per group). Data represented as mean \pm SEM. P values computed using an unpaired t-test.



Supplemental figure 2. Insulinitis scores in NOR and NOD mice. Insulinitis was determined by evaluating H&E stained sections of the pancreas for islet infiltration, scoring each islet from grade 0 (no infiltration) to grade 4 (end-stage insulitis with <20% of islet mass remaining). An average insulinitis score was determined for each group of mice. (A) 6-week old NOD mice, (B) 14-week old NOD mice, and (C) 14-week old NOR mice. Original magnification, x10. (D) Quantification of insulinitis index for multiple mice (n=8 per group). Data represented as mean \pm SEM. P values computed using an ANOVA with a post-hoc test for multiple comparisons.



Supplemental figure 3. Intestinal barrier function in NOR and NOD mice. Sections of small intestine were mounted in Ussing chambers and tissue conductance (mS/cm²) was measured in 6-week old NOD mice, 14-week old NOD mice, and NOR mice. Each dot represents an individual mouse. P values computed using an unpaired

CHAPTER 4

**MICROBIOTA MODULATES THE RESPONSE TO DIETARY GLUTEN IN
GNOTOBIOTIC MICE**

Microbiota modulates the response to dietary gluten in gnotobiotic mice.

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Preface: The work in this manuscript was performed from Sept 2012 to Sept 2014. I am a primary co-author of the manuscript along with J.L. McCarville. The manuscript was written by my supervisor, E. Verdu, and myself. Experiments were designed by myself, J.L. McCarville and my supervisor. Data was analyzed by J. L. McCarville and myself. I performed *in vivo* gnotobiotic and conventional SPF experiments and J.L. McCarville performed antibiotic experiments. S. Huebener and A. Alaedini assisted with antibody determinations. O. Litwin assisted with TUNEL staining. M. Meisel assisted and provided technical support for IL-15 mRNA measurements. Y. Sanz provided the *E. coli* ENT CA15. B. Jabri, Y. Sanz, J.A. Murray, M. Jordana, A. Alaedini, and F.G. Chirido contributed scientific input and critically appraised the manuscript.

Summary and significance: The microbiota has been suggested to influence celiac disease (CD) development, but *in vivo* evidence to support a causative role is lacking. Therefore, this article utilized NOD/DQ8 mice, which were characterised in the manuscript presented in **chapter 3**, to investigate the role of the microbiota in host responses to gluten. The absence of a microbiota as well as the composition of the microbiota was shown to influence gluten-induced pathology. Specifically, mice maintained in a germ-free environment or treated perinatally with antibiotics developed enhanced gluten-induced pathology, while mice maintained with a benign microbiota free from any opportunistic pathogens were protected. The presence or expansion of opportunistic bacteria, such as *Escherichia*, was associated with more severe gluten-induced pathology. The findings in this article demonstrate for the first time that the microbiota can modulate host responses to gluten *in vivo*, and support the hypothesis that the microbiota may represent an environmental factor involved in CD development or progression. In addition, the data also provide mechanistic evidence underlying the reported clinical association between antibiotic use and subsequent increased CD risk.

Title: Microbiota modulates the response to dietary gluten in gnotobiotic mice.

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Conflict of interest: none to declare

Abstract

Celiac disease (CD) is an immune-mediated enteropathy triggered by the ingestion of gluten in genetically susceptible individuals. Dysbiosis has been described in patients with CD, but it is unknown whether these microbial changes are a CD-promoting factor. To investigate a potential influence of the microbiota on host responses to gluten, germ-free, clean specific pathogen free (SPF) and conventional SPF NOD/DQ8 mice were sensitized with a gliadin digest and challenged with gluten. Clean SPF mice harbour a defined microbiota absent of opportunistic pathogens and Proteobacteria. Conventional SPF mice harbour a complex microbiota with opportunistic pathogens, including *Helicobacter* and *Escherichia*. Mice colonized with a clean SPF microbiota had attenuated responses to gluten compared to germ-free mice that developed increased intraepithelial lymphocyte (IEL) cytotoxicity. Clean SPF mice also had less severe gluten-induced responses compared to conventional SPF mice. Supplementation of clean SPF mice with *E. coli* ENT CA15, isolated from CD patients, enhanced gluten-induced pathology. Finally, we disrupted the colonization process of conventional SPF mice using perinatal antibiotic therapy which resulted in increased Proteobacteria, and more severe gluten-induced pathology in adulthood. The results provide evidence that distinct changes in the microbiota can either ameliorate or worsen responses to gluten in genetically susceptible hosts.

Introduction

Celiac disease (CD) is an immune-mediated enteropathy triggered by the ingestion of gluten in genetically susceptible individuals. Gluten peptides are resistant to degradation by proteolytic enzymes due to their high proline content, leaving large, immunogenic fragments that can translocate across the intestinal barrier.^{1,2} There is a key interplay between these gluten peptides, HLA-DQ2/DQ8 and tissue transglutaminase (TG-2) in the lamina propria leading to a pro-inflammatory adaptive immune response. The development of CD also requires innate immune activation. The upregulation of stress markers on epithelial cells and the activation of intraepithelial lymphocytes (IELs) contribute to intestinal epithelial cell damage and apoptosis.^{3,4} The exact triggers of innate immunity in CD remain unclear. The major CD susceptibility genes, HLA-DQ2/DQ8, are expressed in 30% of the general population; however, only 2-5% of genetically susceptible individuals will develop CD.⁵ This, in conjunction with the rapid rise in CD prevalence over the past 50 years, argues for other precipitating factors involved in the development of CD pathogenesis.⁶⁻⁸

The intestinal microbiota plays an important role in immune maturation and homeostasis. Studies using gnotobiotic technology have demonstrated that germ-free mice have a number of innate and adaptive immunological differences compared to conventionally raised mice.⁹⁻¹¹ Clinical and experimental evidence has also suggested that altered colonization early in life may increase susceptibility to chronic inflammatory diseases and food intolerances.¹²⁻¹⁶ CD is associated with alterations in intestinal microbial composition, some of which are normalized after treatment with a gluten-free

diet (GFD).^{17,18} Recent clinical evidence has proposed a link between antibiotic use, elective caesarean section (c-section) and CD development.¹⁹⁻²² However, it is unclear whether altered colonization instigates CD in an individual at risk of developing CD. Therefore, we investigated whether microbial colonization modulates host responses to gluten using transgenic DQ8 mice on the non-obese diabetic background (NOD/DQ8), a model to study gluten-sensitivity.²³⁻²⁵ We used germ-free and gnotobiotic clean specific pathogen free (SPF) mice, which are strictly monitored for the absence of a variety of pathogens, opportunistic and segmented filamentous bacteria. We also used conventional SPF mice, which harbour a complex microbiota containing opportunistic pathogens, including members of the phylum Proteobacteria, to determine the role of the background microbiota on gluten-induced pathology and immune responses. Finally, we evaluated whether disruption of gut colonization at birth by antibiotic treatment influences responses to gluten in adult conventional SPF mice.

Results

Colonization with a “clean” microbiota attenuates gluten-induced enteropathy in germ-free NOD/DQ8 mice.

To test the hypothesis that the background microbiota modulates host responses to gluten, we compared gluten-induced responses under strict gnotobiotic conditions, i.e., in germ-free and clean SPF NOD/DQ8 mice. Clean SPF mice, generated by colonizing germ free mice with an altered Schaedler flora (ASF) microbiota and bred in a gnotobiotic environment, harbour a limited, benign microbiota that is dominated by members of the

Bacteroidetes and Firmicutes phyla that is free from any pathogens, opportunistic bacteria, or bacteria from the Proteobacteria phylum (Table 1). Mice were sensitized with a pepsin-trypsin digest of gliadin (PT-gliadin) and cholera toxin, followed by challenges with gluten. For simplicity, this group will be called “gluten-treated” hereafter. Non-sensitized (control) mice received cholera toxin alone during the sensitization and were maintained gluten-free. Gluten treatment in germ-free, but not clean SPF NOD/DQ8 mice, led to a reduction in villus-to-crypt (V/C) ratios ($p < 0.001$; Figure 1a). Since germ-free mice have longer and thinner villi compared to colonized mice (Supplementary Figure S1)²⁶, V/C ratios were expressed as a percentage of their respective controls (i.e. non-sensitized germ-free or clean SPF respectively; Figure 1b). The analysis showed that the change in V/C ratios was greater under germ-free versus clean SPF conditions ($p < 0.001$; Figure 1c, e). IEL counts in villi tips, normalized *per* 100 enterocytes, were greater in germ-free mice following gluten treatment compared to clean SPF mice ($p < 0.001$; Figure 1d-f). These data suggest that commensal colonization with a balanced microbiota down regulates host responses to gluten.

Table 1: Clean SPF NOD/DQ8 mice have a limited, homeostatic microbiota devoid of bacterial pathogens and opportunists.

Phylum	Class	Order	Family	Genus	Clean SPF	SPF
Bacteroidetes	Bacteroidia	Bacteroidales	Unclassified	Unclassified	0%	3.7%
			Bacteroidaceae	Bacteroides*	0%	62.2%
			Porphyromonadaceae	Unclassified	0%	0.5%
				Parabacteroides	73.9%	14.3%
			Rikenellaceae	Unclassified	0%	0.3%
Alistipes	0%	0.1%				
Deferribacteres	Deferribacteres	Deferribacterales	Deferribacteraceae	Mucispirillum	1.9%	0%
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus*	0%	0.1%
		Gemellales	Gemellaceae	Gemella	0%	0.2%
		Lactobacillales	Aerococcaceae	Aerococcus	0%	0.1%
			Lactobacillaceae	Lactobacillus	0.1%	1.7%
			Streptococcaceae	Streptococcus*	0%	0.1%
		Turicibacterales	Turicibacteraceae	Unclassified	2.4%	0.1%
	Clostridia	Clostridiales	Unclassified	Unclassified	0.2%	0%
			ClostridialesFamilyXIII. IncertaeSedis	Eubacterium	0%	0.1%
			Lachnospiraceae	Unclassified	21%	13.1%
				Blautia	0.2%	0.2%
				Clostridium	0%	0.1%
			Ruminococcaceae	Unclassified	0.2%	0.2%
				Anaerotruncus	0%	0.1%
Proteobacteria	Betaproteobacteria	Burkholderiales	Unclassified	Unclassified*	0%	0.1%
	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	Helicobacter*	0%	0.1%
	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Escherichia*	0%	2.0%
Tenericutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Allobaculum	0%	0.4%
				Coprobacillus	0%	0.1%

Numbers indicate relative proportions at the genus level

*Contains pathogenic or opportunistic bacteria associated with inflammation

SPF, specific pathogen free; Cutoff- 0.1% abundance

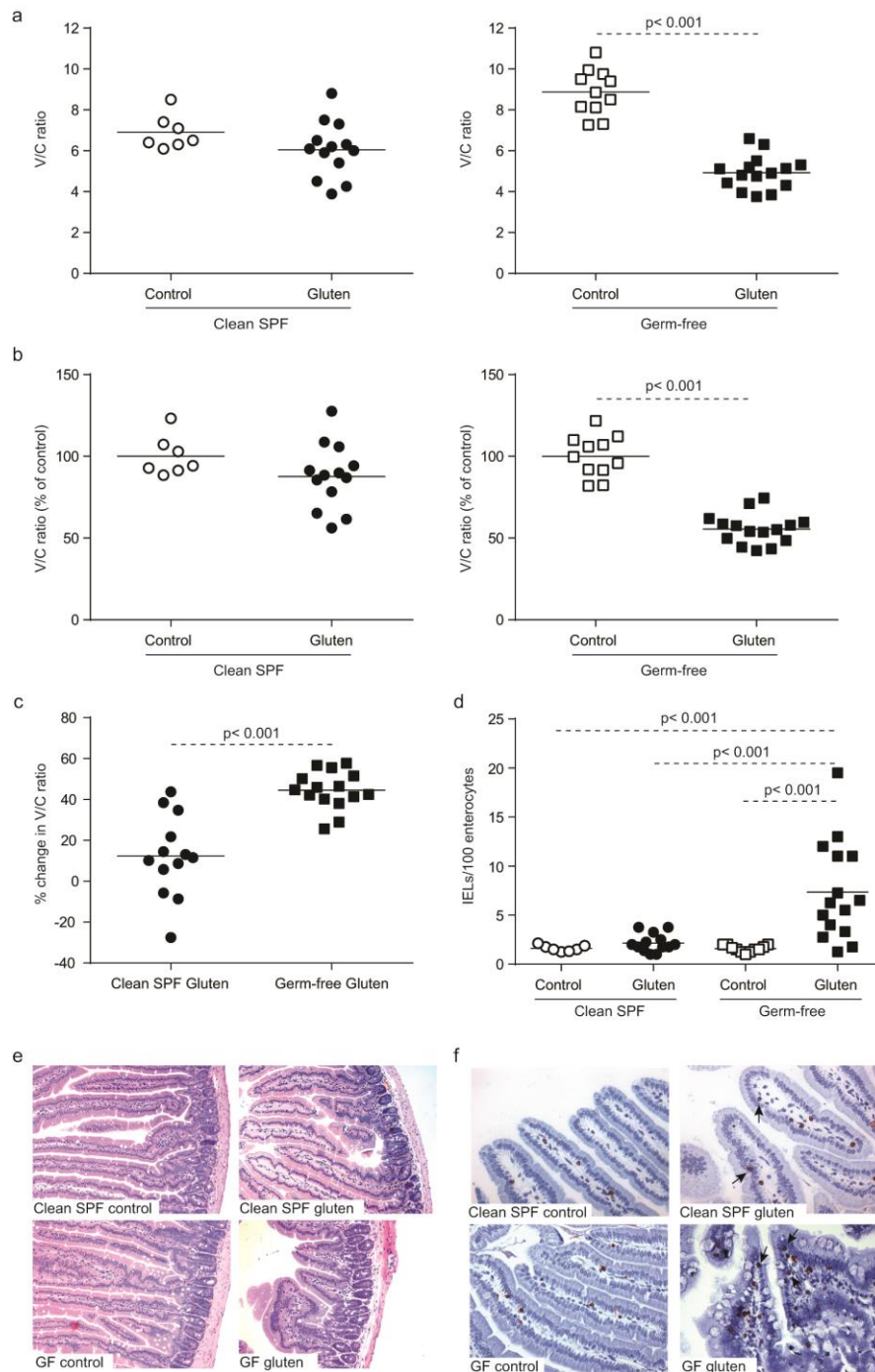


Figure 1: Colonization with a clean specific pathogen free (SPF) microbiota attenuates gluten-induced intraepithelial lymphocytosis and enteropathy in germ-free NOD/DQ8 mice. Gluten-treated germ-free and clean SPF NOD/DQ8 mice were sensitized with a pepsin-trypsin digest of gliadin (PT-gliadin) and challenged with gluten. Non-sensitized

controls were maintained gluten-free. Sections of jejunum collected 18-24 hours for following the final challenge for histology. (a) Quantification of villus-to-crypt (V/C) ratios in control and gluten-treated clean SPF and germ-free mice. (b) V/C ratios for clean SPF and germ-free control and gluten-treated mice, expressed as a percent of controls. (c) Percent change in V/C ratios in clean SPF and germ-free mice following gluten treatment. (d) Quantification of CD3⁺ cells in villi tips, expressed as intraepithelial lymphocytes (IEL)/100 enterocytes. Each dot represents an individual mouse. (e) Representative hematoxylin and eosin (H&E) stained sections of jejunum. Original magnification 10x. (f) Representative CD3⁺ stained sections of jejunum. Black arrows indicate examples of IELs. Original magnification 20x. Statistical significance determined by (a, b, c) unpaired T-test or (d) ANOVA with Bonferroni post-hoc test for multiple comparisons.

Colonization with a “clean” microbiota attenuates gliadin-specific responses.

Serum and intestinal washes were tested for the presence of anti-gliadin IgA and IgG antibodies. No clean SPF or germ-free control mouse developed anti-gliadin antibodies (AGA) (Figure 2a-c). In germ-free conditions, 4/10 gluten-treated mice developed positive AGA in serum (IgG and IgA) and intestinal washes (IgA; Figure 2a-c); in contrast, only 1/16 clean SPF did. Germ-free gluten-treated mice exhibited higher serum and intestinal IgA reactivity to gliadin when compared to either clean SPF controls ($p < 0.01$) or gluten-treated mice ($p < 0.01$, serum AGA; $p < 0.05$, intestinal AGA; Figure 3b, c). Increased antibody reactivity to specific gluten proteins of wheat was confirmed by Western blot analysis (Figure 3d).

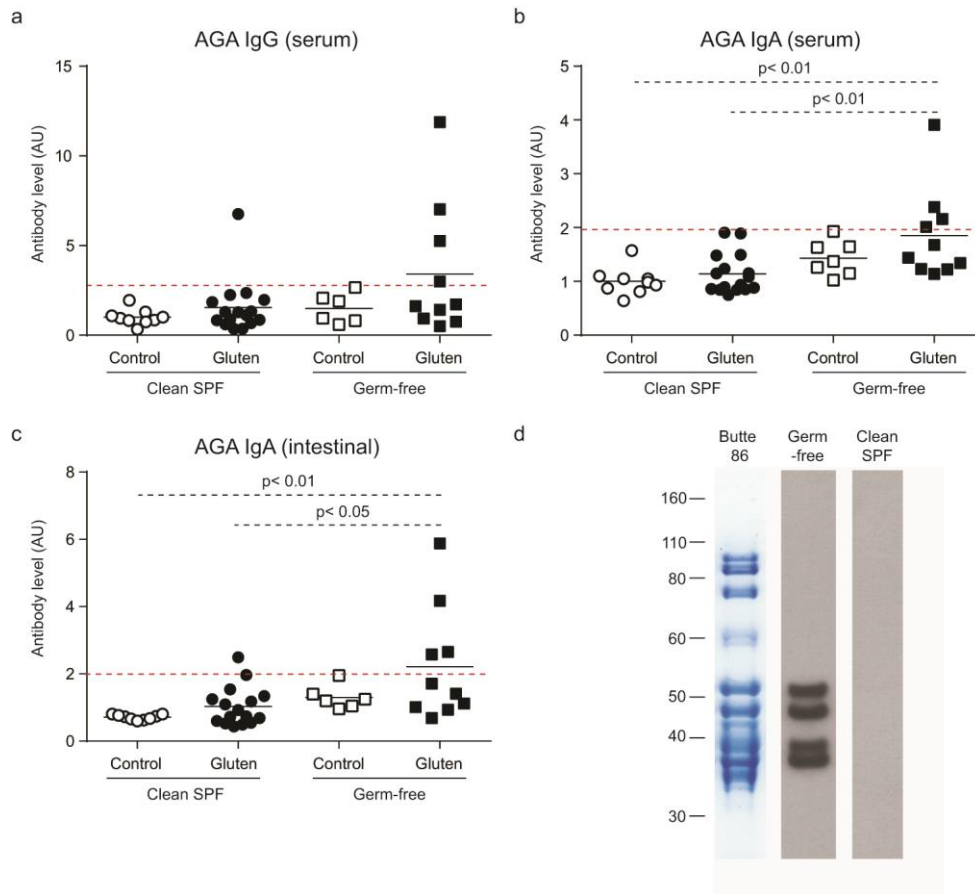


Figure 2: Colonization with a clean specific pathogen free (SPF) microbiota attenuates gliadin-specific antibody responses in germ-free NOD/DQ8 mice. Gluten-treated germ-free and clean SPF NOD/DQ8 mice were sensitized with a pepsin-trypsin digest of gliadin (PT-gliadin) and challenged with gluten. Non-sensitized controls were maintained gluten-free. Serum and intestinal washes were collected 18-24 hours following the final gluten challenge for determination of (a) serum anti-gliadin IgG, (b) serum anti-gliadin IgA, and (c) intestinal anti-gliadin IgA. Each dot represents an individual mouse. Positive reactivity was determined by using a positive cut-off value of ≥ 2 SD above the mean of control mice. (d) Confirmation of antibody reactivity to gluten proteins by Western blotting. SDS-PAGE profile of the gluten extract from the 'Butte 86' cultivar used for immunoblotting assays. Western blot reactivity of serum antibodies from a representative germ-free gluten-treated mouse, and a representative clean SPF control mouse. Molecular weight markers on the left are in kDa. Statistical significance determined by ANOVA with Bonferroni post-hoc test for multiple comparisons.

To further assess the immune reactivity to gliadin, we performed an *in vitro* T cell proliferation assay using CD4⁺ T cells isolated from the mesenteric lymph nodes (MLNs) of germ-free control and gluten-treated mice. We focused on germ-free mice due to their heightened gluten-induced responses compared to SPF (clean) mice. T cells were labelled with carboxyfluorescein succinimidyl ester (CFSE), co-cultured with dendritic cells (DCs) and stimulated with PT-gliadin, PT-zein, or medium alone. After three days, proliferation was determined by measuring CFSE intensity in CD4⁺ T cells. T cells isolated from gluten-treated mice responded to PT-gliadin stimulation significantly more than T cells stimulated with PT-zein ($p < 0.01$), medium ($p < 0.001$) or T cells isolated from non-sensitized mice stimulated with PT-gliadin ($p < 0.01$; Supplementary Figure S2). The results suggest gliadin-specific immune responses are attenuated by colonization with a clean SPF microbiota.

Protection from gluten treatment in clean SPF mice is not associated with changes in small intestinal T regulatory cells.

Next, we investigated regulatory T cell frequency in the small intestine of germ-free and clean SPF NOD/DQ8 mice before and after gluten treatment. It has previously been reported that there is a lower proportion of Tregs in the colon of germ-free mice.¹⁰ However, we found that, Tregs, defined as CD4⁺CD25⁺FOXP3⁺ T cells, were increased in the small intestine of naïve germ-free NOD/DQ8 mice compared to naïve clean SPF mice ($p < 0.01$; Supplementary Figure S3a, c). Furthermore, gluten treatment had no effect on Treg proportions regardless of microbial background (Supplementary Figure S3b, c).

In addition, we did not find significant differences in the levels of the regulatory cytokine IL-10 in the small intestine between groups (Supplementary Figure 3d).

Colonization with a “clean” microbiota attenuates gluten-induced IEL cytotoxicity and enterocyte cell death in germ-free NOD/DQ8 mice.

We evaluated different subsets of IELs and their cytotoxic activity through granzyme B and NKG2D expression by flow cytometry. Consistent with the literature,^{27,28} naïve germ-free mice had a higher proportion of $\gamma\delta$ T cell receptor (TCR)⁺ IELs compared to clean SPF mice (Supplementary Figure S4a). The proportions of $\gamma\delta$ TCR⁺ IELs or β TCR⁺ IELs did not change following gluten treatment in germ-free or clean SPF conditions (Supplementary Figure S4a-c). However, in germ-free mice, there was an increase in NKG2D⁺ β TCR⁺CD3⁺ IELs ($p < 0.05$; Figure 3a, b) and granzyme B⁺ β TCR⁺CD3⁺ IELs ($p < 0.05$; Figure 3c, d) following gluten treatment. No changes in NKG2D or granzyme B expression were observed in gluten-treated clean SPF mice compared to controls (Figure 3a-d). No changes in NKG2D or granzyme B were detected on $\gamma\delta$ TCR⁺ IELs in germ-free or clean SPF mice following gluten treatment (data not shown). In addition, no changes in TNF α , IFN γ , MCP-1, IL-6 or IL-12p70 were detected in clean SPF or germ-free gluten-treated mice (data not shown). IL-15 mRNA was expressed at very low levels in all groups, and did not change following gluten treatment in either clean SPF or germ-free mice (Supplementary Figure S5).

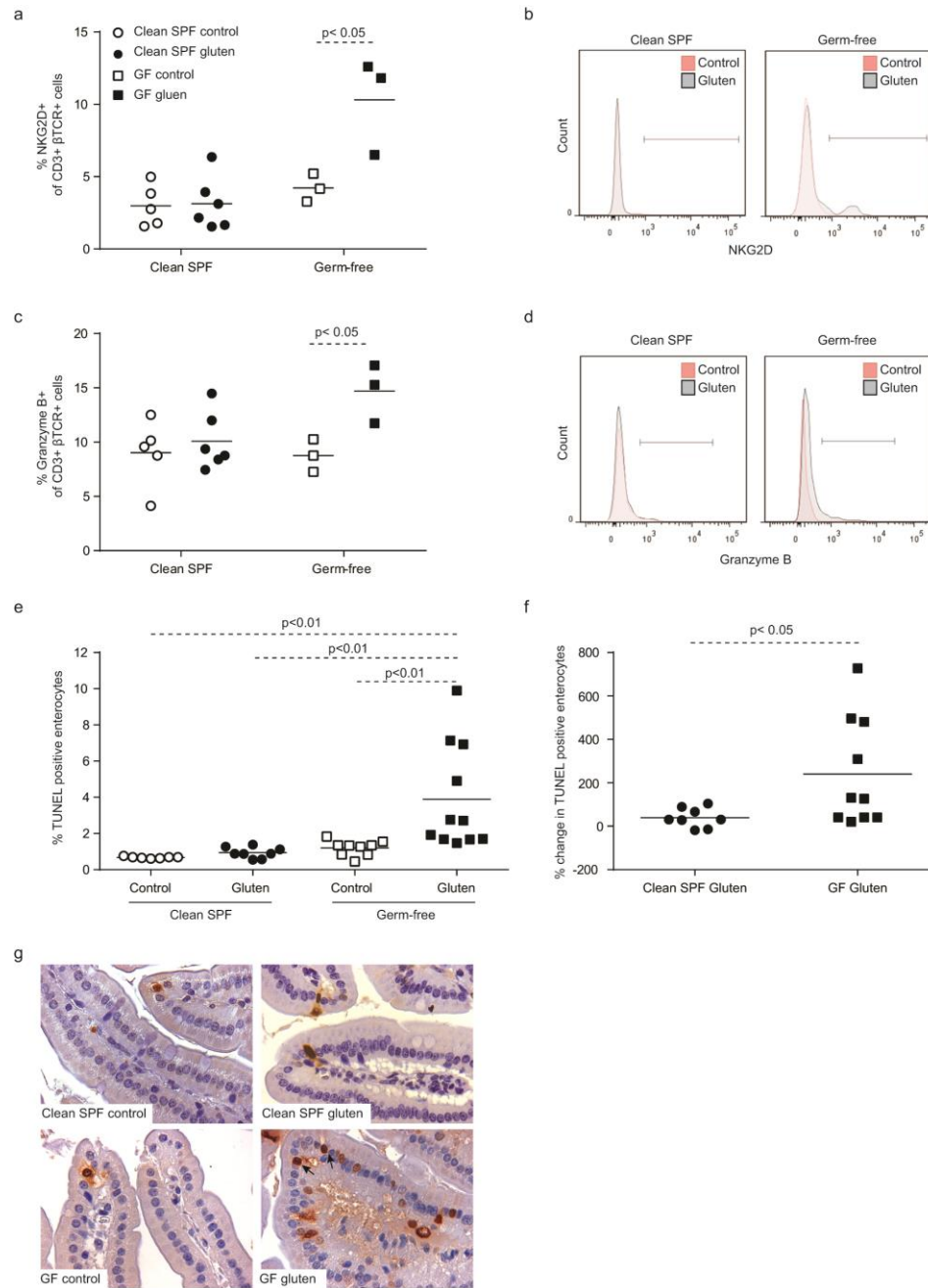


Figure 3: Colonization with a clean specific pathogen free (SPF) microbiota attenuates gluten-induced intraepithelial lymphocyte (IEL) cytotoxicity and enterocyte cell death in germ-free NOD/DQ8 mice. Intraepithelial lymphocytes (IELs) were isolated from the small intestine of non-sensitized controls and gluten-treated clean SPF and germ-free NOD/DQ8 mice and the expression of activation and cytotoxicity markers assessed by flow cytometry. (a) Quantification of NKG2D+ cells gated on β TCR+ CD3+ and (b) corresponding histograms of NKG2D+ cells gated on β TCR+ CD3+ from control (pink)

and gluten-treated (grey) mice. (c) Quantification of granzyme B+ cells gated on β TCR+ CD3+ lymphocytes and (d) corresponding histograms of granzyme B+ cells gated on β TCR+ CD3+ from control (pink) and gluten-treated (grey) mice. Each dot represents an individual mouse. Open circles/squares represent non-sensitized control mice and closed circles/squares represent gluten-treated mice. (e) Sections of jejunum were collected from gluten-treated and non-sensitized control clean SPF and germ-free mice and stained for TUNEL positive enterocytes. Quantification of the percent of TUNEL positive enterocytes in the villi of jejunum sections. (f) The percent change in TUNEL positive enterocytes in gluten-treated mice relative to non-sensitized controls. Each dot represents an individual mouse. (g) Representative TUNEL stained sections of the jejunum. Black arrows indicate examples of positive cells. Original magnification 20x. Statistical significance determined by (a, c, f) unpaired T-test or (e) ANOVA with Bonferroni post-hoc test for multiple comparisons.

Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) staining was performed on sections of the jejunum to determine if these changes in cytotoxicity were associated with enterocyte cell death in small intestinal villi. The percentage of TUNEL positive enterocytes was increased in gluten-treated germ-free, but not clean SPF mice compared to non-sensitized controls ($p < 0.01$; Figure 3e-g). The increase in enterocyte cell death following gluten treatment was greater in germ-free mice than in clean SPF mice ($p < 0.05$; Figure 3f, g). These data suggest that under germ-free conditions, gluten induces a cytotoxic IEL response that is associated with increased enterocyte cell death that is not observed under clean SPF conditions.

Conventional SPF mice develop more severe gluten-induced pathology compared to clean SPF NOD/DQ8 mice

Conventional SPF mice, harbour several members from the Proteobacteria phylum such as *Helicobacter* and *Escherichia* species (Table 1; Figure 4a). We found that gluten treatment induced a significant reduction in V/C ratios in conventional SPF ($p < 0.05$; Figure 4b-d), which was greater compared to clean SPF mice ($p < 0.05$; Figure 4c). Gluten treatment also increased IELs in villi tips in conventional SPF mice ($p < 0.01$; Figure 4e, f). In sharp contrast, no increase in IELs was detected in clean gluten-treated SPF mice (Figure 4e, f). The blunted pathological reactivity to gluten in clean SPF mice compared to conventional SPF reveals a complex modulatory role of the microbiota to gluten, which may include exacerbation of responses due to the presence of opportunists in a conventional SPF microbiota.

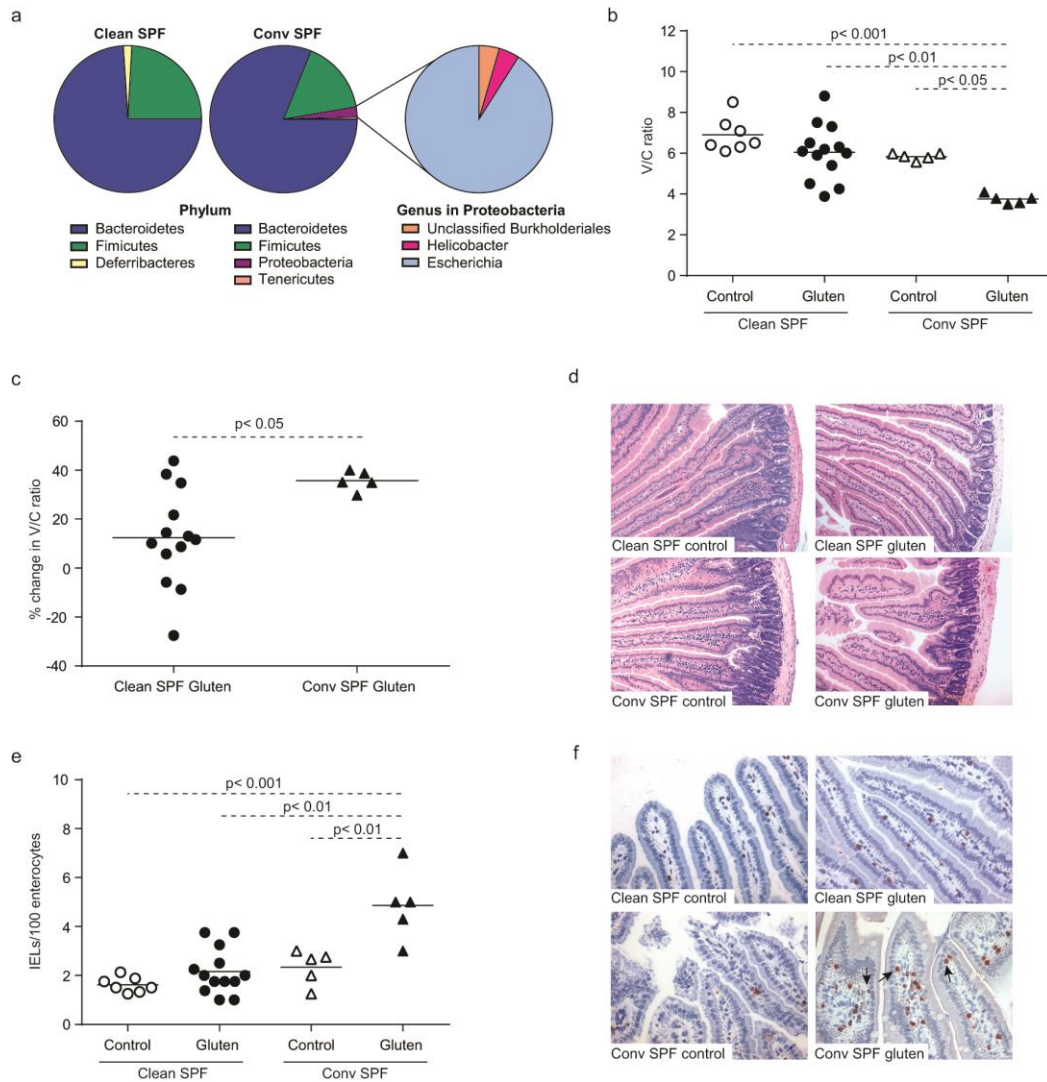


Figure 4: Conventional specific pathogen free (SPF) mice harbour opportunistic bacteria and develop more severe gluten-induced pathology compared to clean SPF NOD/DQ8 mice, which are bred under gnotobiotic conditions and are free from any opportunists. (a) Microbial composition from gnotobiotic clean SPF and conventional (conv) SPF NOD/DQ8 mice by 16s rRNA sequencing at the phylum and genus level. Gluten-treated clean and conv SPF NOD/DQ8 mice were sensitized with a pepsin-trypsin digest of gliadin (PT-gliadin) and challenged with gluten. Non-sensitized controls were maintained gluten-free. Sections of jejunum were collected for histology. (b) Quantification of villus-to-crypt (V/C) ratios in control and gluten-treated clean SPF and conv SPF mice. (c) Percent change in V/C ratios in clean SPF and conv SPF mice following gluten treatment, relative to controls. Each dot represents an individual mouse. (d) Representative

hematoxylin and eosin (H&E) stained sections of jejunum. Original magnification 10x. (e) Quantification of CD3⁺ cells in villi tips, expressed as intraepithelial lymphocytes (IEL)/100 enterocytes. Each dot represents an individual mouse. (f) Representative CD3⁺ stained sections of the jejunum are shown for gluten-treated and control clean and conv SPF mice. Black arrows indicate examples of IELs. Original magnification 20x. Statistical significance determined by (b, e) ANOVA with Bonferroni post-hoc test for multiple comparisons or (c) unpaired T-test.

Supplementation of clean SPF microbiota with E. coli ENT CA15 increases severity of gluten-induced pathology in NOD/DQ8 mice.

Since conventional SPF microbiota harbours bacteria from the Proteobacteria phylum, including *Escherichia spp* absent in clean SPF microbiota (Table 1), we investigated whether supplementation of clean SPF microbiota with an enteroadherent *E. coli* ENT CA15, isolated from a CD patient and carrying several virulence genes (*fimA* and *kfiC*)²⁹ reversed its protective effect (Figure 5a). *E. coli*-supplemented clean SPF mice developed reduced V/C ratios ($p < 0.01$; Figure 5b, c) and increased IEL counts ($p < 0.01$; Figure 5d, e) after gluten treatment compared to clean SPF mice, supporting the notion that presence of specific groups of bacteria contribute to exacerbate responses to gluten.

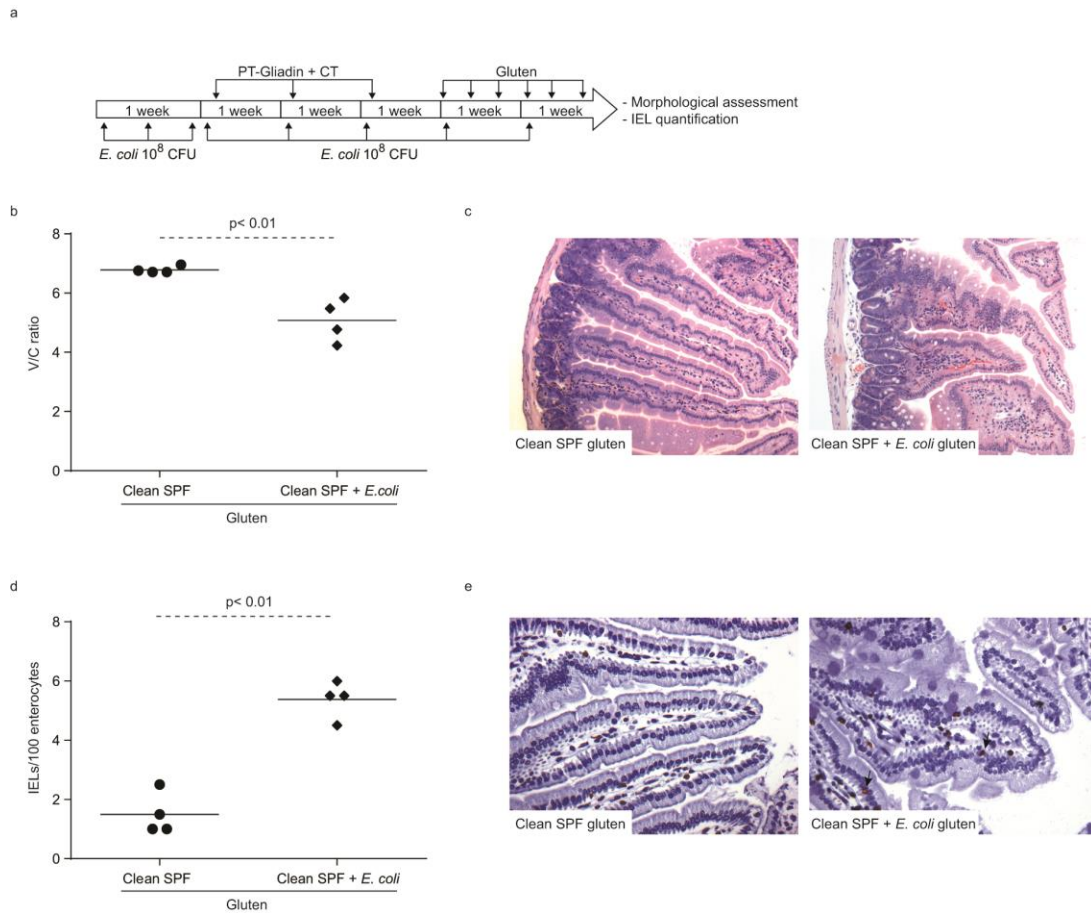


Figure 5: Supplementation of clean specific pathogen free (SPF) microbiota with *E. coli* ENT CA15 increases severity of gluten-induced pathology in NOD/DQ8 mice. (a) *E. coli*-supplemented clean SPF and clean SPF mice were sensitized with a pepsin-trypsin digest of gliadin (PT-gliadin) and challenged with gluten. Sections of jejunum were collected for histological analysis. (b) Quantification of villus-to-crypt (V/C) ratios in gluten treated clean SPF and *E. coli*-supplemented clean SPF mice. Each dot represents an individual mouse. (c) Representative hematoxylin and eosin (H&E) stained sections of jejunum. Original magnification 10x. (d) Quantification of CD3+ cells in villi tips, expressed as intraepithelial lymphocytes (IEL)/100 enterocytes. Each dot represents an individual mouse. (e) Representative CD3+ stained sections of the jejunum. Black arrows indicate examples of IELs. Original magnification 20x. Statistical significance determined by unpaired T-test.

Perinatal antibiotic treatment of conventional NOD/DQ8 mice increases responses to gluten in adulthood

Conventional SPF NOD/DQ8 mice were treated with antibiotics *in utero* and as neonates to determine whether altered intestinal colonization modifies the host response to gluten-sensitization later in life. Pregnant mice received vancomycin (200 mg/L) in drinking water until pups were weaned at 3 weeks of age, after which gluten-treated pups were sensitized with PT-gliadin and cholera toxin and challenged with gluten. Non-antibiotic-treated mice received sterile water alone (Figure 6a). Additional non-sensitized (control) mice received cholera toxin alone and no gluten challenges. Sequencing analysis revealed antibiotic-treatment resulted in an increase in phyla Proteobacteria ($p < 0.01$; Figure 6b, d) and Firmicutes ($p < 0.05$; Figure 6b) and reductions in phyla Actinobacteria ($p < 0.01$; Figure 6b), Bacteroidetes ($p < 0.01$; Figure 6b, d) and Tenericutes ($p < 0.01$; Figure 6b). Overall, there was a lower fecal microbiota diversity ($p < 0.01$; Figure 6c). Antibiotic-treated mice had greater abundances of *Escherichia* ($p < 0.01$; Figure 6e), *Helicobacter* ($p < 0.01$; Figure 6e), *Pasteurella* ($p < 0.01$; Figure 6e) and an unclassified Betaproteobacteria ($p < 0.01$; Figure 6e). The increase in Firmicutes was due primarily to increased *Lactobacillus* genus ($p < 0.01$; Figure 6f). Additionally, there was loss of the family Lachnospiraceae ($p = 0.056$; Figure 6f). Within the Bacteroidetes phylum, significant reductions were attributed to loss of *Bacteroides* ($p < 0.01$; Figure 6g) and *Parabacteroides* genera ($p < 0.01$; Figure 6g).

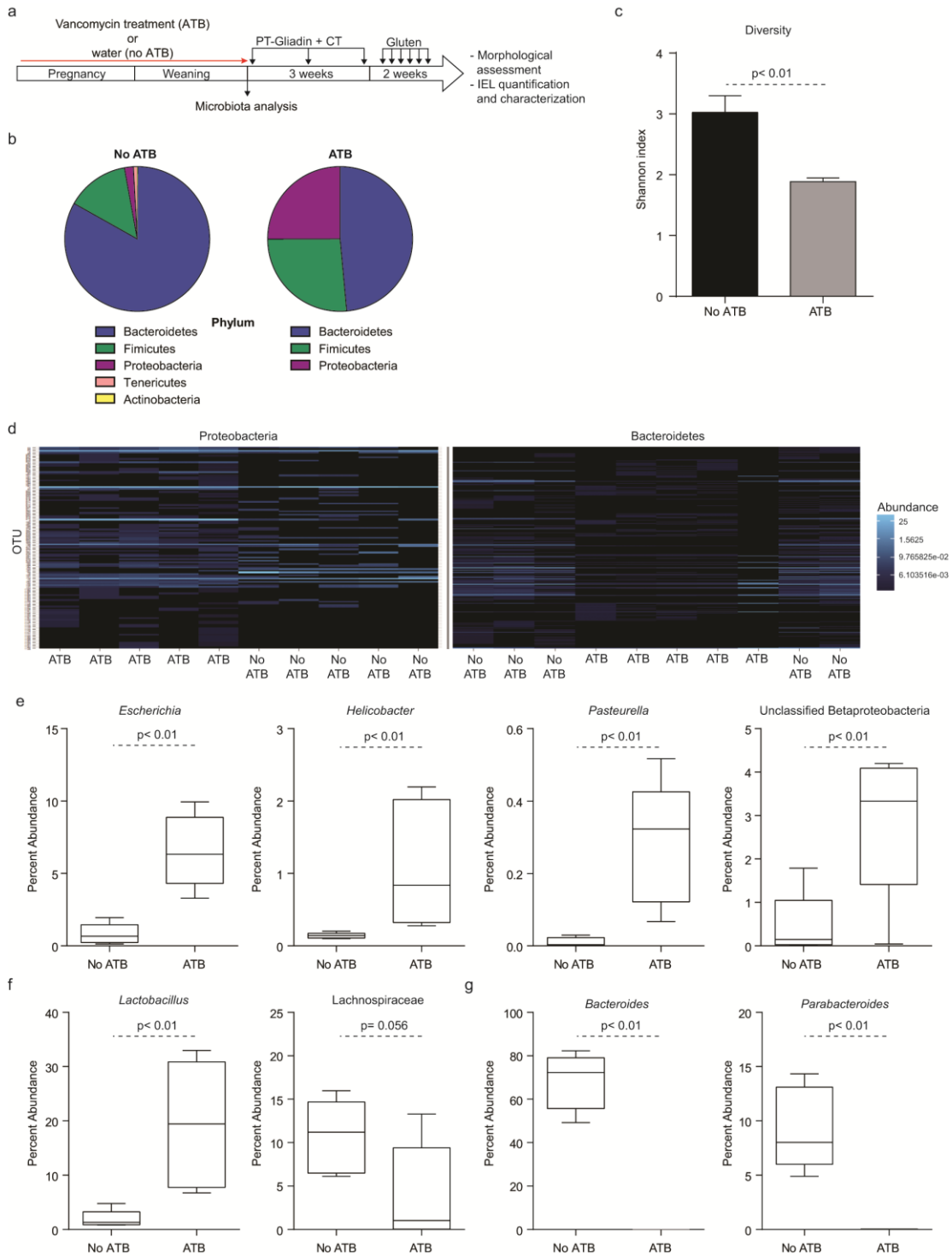


Figure 6: *In utero* and neonatal vancomycin treatment induces long term changes in microbial composition in conventional specific pathogen free (SPF) NOD/DQ8 mice. (a)

Pregnant conventional SPF mice received vancomycin in drinking water until pups were weaned at 3 weeks of age. Non-antibiotic (ATB) treated mice received water alone. Gluten-treated mice were then sensitized with a pepsin-trypsin digest of gliadin (PT-gliadin) plus cholera toxin (CT) and challenged with gluten. Additional non-sensitized mice that received CT alone and no gluten challenges served as controls. Fecal pellets were collected from mice at 3 weeks of age for microbial analysis by 16s rRNA sequencing. Histological changes were assessed following the final gluten challenge. (b) Microbial composition of mice that received water alone (no ATB) or ATB-containing water at the phylum level. 0.01% abundance used as cut-off. (c) Microbial diversity of mice that received water alone (no ATB) or ATB-containing water, expressed via Shannon Index. (d) Heat map of Proteobacteria and Bacteroidetes Phyla, each band representing a unique OTU. (e) Percent abundance of genera *Escherichia*, *Helicobacter*, *Pasteurella* and unclassified Betaproteobacteria. (e) Percent abundance of genera *Bacteroides* and *Parabacteroidetes*. (f) Percent abundance of genera *Lactobacillus* and family Lachnospiraceae. Data represented as mean \pm standard error of the mean (s.e.m; c) or as median, with each box extending from the 25th to 75th percentile and whiskers extending from the minimum to maximum value (e, f, g). N=5 per group. Statistical significance was determined by unpaired T-test (c) or Mann–Whitney test (e, f, g).

Antibiotic-induced changes in microbiota composition were associated with heightened gluten-induced responses following sensitization. V/C ratios decreased in gluten-treated mice treated with (p<0.001) or without antibiotic (p< 0.05; Figure 7a, c). However, the gluten-induced decrease in V/C ratios was greater in vancomycin-treated mice compared to non-antibiotic-treated mice (p<0.01; Figure 7b). IELs increased in gluten-treated mice compared to non-sensitized controls treated with (p<0.001) or without antibiotic (p<0.01). However, IEL counts were greater in gluten and antibiotic-treated mice compared to gluten, non-antibiotic-treated mice (p<0.05; Figure 7d, e). Furthermore, antibiotic treatment led to an increased proportion of β TCR⁺ IELs (p<0.05; Figure 7f, g). No changes in NKG2D or granzyme B expression were detected following gluten-treatment in either group (data not shown). Together, these studies demonstrate that

perturbation of early colonization in life and induction of dysbiosis, characterized by increased Proteobacteria, enhanced the severity of gluten-induced responses in NOD/DQ8 mice.

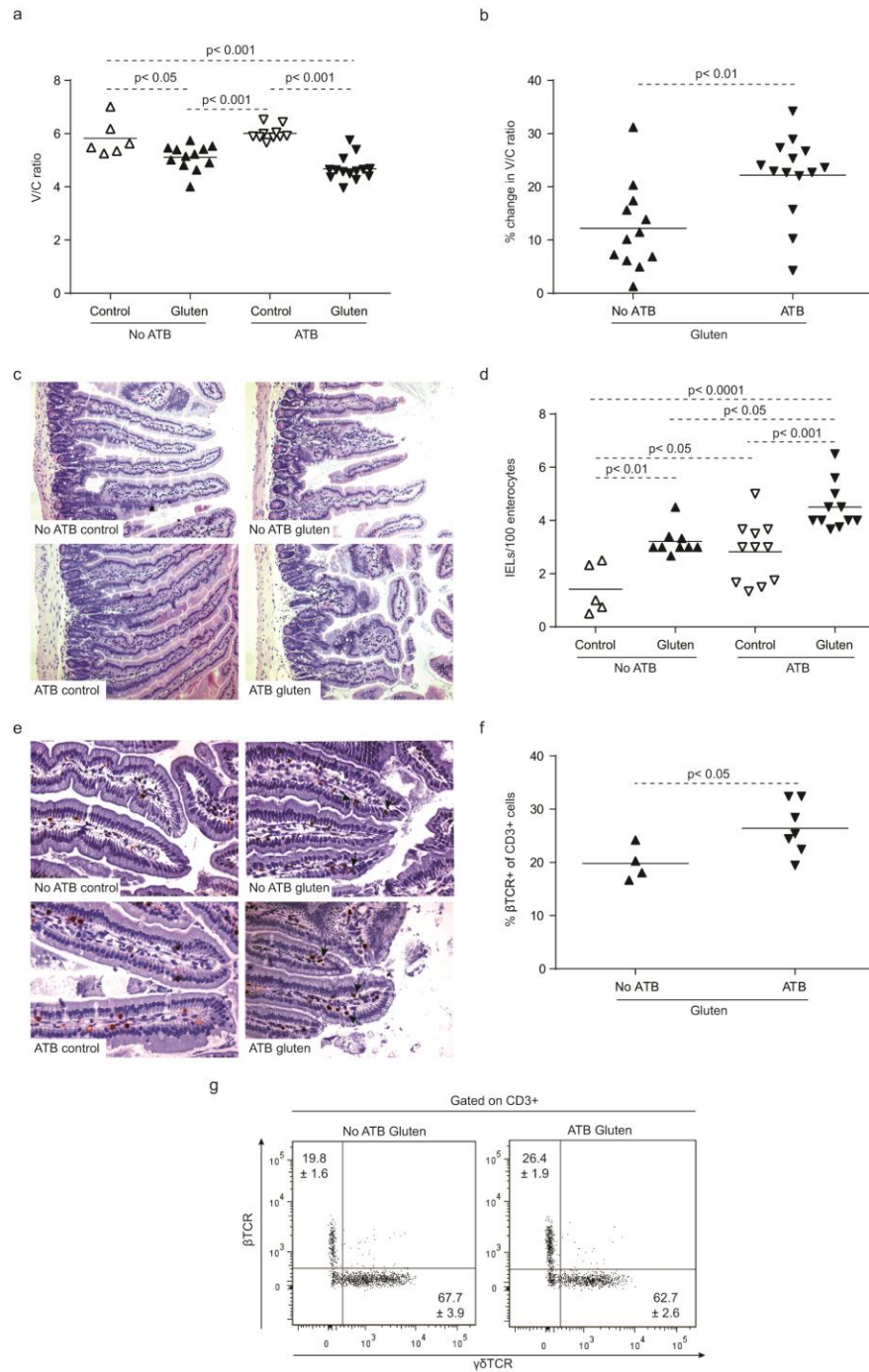


Figure 7: Perturbation of the colonization process in NOD/DQ8 mice increases severity of gluten-induced pathology. Pregnant conventional specific pathogen free (SPF) NOD/DQ8 females were placed on vancomycin-containing water or water alone. Pups were reared on antibiotic (ATB)-containing water or water alone (no ATB) until weaning, after which gluten-treated mice were sensitized with a pepsin-trypsin digest of gliadin (PT-gliadin) and challenged with gluten. Additional non-sensitized mice served as controls. Sections of jejunum were collected 18-24 hours following the final gluten challenge for histological analysis. (a) Quantification of villus-to-crypt (V/C) ratios in sections of the jejunum of control and gluten-treated mice that received water alone (no ATB) or ATB-containing water. (b) The percent change in V/C ratios in gluten-treated mice relative to non-sensitized controls. Each dot represents an individual mouse. (c) Representative hematoxylin and eosin (H&E) stained sections of the jejunum. Original magnification 10x. (d) Quantification of CD3⁺ cells in villi tips, expressed as intraepithelial lymphocytes (IEL)/100 enterocytes. Each dot represents an individual mouse. (e) Representative CD3⁺ stained sections of jejunum. Black arrows indicate examples of IELs. Original magnification 20x. IELs were isolated from the small intestine of gluten-treated that received water alone (no ATB) or ATB-containing water and the expression of T cell receptors was determined by fluorescence-activated cell sorting (FACS). (f) Quantification of the percentage of β TCR⁺ cells gated on CD3⁺ lymphocytes. Each dot represents an individual mouse. (g) Representative FACS plots for $\gamma\delta$ TCR⁺ and β TCR⁺ CD3⁺ IELs are shown with the mean \pm standard error of the mean (s.e.m) indicated. Statistical significance determined by (a, d) ANOVA with Bonferroni post-hoc test for multiple comparisons or (b, f) unpaired T-test.

Discussion

The incidence of CD has risen dramatically over the last five decades suggesting an important role for environmental factors in disease development.^{6,7} A role for the intestinal microbiota as an adjuvant factor has been suggested based on studies demonstrating changes in intestinal microbial composition in CD patients compared to healthy controls.¹⁸ Some case control and cohort studies investigating the association between antibiotic use and birth delivery mode have further implicated altered intestinal

colonization in CD development.¹⁹⁻²² However, a modulatory role of the microbiota on gluten induced responses has remained elusive. We used a gnotobiotic approach to test the hypothesis that the background microbiota constitutes an environmental factor that modulates host responses to gluten. We report here that NOD/DQ8 mice colonized with a “clean” microbiota free from any opportunistic bacteria and bred in gnotobiotic conditions (clean SPF) developed an attenuated gluten-induced response compared to germ-free mice. On the other hand, conventional SPF mice, which harbour a diverse microbiota containing opportunistic pathogens belonging to the Proteobacteria phylum developed stronger gluten-induced responses compared to clean SPF mice. The data indicate a complex modulatory effect of the microbiota to the dietary protein gluten. While a protective effect of colonization was observed using a balanced commensal microbiota (clean SPF), presence of Proteobacteria and other opportunists in a conventional microbiota as well as supplementation of clean SPF microbiota with *E. coli* ENT CA15, a component of Proteobacteria phylum that was isolated from a CD patient, led to more severe gluten-induced pathology. Finally, we disrupted the colonization process of conventional SPF mice using perinatal antibiotic therapy. Antibiotic-induced changes of the microbiota were associated with increased Proteobacteria and an increased severity of gluten-induced pathology and immune responses later in life, supporting a contributory role of the microbiota in the modulation of host responses to gluten.

The microbiota of our clean SPF colony is derived from ASF³⁰ and is primarily composed of bacteria that are important for intestinal health. These include Porphyromonadaceae and Lachnospiraceae families, which account for the majority of

the “clean SPF” microbiota, and have been shown to possess anti-inflammatory properties.^{16,31,32} Colonization with clean SPF microbiota³³ or with the original 8-strain ASF¹¹ has previously been shown to induce a balanced maturation of the murine immune system. Germ-free NOD/DQ8 mice developed increased intraepithelial lymphocytosis and enteropathy compared to clean SPF mice following gluten treatment. Increased enterocyte apoptosis of intestinal villi and increased proliferation of small intestinal crypts have been reported in CD.³⁴ The finding is also in agreement with an earlier study, which demonstrated that long-term gliadin feeding to germ-free rats induced moderate small intestinal damage, including crypt hyperplasia, villous atrophy, and increased IELs. Unfortunately, interpretation of this study is limited by the lack of appropriate colonized control rats.³⁵

More severe enteropathy in gluten-treated germ-free mice compared to clean SPF was accompanied by a heightened gliadin-specific adaptive response as well as a more cytotoxic IEL phenotype and increased enterocyte cell death. IELs from gluten treated germ-free mice had increased expression of NKG2D and granzyme B, markers that mediate epithelial cell death³⁶ and are increased in IELs from active CD patients. Our findings indicate that colonization with a clean microbiota, free from opportunistic pathogenic bacteria, protected from both innate and adaptive gluten-induced responses. The results, in concert with a recent report in a peanut allergy model of which commensal bacteria down regulated cholera-toxin driven responses to this protein,¹⁶ establish the ability of the commensal microbiota to pervasively modulate immune responses to dietary antigens.

Increased expression of IL-15 has been found in a proportion of CD patients,³⁷ and animal models have reported IL-15-mediated gluten or OVA induced-enteropathy.^{37,38} In addition, there is evidence that IL-15 can induce IEL activation.³⁹ We found that IL-15 expression was low and no changes were detected between germ-free or clean SPF groups at the transcriptional level. There may be several reasons to explain these apparently divergent findings. First, the biological role and regulation of IL-15 is very complex,^{40,41} methodological issues have been raised regarding its measurement⁴² and IL-15 mRNA levels may not always correlate with protein levels.⁴³ Second, and perhaps more importantly, NOD mice have been reported to have reduced IL-15 gene expression.⁴⁴ This suggests that IL-15 may not mediate the increased susceptibility to gluten in this model. Future studies using HLA-DQ8 gnotobiotic mice (without NOD background) and mice that are transgenic for IL-15 overexpression³⁷ may help clarify the interplay between the microbiota, gluten, and IL-15.

Tregs play a central role in oral tolerance.⁴⁵ Although the role of the microbiota in modulating oral tolerance is controversial,^{46,47} it has been demonstrated that colonic Tregs are reduced in germ-free mice.^{10,32} However, less is known about microbiota-induced modulation of small intestinal Tregs. In contrast with what has previously been demonstrated in the colon of germ free mice, the proportion of small intestinal lamina propria Tregs was higher in naïve germ-free NOD/DQ8 mice compared to clean SPF mice. Furthermore, there was no change in Tregs following gluten-treatment in germ-free or clean SPF mice. Thus, changes in Treg frequency do not explain the different reactivity to gluten between germ-free and clean SPF mice. Although we did not detect changes in

small intestinal levels of the regulatory cytokine IL-10 between groups, the results do not rule out that differences in Treg function may underlie this observation. However, studies have demonstrated that small intestinal Tregs from patients with CD⁴⁸ or IL-15 transgenic mice³⁸ are suppressive, but that effector T cells become unresponsive to Tregs. The responsiveness of effector T cells to Treg suppression in our model remains to be tested.

Conventional SPF NOD/DQ8 mice have previously been shown to respond to gliadin-sensitization and challenge, developing moderately decreased V/C ratios, increased IEL counts in villi tips and increased intestinal permeability compared to non-sensitized controls.^{23,24} The increased IEL response in conventional SPF compared to clean SPF conditions suggests that some opportunistic pathogenic bacteria of the conventional SPF microbiota may modulate IEL proliferation after gluten. Bacteria belonging to the Proteobacteria phylum were found in the conventional SPF microbiota, while they were absent in the clean SPF colony. Supplementation of clean SPF microbiota with *E. coli* ENT CA15, exacerbated gluten-induced intestinal damage and IEL responses, suggesting that particular bacterial species may modulate host-responses to gluten. In line with this, increased abundance of Proteobacteria, including *E. coli*, have been reported in CD children.^{49,50} Furthermore, *E. coli* isolated from CD children was shown to carry higher number of virulence genes²⁹ and induce pro-inflammatory cytokine production and activation markers in responses to gluten in PBMC cultures, dendritic cell cultures, and intestinal loops.⁵¹⁻⁵³ Further studies are needed to determine the mechanisms through which certain strains of *E. coli* may enhance host responses to gluten.

Our experiments indicated that commensal bacterial colonization influenced immune activation and host responses to gluten. More importantly, both the absence of commensal colonization and the type of bacteria colonizing the gut led to differential responses to gluten. In order to further investigate this, we performed additional experiments using perinatal antibiotic treatment of conventional NOD/DQ8 mice to deliberately perturb the normal colonization process. Early-life antibiotic treatment led to significant changes in the microbial profiles at 3 weeks of age in NOD/DQ8 mice. The most altered phyla were Proteobacteria, Firmicutes and Bacteroidetes. Antibiotic treatment led to increased Proteobacteria and Firmicutes. The increase in Proteobacteria was primarily due to increases of the genera *Escherichia*, *Helicobacter*, *Pasteurella* and unclassified Betaproteobacteria. Expansion of opportunistic pathogens in this phylum has previously been reported in response to vancomycin treatment.⁵⁴ Interestingly, infants with a high genetic risk for CD have elevated levels of Proteobacteria, including *Escherichia*.⁵⁵ The antibiotic-induced changes in the microbiota early in life were associated with increased total IELs, with or without gluten, as well as increased β TCR⁺ IELs in adult gluten-treated mice, an IEL subset that has been shown to be responsible for small intestinal enteropathy associated with CD.⁵⁶ By comparison, $\gamma\delta$ TCR IELs have been shown to have protective roles in the disease.⁵⁷ It has been demonstrated that $\alpha\beta$ TCR IELs are induced by Gram-negative bacteria via TLR-4 signaling,⁵⁸ and we show that antibiotic treatment led to increased Gram-negative bacteria. Combinatory effects of both an increase in Proteobacteria and presence of gliadin could explain the higher IEL numbers in antibiotic and gluten-treated mice, leading to more severe enteropathy. This is

in agreement with our findings where *E. coli* supplementation to a benign microbiota exacerbated host-responses to gluten. However, unlike germ-free mice, changes in NKG2D or granzyme B expression were not detected in IELs following gluten-treatment in antibiotic treated mice. Germ-free mice have immature immune systems, and the mechanisms leading to heightened host-responses to gluten and increased IEL activation in germ-free versus colonized mice may be different. The results indicate that several pathways may be involved in the microbial modulation of gluten-induced host responses.

Interventions that impact early life colonization, such as delivery by c-section and antibiotic use,^{59,60} have been associated with increased CD risk.^{19,20} Our study provides a potential causative link to these epidemiological observations as it demonstrates that distinct changes in the microbiota can either ameliorate or enhance responses to the dietary protein, gluten, in NOD/DQ8 mice and suggest that changes in microbiota composition may be part of the initial events triggering CD in humans. These findings also support that specific microbiota-directed therapies such as probiotics or selective antimicrobials, may aid in the prevention or treatment of gluten-related disorders in genetically susceptible individuals.

Methods

Mice and colonization procedures

Female and male conventional SPF, clean SPF and germ-free NOD AB^o DQ8 (NOD/DQ8) transgenic mice were used for experiments.⁶¹ Mice had unlimited access to autoclaved gluten-free food and water. NOD/DQ8 mice were re-derived germ-free at the

McMaster University Axenic Gnotobiotic Unit (AGU) by two-cell embryo transfer technique as previously described.³³ Germ-free mice were maintained in flexible film isolators in the AGU. Additional germ-free NOD/DQ8 mice were naturally colonized by co-housing with female mouse colonizers harbouring ASF microbiota. These mice were subsequently bred in individually ventilated cage racks within the AGU and regularly monitored for pathogen contamination or microbiota diversification by culture and non-culture techniques, and referred to as clean SPF (see supplementary methods for more details). Conventional SPF mice were bred and maintained in a conventional SPF facility at McMaster University. For *E. coli* supplementation experiments, additional 8-12 week old clean SPF mice were supplemented with *E. coli* ENT CA15. *E. coli* ENT CA15 was originally isolated from fecal microbiota of CD patients as previously described and is known to carry pathogenic-associated genes.²⁹ Mice were gavaged with 100ul of 10⁹ CFU/mL of *E. coli* ENT CA15 three times a week, one week prior to the start of sensitization. During the sensitization and challenge, mice were gavaged with 10⁸ CFU of *E. coli* ENT CA15 once per week. All mice were weaned and maintained on a gluten-free diet. All experiments were conducted with approval from the McMaster University Animal Care Committee.

Gluten-sensitization and challenge

NOD/DQ8 mice were sensitized with sterilized PT-gliadin (500µg) and cholera toxin (25µg; Sigma-Aldrich, St. Louis, MO) by oral gavage once a week for three weeks, as previously described.²³ PT-gliadin was prepared as previously described.²³ In antibiotic experiments, mice were sensitized at 3 weeks of age, following weaning. For all other

experiments, 8-12 week old mice were used for sensitizations. Following PT-gliadin sensitization, “gluten-treated” mice were challenged three times a week for two weeks with 2mg of sterile gluten (Sigma-Aldrich) dissolved in acetic acid by oral gavage. Non-sensitized control mice received cholera toxin alone during the sensitization phase and acetic acid alone during challenges. All mice were maintained on a gluten-free diet. Mice were sacrificed 18-24 hours following the final gluten challenge. All preparations were tested for LPS contamination using the E-Toxate kit (Sigma-Aldrich).

Microbial analysis

Fecal and cecal samples were collected from mice under sterile conditions and subsequently flash frozen on dry ice. DNA was extracted from samples using methods that have been previously described.⁶² Extracted DNA then underwent amplification for the hypervariable 16S rRNA gene v3 region as previously described⁶³ and sequenced on the Illumina MiSeq platform. Generated data was analyzed as described previously.⁶² Briefly, sequences were trimmed using Cutadapt,⁶⁴ aligned using PANDAseq,⁶⁵ OTUs selected via AbundantOTU,⁶⁶ and taxonomy assigned against the Greengenes reference database.⁶⁷ α -diversity was calculated using Quantitative Insights Into Microbial Ecology (QIIME)⁶⁸ and heat maps were generated using R.⁶⁹

Evaluation of small intestinal inflammation

Cross sections of the jejunum were fixed in 10% formalin, embedded in paraffin and stained with hematoxylin and eosin (H&E) for histological evaluation by light microscopy (Olympus, Richmond Hill, ON) using Image Pro Plus (Media Cybernetics,

Rockville, MD). Signs of enteropathy were determined by measuring villus-to-crypt ratios, as previously described.²³ Briefly, twenty villus-to-crypt ratios were measured for each mouse in a blinded fashion. Intraepithelial lymphocytosis in jejunum sections was determined by counting the number CD3⁺ IELs per 20 enterocytes in five randomly chosen villus tips as previously described and expressed as IEL/100 enterocytes.⁷⁰ CD3⁺ immunostaining was performed on paraffin embedded sections of the jejunum as previously described.²³

Anti-gliadin ELISA

Serum IgA and IgG antibodies to gliadin were measured by ELISA as previously described,^{71,72} with minor modifications, as detailed in supplementary methods. In addition, intestinal wash IgA antibodies to gliadin were measured similarly. Intestinal wash IgG antibody reactivity was too low to be detected reliably and was not measured.

Anti-gliadin Western blots

Antibody reactivity to gluten in sera was confirmed by Western blot. The 'Butte 86' gluten extract was dissolved in sample buffer, heated for 10 min at 75°C, and separated by SDS-PAGE (0.66 µg protein/lane) using NuPAGE 4-20% bis-tris gels (Life Technologies, Grand Island, NY). Protein transfer onto nitrocellulose membranes was carried out with the iBlot Dry Blotting System (Life Technologies). The membrane was incubated for 2 h in blocking solution (5% milk + 0.5% BSA in TBS-T). Incubation with serum specimens (1:500) in dilution buffer (10% blocking solution + 10% fetal bovine serum in TBS-T) was done for 1 h. The secondary antibody used was HRP-conjugated

anti-mouse IgG or IgA. Detection of bound antibodies was by the ECL system (Millipore, Billerica, MA) and autoradiography film (Fuji, Valhalla, NY).

Small intestinal lamina propria and IEL preparation

Small intestines from mice were flushed to remove intestinal contents, Peyer's patches and mesentery were removed, intestines opened longitudinally, and cut into 3-5mm pieces. Intestinal pieces were incubated 5-6 times in 25mLs of EDTA/HEPES/DPBS for 15 minutes in a 37°C shaker. After each 15 minute incubation intestines were vigorously vortexed and the IELs were collected by passing the supernatants through a 40µm cell strainer. Intestinal pieces were further digested in 25 mL of IMDM containing DNase I (Roche, Mississauga, ON), Collagenase Type VIII (Sigma-Aldrich) and HEPES for 50 minutes in a 37°C shaker to collect lamina propria lymphocytes. IELs and lamina propria lymphocytes were enriched on a Percoll gradient. Cells were collected from the 30%/100% interface, washed, and resuspended in FACS buffer for cell staining.

Flow cytometry

Single cell suspensions of lamina propria preparations were stained with fluorochrome-labeled cell-surface antibodies including CD4-APC (RM4-5), CD8a-PerCP (53-6.7), and CD25-PE (7D4) purchased from BD Biosciences. IEL cell suspensions were stained with fluorochrome-labeled cell-surface antibodies including CD3-Alexa Fluor-700 (ebio500AZ; eBioscience, San Diego, CA), CD3-Pacific Blue (RM4-5; BD), CD8-PerCP (53-6.7; BD), TCRβ (H57-597; eBioscience), TCRγδ-APC (eBioGL3;

eBioscience), NKG2D-PE (CX5; eBioscience), and CD69-PE-CF594 (H1.2F3; BD). For intracellular staining, cells were permeabilized using the Foxp3 staining buffer set (eBioscience). Lamina propria cells were incubated with FITC-conjugated Abs toward Foxp3 (FJK-16s; eBioscience) and IELs were incubated with PE-CY7 conjugated Granzyme-B (NGZB; eBioscience) for 90 min at 4°C. Stained cells were acquired using the LSR II (BD Biosciences) and analyzed with FlowJo software (TreeStar).

TUNEL staining

Enterocyte cell death in jejunum sections was determined by TUNEL staining using the ApopTag® Peroxidase *In Situ* Apoptosis Detection Kit (Millipore, Billerica, MA) according to the manufacturer's instructions. Slides were viewed by light microscopy (Olympus). The percentage of TUNEL-positive enterocytes in 20 villi was determined for each mouse.

Antibiotic treatment

Pregnant SPF NOD/DQ8 mice were placed on 200 mg/L vancomycin (Sigma-Aldrich) in sterile drinking water and continued after birth until pups were weaned at 3 weeks of age. Vancomycin-containing water was replaced every 3 days. Fecal pellets were collected at 3 weeks of age for microbial analysis by 16s rRNA sequencing. Additional mice originating from non-antibiotic-treated NOD/DQ8 mice served as controls.

Statistics

Statistical significance was determined by ANOVA with the Bonferroni post-hoc test for multiple comparisons when comparing more than two groups. Unpaired T-test was used to compare two groups. For all microbial analysis, Mann–Whitney *U* test was used. Differences were considered significant when $p < 0.05$. All statistical analysis was performed in GraphPad Prism Software (Version 6).

Supplementary material is linked to the online version of the paper.

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Disclosure: none to declare

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Supplemental Methods

Bacteriology monitoring of germ-free and clean SPF mice.

Microbiota diversification in mouse cecum contents of “clean SPF” mice was evaluated every 2 weeks in cage sentinels and at the end of the study in the experimental mice by PCR for *Helicobacter bilis*, *Helicobacter ganmani*, *Helicobacter hepaticus*, *Helicobacter mastomyrinus*, *Helicobacter rodentium*, *Helicobacter spp.*, *Helicobacter typhlonius* and *Pneumocystis murina*. Mouse serum was also tested for murine viral pathogens by MFIA/ELISA/IFA. Germ free status was monitored in sentinels and, at the end of the study in the experimental mice, by immunofluorescence (SYTOX® Green), anaerobic and aerobic culture as well as PCR technique.

Anti-gliadin ELISA

One hundred mg of the U.S. hard red spring wheat *Triticum aestivum* ‘Butte 86’ variety flour was suspended in 1 mL of phosphate-buffered saline (PBS) and mixed for 1 h at 4 °C. The suspension was centrifuged at 10,000 g for 20 min. The supernatant containing mostly non-gluten proteins, was removed. The pellet was washed with phosphate buffered saline (PBS), resuspended in 50% isopropanol, and mixed for 1 h at room temperature. The suspension was centrifuged at 10,000 x g for 20 min and the supernatant, containing gliadin and glutenin proteins, was stored at -20 °C. 96-well Maxisorp round-bottom polystyrene plates (Nunc, Roskilde, Denmark) were coated with 50 µL/well of a 0.01 mg/mL solution of the gliadin gluten extract in 0.1 M carbonate

buffer (pH 9.6) or were left uncoated to serve as control wells. After incubation at 37 °C for 1 h, all wells were washed and blocked by incubation with 1% bovine serum albumin (BSA) in phosphate buffered saline containing 0.05% Tween-20 (PBS-T) for 1.5 h at room temperature. Serum samples were diluted at 1:100, while intestinal wash samples were diluted at 1:10. The samples were added at 50 µL/well in duplicates and incubated for 1 h. Each plate contained a positive control sample. After washing the wells, they were incubated with a 1:2000 dilution of either HRP-conjugated anti-mouse IgG (GE Healthcare, Piscataway, N.J.) or IgA (Abcam, Cambridge, MA) secondary antibodies for 50 min. The plates were washed and 50 µL of developing solution, comprising of 27 mM citric acid, 50 mM Na₂HPO₄, 5.5 mM *o*-phenylenediamine, and 0.01% H₂O₂ (pH 5), was added to each well. After incubating the plates at room temperature for 20 min, absorbance was measured at 450 nm. Absorbance values were corrected for non-specific binding by subtraction of the mean absorbance of the associated BSA-coated control wells. The corrected values were first normalized according to the mean value of the positive control duplicate on each plate. The mean antibody level for the clean SPF control group was then set as 1.0 AU and all other results were normalized accordingly.

T cell proliferation assay

Cell suspensions of MLN were prepared in RPMI 1640 (1% penicillin/streptomycin, 10% FCS, 2 mM L-glutamine) by passing organs through a 100-µm nylon mesh screen to dissociate cells. CD4⁺ T cells were isolated from MLNs through negative selection (EasySep Mouse CD4⁺ T Cell Enrichment Kit; Stemcell, Vancouver, BC). Cell

yield was assessed by fluorescence-activated cell sorting (FACS) analysis, with an enriched population of 89–96% CD4⁺ T cells. Isolated CD4⁺ T cells were labeled with CFSE as previously described and washed three times with RPMI 1640.¹ Dendritic cells were isolated from spleens through positive selection (Easysep Mouse CD11c Selection Kit, Stemcell). Dendritic cells (5×10^4 cells/well) were co-cultured with isolated CD4⁺ T cells (2×10^5 cells/well) in the presence of PT-gliadin (500 µg/mL), PT-zein (500 µg/mL), or media alone in a round-bottom 96-well plate. For positive controls, cells were cultured with plate-bound anti-CD3 (BD Biosciences, Mississauga, ON) and soluble anti-CD28 (BD Biosciences). Cells were cultured for 3 d at 37°C, 5% CO₂. Cells were harvested on day 3 to assess proliferation by FACS analysis. Cells were resuspended in FACS buffer and stained with flouochrome conjugated antibodies to CD3 and CD4 and a viability stain. CFSE-labeled cells were acquired using the LSR II (BD Biosciences). Viable cells were gated on CD4⁺ T cells. CFSE intensity for this population was determined using FlowJo software (TreeStar, Ashland, OR).

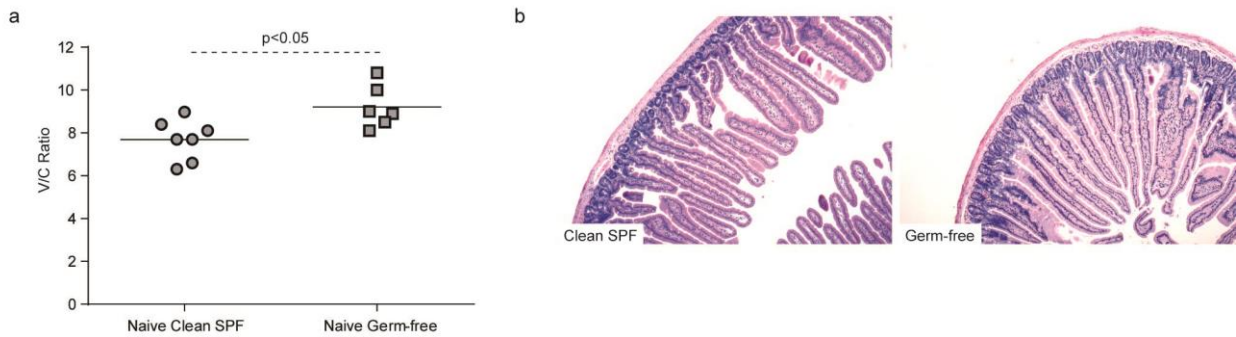
Cytokine measurement

Sections of the jejunum were collected 18-24 hours following the final gluten challenge and homogenized. Cytokines were measured in tissue supernatants using the Mouse Inflammatory CBA kit (BD Biosciences) and analyzed using FACSarray Bioanalyzer System (BD Biosciences).

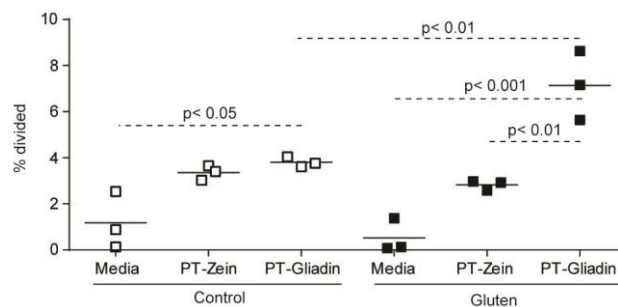
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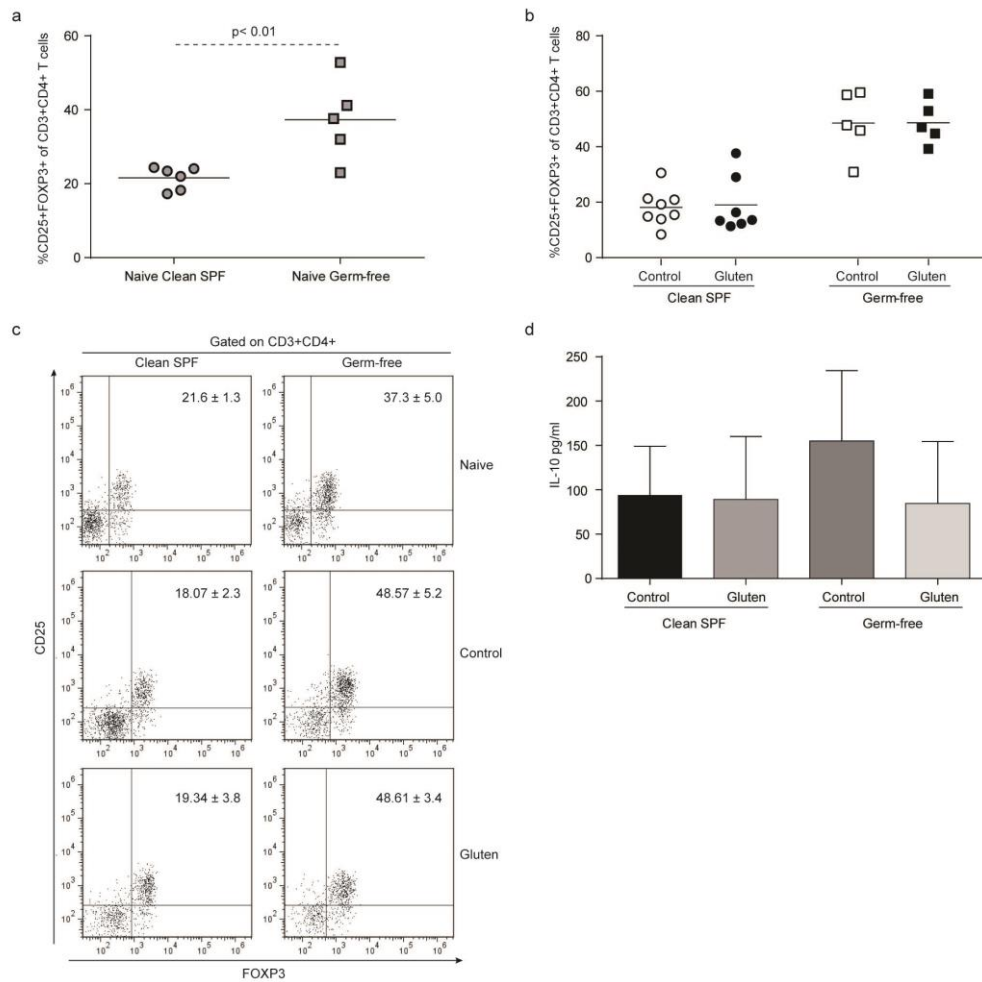
Supplementary Figures



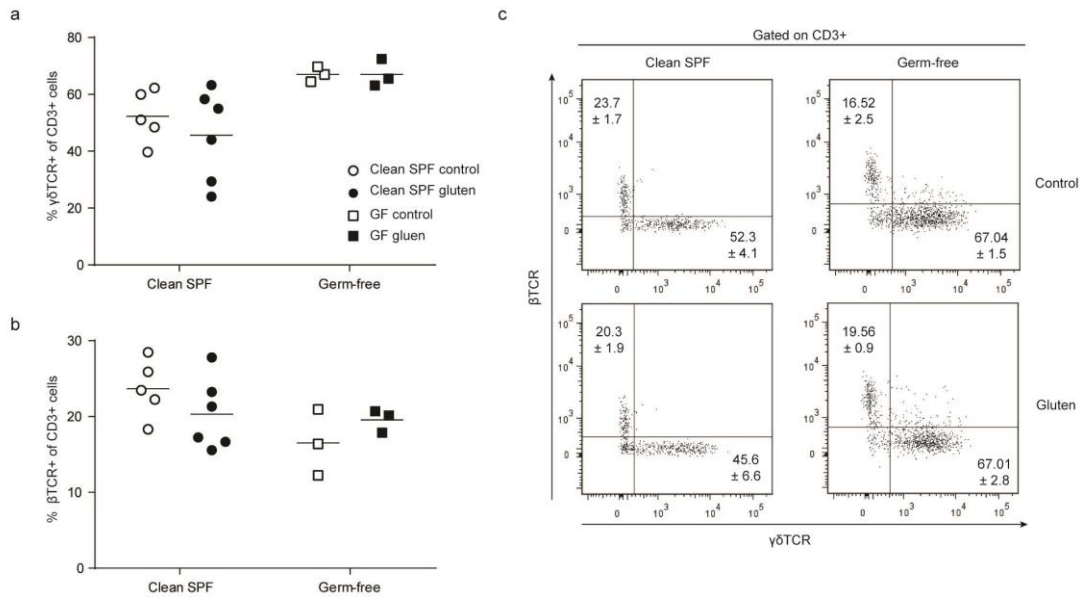
Supplementary Figure S1: Naïve germ-free NOD/DQ8 mice have greater villus-to-crypt (V/C) ratios compared to clean specific pathogen free (SPF) NOD/DQ8 mice. (a) Quantification of V/C ratios in jejunum sections from naïve clean SPF and germ-free NOD/DQ8 mice. Each dot represents an individual mouse. (b) Representative hematoxylin and eosin (H&E) stained jejunum sections from naïve clean SPF and germ-free NOD/DQ8 mice. Original magnification 4x. Statistical significance determined by unpaired T-test.



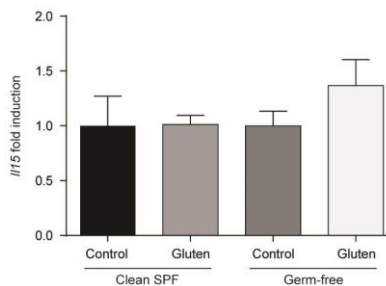
Supplementary Figure S2: Gluten-treatment in germ-free NOD/DQ8 mice induces a gliadin-specific mucosal T cell response. CD4⁺ T cells were isolated from mesenteric lymph nodes (MLNs) of non-sensitized control and gluten-treated germ-free NOD/DQ8 mice, labelled with CFSE, and stimulated with pepsin-trypsin (PT)-gliadin, PT-zein, or medium. T cell proliferation was determined by flow cytometry. Statistical significance determined by ANOVA with Bonferroni post-hoc test for multiple comparisons.



Supplementary Figure S3: Gluten treatment in clean specific pathogen free (SPF) and germ-free NOD/DQ8 mice is not associated with changes in small intestinal regulatory T cells (Tregs). Lymphocytes were isolated from the small intestinal lamina propria of naïve, non-sensitized control and gluten-treated clean SPF and germ-free NOD/DQ8 mice and the expression of regulatory T cell markers was assessed by flow cytometry. Quantification of CD25+Foxp3+ cells gated on CD3+CD4+ lymphocytes and in the small intestine of (a) naïve, (b) controls and gluten-treated clean SPF and germ-free mice. Each dot represents an individual mouse. (c) Representative fluorescence-activated cell sorting (FACS) plots of small intestinal CD25+FOXP3+ cells gated on CD3+CD4+ cells in naïve, non-sensitized control, and gluten-treated clean SPF and germ-free mice are shown with the mean ± standard error of the mean (s.e.m) indicated. (d) Quantification of IL-10 in small intestinal tissue samples of non-sensitized control and gluten-treated clean SPF and germ-free mice. N=5-6 per group. Statistical significance determined by (a) unpaired T-test or (d) ANOVA.



Supplementary Figure S4: Gluten treatment does not induce changes in β TCR+ or $\gamma\delta$ TCR+ IEL frequency in clean specific pathogen free (SPF) or germ-free NOD/DQ8 mice. Intraepithelial lymphocytes (IELs) were isolated from the small intestine of non-sensitized controls and gluten-treated clean SPF and germ-free NOD/DQ8 mice and the expression of β TCR and $\gamma\delta$ TCR was determined by flow cytometry. Quantification of (a) $\gamma\delta$ TCR+ cells gated on CD3+ lymphocytes and (b) β TCR+ cells gated on CD3+ lymphocytes. Each dot represents an individual mouse. (c) Representative fluorescence-activated cell sorting (FACS) plots for $\gamma\delta$ TCR+ and β TCR+ CD3+ IELs are shown with the mean \pm standard error of the mean (s.e.m) indicated.



Supplementary Figure S5: Gluten treatment in clean specific pathogen free (SPF) and germ-free NOD/DQ8 mice is not associated with increased IL-15. *I/15* mRNA expression in the small intestine, normalized to GAPDH, and expressed as fold induction relative to controls. N=6-10 per group, mean \pm standard error of the mean (s.e.m). Statistical significance determined by ANOVA.

CHAPTER 5

**THE COPOLYMER P(HEMA-CO-SS) BINDS GLUTEN
AND REDUCES IMMUNE RESPONSE IN GLUTEN-SENSITIZED MICE
AND HUMAN TISSUES.**

**The copolymer P(HEMA-co-SS) binds gluten and reduces immune response in
gluten-sensitized mice and human tissues.**

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Preface: The work in this manuscript was performed from 2009 to 2012. I am third author of the manuscript. Experiments were designed and conducted by M. Pinier, myself, J.C Leroux and my supervisor, E.F Verdu. *In vitro* and *in vivo* rat experiments were designed and conducted by M. Pinier, G. Fuhmann, J.C Leroux and my supervisor, E.F Verdu. *In vivo* experiments in HLA/DQ8 mice were designed by M. Pinier, myself, J.C, Leroux, and E.F Verdu and conducted by M. Pinier and myself. This thesis will focus on results from HLA/DQ8 mouse experiments. I analyzed data related to chronic *in vivo* mouse experiments. The manuscript was written by M. Pinier, myself, J.C, Leroux, and E.F Verdu. HLA/DQ8 mice were provided by C.S David and J.A Murray. P(HEMA-co-SS) was designed by Jean Cristophe Leroux at the University of Montreal. Nathalie Rivard performed co-culture *in vitro* studies and Hana Drasarova and Ludmila Tuckova provided and conducted *in vitro* studies in patients' samples.

Summary and significance: The current treatment for celiac disease (CD) is a strict adherence to a gluten free diet (GFD). However, the GFD is expensive, difficult to maintain, and is often unsatisfactory to celiac patients. Furthermore, gluten contamination and non-adherence can lead to persistent mucosal damage and symptoms. Therefore, this article utilized *in vitro* assays and HLA/DQ8 mice, previously characterized by our lab, to investigate the potential therapeutic effectiveness of the gluten-binding polymer, P(HEMA-co-SS). Administration of P(HEMA-co-SS) to gluten-sensitive mice reduced increased paracellular permeability, anti-gliadin IgA levels, and systemic T cell responses following an acute gluten-containing meal challenge. Moreover, P(HEMA-co-SS) administration also protected against gluten-induced mucosal damage and barrier dysfunction following a chronic challenge with a gluten-containing meal. These findings expand previous work demonstrating the effectiveness of P(HEMA-co-SS) on ameliorating mild gluten-induced inflammation following a systemic gluten-sensitization protocol. Importantly, the findings support the therapeutic safety and efficacy of P(HEMA-co-SS) when administered with a more clinically relevant mixture of food proteins that includes gluten.

Title: The copolymer P(HEMA-*co*-SS) binds gluten and reduces immune response in gluten-sensitized mice and human tissues.

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Abbreviations used in this paper: BSA, bovine serum albumin; GFD, gluten-free diet; HD, high dose; IL, interleukin; LC-MS, liquid chromatography–mass spectrometry; LD, low dose; MCP-1, monocyte chemotactic protein 1; P(HEMA-*co*-SS), poly(hydroxyethyl methacrylate-*co*-styrene sulfonate); PT, peptic-tryptic; TNF, tumor necrosis factor

Abstract

BACKGROUND & AIMS: Copolymers of hydroxyethyl methacrylate and styrene sulfonate complex with isolated gliadin (the toxic fraction of gluten) and prevent damage to the intestinal barrier in HLA-HCD4/DQ8 mice. We studied the activity toward gluten and hordein digestion and biologic effects of poly(hydroxyethyl methacrylate-*co*-styrene sulfonate (P(HEMA-*co*-SS)). We also investigated the effect of gliadin complex formation in intestinal biopsy specimens from patients with celiac disease.

METHODS: We studied the ability of P(HEMA-*co*-SS) to reduce digestion of wheat gluten and barley hordein into immunotoxic peptides using liquid chromatography–mass spectrometry. The biodistribution and pharmacokinetic profile of orally administered P(HEMA-*co*-SS) was established in rodents using tritium-labeled polymer. We assessed the capacity of P(HEMA-*co*-SS) to prevent the immunologic and intestinal effects induced by a gluten-food mixture in gluten-sensitized HLA-HCD4/DQ8 mice after short-term and long-term administration. We measured the effects of gliadin complex formation on cytokine release *ex vivo* using intestinal biopsy specimens from patients with celiac disease.

RESULTS: P(HEMA-*co*-SS) reduced digestion of wheat gluten and barley hordein *in vitro*, thereby decreasing formation of toxic peptides associated with celiac disease. After oral administration to rodents, P(HEMA-*co*-SS) was predominantly excreted in feces, even in the presence of low-grade mucosal inflammation and increased intestinal permeability. In gluten-sensitized mice, P(HEMA-*co*-SS) reduced paracellular

permeability, normalized anti-gliadin immunoglobulin A in intestinal washes, and modulated the systemic immune response to gluten in a food mixture. Furthermore, incubation of P(HEMA-*co*-SS) with mucosal biopsy specimens from patients with celiac disease showed that secretion of tumor necrosis factor- α was reduced in the presence of partially digested gliadin.

CONCLUSIONS: The copolymer P(HEMA-*co*-SS) reduced digestion of wheat gluten and barley hordein and attenuated the immune response to gluten in a food mixture in rodents. It might be developed to prevent or reduce gluten-induced disorders in humans.

Introduction

Celiac disease is an inflammatory condition of the small intestine triggered by the ingestion of gluten in genetically susceptible individuals. Contrary to previous beliefs, celiac disease is a common disorder with a worldwide prevalence of approximately 1%.^{1, 2 and 3} Genetic predisposition is conferred by HLA, since 90%–95% of affected people exhibit HLA-DQ2 molecules and the remainder exhibit HLA-DQ8.⁴ Recent genome-wide association studies have revealed that non-HLA genetic factors are involved in celiac disease.^{5 and 6} During the past 2 decades, tremendous progress has been made toward understanding the pathophysiologic processes in celiac disease and its clinical presentation.⁷ Some patients without villous atrophy but reporting symptomatic responses to a gluten-free diet (GFD) are labeled “gluten sensitive.”⁸ Both patients with untreated celiac disease and gluten-sensitive patients with low-grade inflammation have been reported to have increased morbidity and mortality.^{9 and 10}

No pharmacologic therapies are available to gluten-intolerant patients, and a GFD remains the cornerstone treatment. However, strict gluten restriction for life is far from simple, because it can carry a significant psychosocial and financial burden.¹¹ Nonadherence to a GFD is common because gluten is often used as an additive in processed foods.¹² Therefore, complementary therapeutic options are required. Several attractive strategies are currently under investigation.¹³ Research is being performed to develop celiac-safe wheat by enzymatic treatment¹⁴ or by wheat gene modulation¹⁵ and bacterial fermentation.¹⁶ Another strategy using oral exogenous enzyme intake aims at

reducing gluten toxicity by cleaving immunogenic peptide sequences before gluten ingestion or directly in the gut lumen.^{17, 18 and 19} Modulators of intestinal permeability have been developed to diminish uptake of gluten peptides into the gastrointestinal mucosa.^{20 and 21} Other experimental therapies include restoration of oral tolerance by administration of gluten peptides secreted by *Lactococcus*²² and immunomodulation by helminths.²³ Finally, tissue transglutaminase inhibitors^{24 and 25} to prevent peptide selective deamidation, HLA-DQ groove antagonists²⁶ to block the T-CD4 lymphocyte recognition, and inhibitors of adhesion molecules to hinder inflammatory cell recruitment²³ are also under development.

We have recently explored a novel approach to block gluten toxicity, based on the use of the polymeric binder poly(hydroxyethyl methacrylate-*co*-styrene sulfonate) (P(HEMA-*co*-SS)). Such an approach could prove useful as a supportive therapy for celiac disease when the gluten-free quality is not ascertained or when gluten exposure is low (a few milligrams). Polymeric binders are already used in various clinical disorders to sequester compounds in the gastrointestinal tract.²⁷ P(HEMA-*co*-SS) was shown to complex α -gliadin (the gluten protein fraction predominantly involved in celiac disease) in a relatively selective fashion. The binder counteracted the toxic effects of gliadin on intestinal epithelial cells in vitro. It also reduced the jejunum mucosal toxicity induced by oral gavage of isolated gliadin in mice that had been sensitized with gliadin. Complementary experiments on the ileum mucosa suggested that the deleterious effects of gliadin did not occur farther down in the gastrointestinal tract in the presence of P(HEMA-*co*-SS). Furthermore, these studies indicated no detectable adverse effects, even

at high doses.²⁸ In this report, we provide evidence that P(HEMA-*co*-SS) has potential for the treatment of gluten-induced disorders by decreasing the formation of pathogenic peptide sequences from wheat and barley. The efficacy of P(HEMA-*co*-SS) was tested by measuring jejunum permeability parameters, the mucosal and systemic immune activation after administration of wheat gluten/food mixture to orally gluten-sensitized mice. We further show that the polymer remains mainly in the gastrointestinal tract because it was almost completely recovered in the feces. Lastly, the capacity of P(HEMA-*co*-SS) to reduce the production of immunogenic gliadin peptides was established by measuring cytokine secretion in mucosal biopsy specimens of patients with celiac disease.

Materials and Methods

*Effect of P(HEMA-*co*-SS) on Wheat Gliadin, Gluten, and Barley Hordein Digestion*

All reagents were purchased from Sigma–Aldrich (St Louis, MO) unless otherwise stated. P(HEMA-*co*-SS) (47:53 ± 2%, 45 and 59 kilodaltons) was synthesized by atom transfer radical polymerization as described elsewhere.²⁸ After completion of the digestion process, samples were analyzed at least in triplicate by liquid chromatography–mass spectrometry (LC-MS) (Supplementary Materials and Methods).

Absorption Experiments

All animal experiments were approved by the McMaster University and University of Montreal Animal Care Committees, in accordance with the Canadian Council on Animal Care guidelines. [³H]-P(HEMA-*co*-SS) (1 mCi/mg) was prepared by

tritium gas exchange chromatography by American Radiolabeled Chemicals (St Louis, MO). Study design and analytical methods are provided in Supplementary Materials and Methods.

In Vivo Experiments on Transgenic Mice

HLA-HCD4/DQ8 male mice (~26 g), 6 to 8 weeks old, were used.²⁹ Mice were mucosally sensitized with gluten (500 µg) and cholera toxin (25 µg) as mucosal adjuvant once a week for 3 weeks. One week after the last sensitization, mice were randomly assigned to one of the study groups and orally challenged once. A positive control group received a mixture composed of 2 mg bovine serum albumin (BSA) and 2 mg wheat starch with 2 mg of gluten dissolved in 0.02 mol/L acetic acid. The treatment group with high dose (HD) of P(HEMA-*co*-SS) consisted of gluten-sensitized mice gavaged with gluten/BSA/starch mixture and P(HEMA-*co*-SS) (4 mg) (gluten polymer 1:2 wt/wt). The treatment group with low polymer dose (LD) received 1 mg of polymer (gluten/polymer 2:1 wt/wt). Mice were killed 24 hours after the oral challenge. In another series of experiments, the dose of P(HEMA-*co*-SS) was kept at 4 mg but the gluten amount increased to 4 mg (higher level of gluten, gluten/polymer 1:1 wt/wt). The negative control group was composed of nonsensitized mice (cholera toxin only) gavaged with BSA/starch mixture but no gluten. P(HEMA-*co*-SS) was administered 5 minutes before the mixture gavage. Finally, the impact of the treatment on long-term gluten exposure was assessed. Mice (10 male and 10 female) were mucosally sensitized as described previously. One week after the last sensitization, they were orally challenged with gluten twice a day, 3

times a week, for 3 weeks. The positive control group (5 male and 5 female) received the mixture composed of BSA (2 mg) and wheat starch (2 mg) with gluten (2 mg) dissolved in 0.02 mol/L acetic acid. The treatment group (5 male and 5 female) consisted of gluten-sensitized mice gavaged with P(HEMA-co-SS) (4 mg), which was administered 5 minutes before the gluten/BSA/starch mixture intake. The negative control group was composed of nonsensitized mice (5 male and 5 female) gavaged only with BSA/starch mixture. Mice were killed 24 hours after the last oral challenge. Histology, hemogram, and biochemical parameters were assessed as described in Supplementary Materials and Methods.

To verify the impact of sensitization on intestinal permeability, an additional negative control group composed of mucosally sensitized mice gavaged with BSA/starch mixture was used. This control group showed identical immunoglobulin (Ig) A level and ⁵¹Cr-EDTA flux as the nonsensitized negative control group gavaged with BSA/starch mixture and a trend toward higher mucosal conductance levels (Supplementary Figure 1).

Effect of P(HEMA-co-SS) on Intestinal Permeability in Gluten-Sensitized Mice

Two sections of the jejunum from each mouse were used for intestinal conductance and paracellular permeability measurements, and the experiments were performed as described elsewhere³⁰ (Supplementary Materials and Methods).

Effect of P(HEMA-co-SS) on Anti-Gliadin Antibodies in Gluten-Sensitized Mice

The small intestine was removed and the content flushed using 6 mL of phosphate-buffered saline containing 0.05 mmol/L EDTA and 0.1 mg/mL soybean trypsin inhibitor into a tube containing 40 μ L of 100 mmol/L phenylmethylsulfonyl fluoride in 95% vol/vol ethanol. After mixing, samples were centrifuged at 2000g at 4°C for 30 minutes, and the supernatants were collected and stored at -20°C. All samples were processed within 1 week (Supplementary Materials and Methods).

Effect of P(HEMA-co-SS) on Splenocyte Proliferation in Gluten-Sensitized Mice

Splenocytes were isolated and a peptic-tryptic digest of gliadin (PT-gliadin) was prepared as described previously.³¹ Detailed methods are provided in Supplementary Materials and Methods.

Effect of P(HEMA-co-SS) in Biopsy Specimens From Patients With Celiac Disease

Patients' biopsy specimens were kindly provided by Dr Pavel Fruhauf (Department of Pediatrics and Adolescent Medicine, 1st Medical Faculty, Charles University, Prague, Czech Republic). Patients were recruited after receiving approval from the local ethics committee. Patients with untreated celiac disease (as diagnosed by serology) attending gastrointestinal endoscopy clinics at various hospitals in the city of Prague were invited to participate in the study. Written informed consent was obtained from all patients. Small intestinal biopsy specimens with confirmed Marsh III lesions were used for cytokine responses to gliadin. In the first set of experiments (5-minute pre-

incubation of gliadin with P[HEMA-*co*-SS] at 37°C before peptic digestion), patients (n = 4) ranging in age from 29 to 49 years (mean, 37 years; female/male, 3/1) were included. In the second set of experiments (pre-incubation of gliadin peptic digest, P-gliadin, with polymer), patients (n = 5) ranging in age from 33 to 49 years (mean, 38 years; female/male, 4/1) were included. Four biopsy specimens (of similar size, 2–3 mm) were obtained from each patient and incubated under 4 different conditions consisting of medium alone, P-gliadin, P(HEMA-*co*-SS), and gliadin + P(HEMA-*co*-SS). The gliadin/P(HEMA-*co*-SS) or P-gliadin/P(HEMA-*co*-SS) ratios were set at 1:3 (wt/wt). The levels of interleukin (IL)-10, tumor necrosis factor (TNF)- α , IL-15 and IL-21 were determined by enzyme-linked immunosorbent assay as described in Supplementary Materials and Methods.

Statistical Analysis

Group values for the comparison of the 2 negative control groups (sensitized vs nonsensitized) were analyzed using a *t*-test. Group values for in vitro and animal experiments were compared using a parametric analysis of variance test, followed by Scheffé, Tukey, or Tukey–Kramer test to determine the significance of all paired combinations (normality was assumed on all experimental data sets). Group values for the biopsy studies were compared using a Kruskal–Wallis test, followed by a Nemenyi test to determine the significance of the combinations.

Results

P(HEMA-co-SS) Decreases the Formation of Gluten-Derived Toxic Peptides

Gliadin, wheat gluten, and barley hordein were subjected to in vitro digestion by gastrointestinal enzymes in the presence or absence of P(HEMA-co-SS), and the digestion products were analyzed by LC-MS. This in vitro test was used to provide insight on the mechanisms of action of the polymer. It is semiquantitative and represents a worst-case experimental setup because the cereal proteins are the sole substrates for digestive enzymes, which is not the case in the gastrointestinal tract. As reported in our first study,²⁸ incubation of gliadin with P(HEMA-co-SS) resulted in a decrease of the overall abundance of generated peptides. P(HEMA-co-SS) greatly reduced (by 70%) the formation, from isolated gliadin, of the 13-mer peptide LGQQQPFPPQQPY (Supplementary Figure 2), which participates in the innate immune response.³² Because both gliadin and glutenin are involved in the pathogenesis of celiac disease,^{33, 34, 35 and 36} in this study further investigations were performed on wheat gluten digests. The analysis of LC-MS traces showed that the signal intensities associated with YPTSPQQSGGQQQL³⁴ and LQPQQPFPPQQPFPPQ, bearing the γ -2 gliadin epitope (QQPFPPQQPFPPQ),³⁷ were reduced by 70% and 25%–30%, respectively (Supplementary Figure 3A and B). This observation is of special interest because those sequences are recognized by T-cell and T-cell clones from patients with celiac disease.^{34 and 37}

The production of a gluten sequence, known to bind to the HLA-DQ2 receptor (ie, QLQPFPPQQLPY),³⁸ was also reduced by 50%–70% in the presence of P(HEMA-co-SS)

(Figure 1A). Besides, the formation of PQQQLPYPQQLPYPQQQPF decreased significantly (Figure 1B). When combined, these 2 peptides (QLQPFQQLPY and PQQQLPYPQQLPYPQQQPF) almost completely make up the known 33-mer sequence (LQLQPFQQLPYPQQLPYPQQLPYPQQQPF), one of the identified initiators of the inflammatory reaction in patients with celiac disease.^{34 and 39} In addition, the glutenin sequence SQQQQPF was also decreased by 60% (Supplementary Figure 3C).

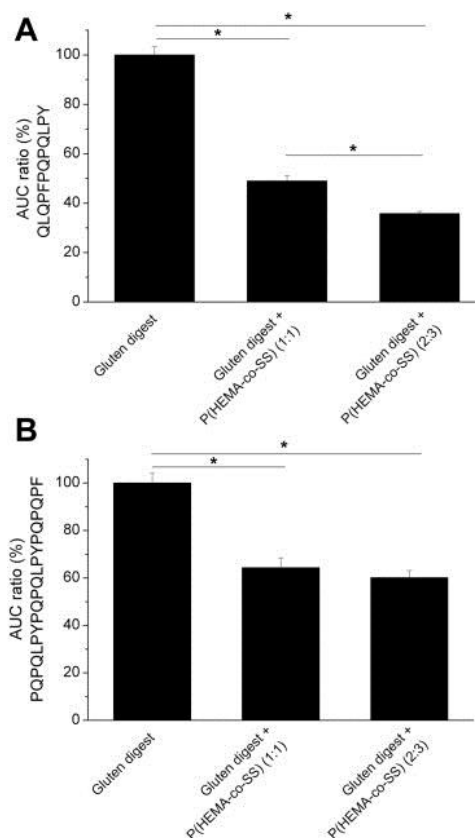


Figure 1. P(HEMA-co-SS) decreases the formation of peptides (A) QLQPFQQLPY and (B) PQQQLPYPQQLPYPQQQPF from wheat gluten. Digested gluten samples were analyzed by LC-MS. Relative abundance was determined by comparison with the gluten digest in the absence of P(HEMA-co-SS). Mean \pm SEM displayed from 3 injections of 3 independent samples, $*P \leq .001$

To test whether the P(HEMA-*co*-SS) could reduce the production of dominant immunogenic peptides from prolamine of barley, additional analyses with hordein digests were performed. The formation of PQQPFPPQQPFPPQQPPFW, bearing a 9-mer (QQPFPPQQPF) sequence, predicted to be a HLA-DQ2 restricted T-cell epitope,⁴⁰ was reduced by 40%–45% (Supplementary Figure 4A). More importantly, 2 sequences, PQQPQQPFPPFQPQQPFRQ and PQQPFPPQQPQQPFPPQQPF,³⁷ reported to stimulate T-cell and T-cell clones from patients with celiac disease,^{34 and 37} were decreased by 40%–45% and by 60%–65%, respectively (Supplementary Figure 4B and C). Finally, a peptide from γ -hordein (ie, LERPQQLFPQWQPLPQQPPFL) exhibited a drastic reduction of 85%–90% (Supplementary Figure 4D).

P(HEMA-co-SS) Is Poorly Absorbed and Mainly Excreted in Feces

The oral absorption of the polymeric binder and its excretion profiles were first examined in Sprague–Dawley rats after single and long-term dosing. The nonfasted animals were gavaged with [³H]-P(HEMA-*co*-SS), and blood, feces, and urine were assayed for radioactivity content. After the animals were killed, selected organs were harvested and the radioactivity levels measured. After a single polymer intake (150 mg/kg), the amount of radioactivity found in the blood over 72 hours was very low, reflecting poor absorption of P(HEMA-*co*-SS) (Supplementary Figure 5A). Indeed, ~98% of the total radioactivity was recovered in the feces. The total amount excreted in the urine was less than 2% of the total dose. Only residual P(HEMA-*co*-SS) activity was detected in harvested organs (Supplementary Figure 5B–D). Similar results were obtained

after a 3-week administration of P(HEMA-*co*-SS) at a dosage of ~100 mg/kg/day, suggesting no permeabilization of the intestinal mucosa on protracted dosing (Supplementary Figure 6).

To determine whether the absorption of P(HEMA-*co*-SS) is increased by mucosal inflammation and changes in intestinal permeability triggered by gluten, ³⁰ [³H]-P(HEMA-*co*-SS) was also administered to nonsensitized and gluten-sensitized HLA-HCD4/DQ8 mice. The amount of tritium recovered in the feces exceeded 93% of the total radioactivity and was comparable in gluten-sensitized and nonsensitized mice (Figure 2A). After 72 hours, less than 1.2% of the administered dose was detected in the urine (Figure 2B). Likewise, the radioactivity levels in the blood, liver, and kidney remained similar to background levels (overall amount, 0.1% of given dose) (Figure 2C).

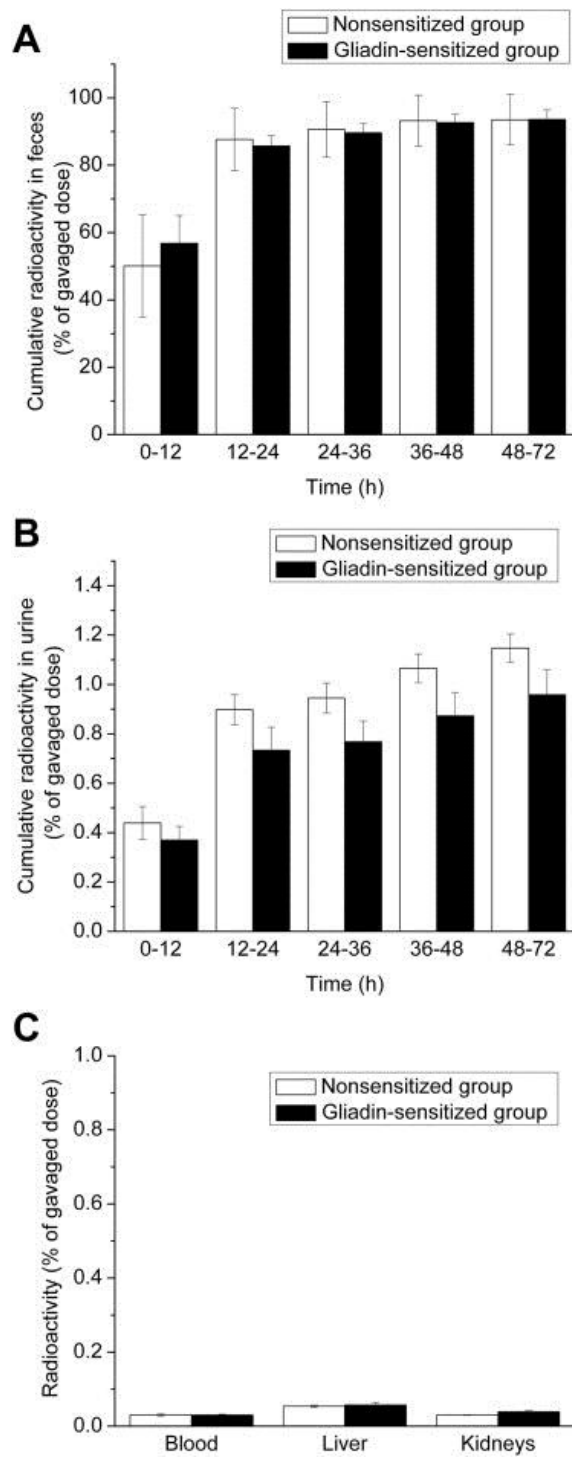


Figure 2. P(HEMA-*co*-SS) is poorly absorbed and mainly excreted in the feces. Excretion profiles and biodistribution of [³H]-P(HEMA-*co*-SS) in nonsensitized (*white bars*) and gluten-sensitized (*black bars*) HCD4/DQ8 mice after single oral dosing (240 mg/kg, 22.4 μCi/kg). (A) Cumulative dose excreted in feces, (B) cumulative dose excreted in urine, and (C) blood, liver, and kidney levels at the time the mice were killed. Mean ± SEM, n = 4/group.

P(HEMA-co-SS) Reduces Mucosal Dysfunction in Gluten-Sensitized Mice When Administered in a Gluten-Containing Meal

To evaluate the therapeutic relevance of the polymeric binder toward a wheat gluten containing meal, an *in vivo* assay was performed on gluten-sensitized HLA-HCD4/DQ8 mice. These mice exhibit increased intestinal permeability and mild mucosal immune activation after gluten or gliadin sensitization.^{28 and 31} We previously reported that P(HEMA-*co*-SS) reversed intestinal barrier abnormalities induced by isolated gliadin.²⁸ However, in the previous study, no other food components were admixed with gliadin, and it was not shown whether the binder was efficient in the presence of whole wheat gluten. Therefore, we measured the activity of P(HEMA-*co*-SS) in gluten-sensitized mice using a mixture of wheat gluten, BSA, and starch for oral challenge. HLA-HCD4/DQ8 mice gavaged with the food mixture exhibited an increase in ⁵¹Cr-EDTA flux, a marker of paracellular permeability (Figure 3A), and in intestinal tissue conductance (Figure 3B) compared with nonsensitized mice receiving the BSA/starch mixture alone. The gluten-induced effects on permeability were normalized in mice treated with P(HEMA-*co*-SS) under the HD regimen. Thus, P(HEMA-*co*-SS) decreased gluten-induced effects on barrier function in HLA-HCD4/DQ8 mice even in the presence of additional food components. The same trend was observed at a lower polymer dose (LD), although

statistical significance could not be achieved (Figure 3, *last bar*). An identical pattern was observed when the dose of polymer was unchanged but the amount of wheat gluten was doubled (higher-level regimen) (Supplementary Figure 7A and B), achieving statistical significance in only one permeability parameter (^{51}Cr -EDTA flux).

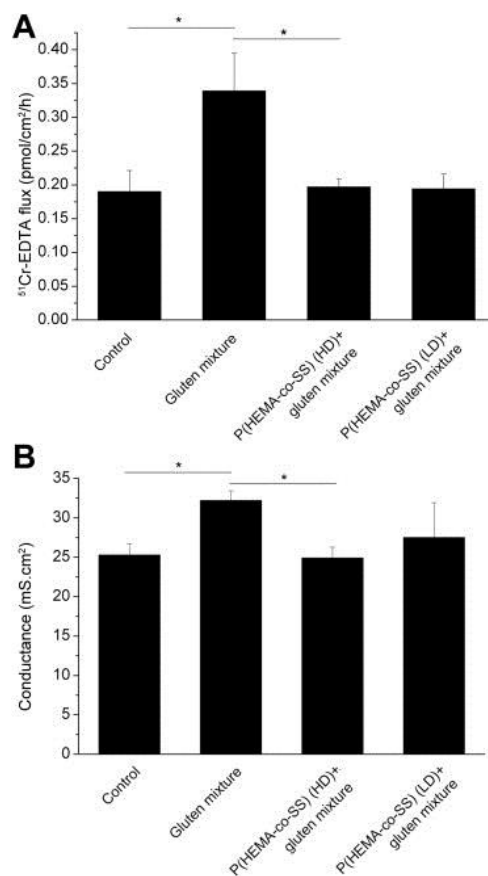


Figure 3. Administration of P(HEMA-*co*-SS) to gluten-sensitized HCD4/DQ8 mice challenged with wheat gluten mixture decreased (A) ^{51}Cr -EDTA flux (paracellular permeability) and (B) intestinal tissue conductance compared with untreated nonsensitized mice. Controls: nonsensitized mice gavaged with BSA and starch (n = 12–13); gluten mixture group: gluten-sensitized mice gavaged with gluten/BSA/starch (n = 9–10); P(HEMA-*co*-SS) (HD) + gluten mixture group (treatment group): gluten-sensitized mice gavaged first with P(HEMA-*co*-SS) and subsequently with gluten/BSA/starch (gluten/polymer 1:2 wt/wt) (n = 9–10); P(HEMA-*co*-SS) (LD) + gluten mixture group (second treatment group): gluten-sensitized mice gavaged first with

P(HEMA-*co*-SS) and subsequently with gluten/BSA/starch (gluten/polymer 2:1 wt/wt) (n = 4). Mean \pm SEM, * $P \leq .02$ for Cr-EDTA and $P \leq .01$ for conductance.

P(HEMA-co-SS) Has Immunomodulatory Effects in Gluten-Sensitized Mice

Mucosal immunity

We measured the effect of P(HEMA-*co*-SS) on anti-gliadin IgA levels in intestinal washes of HLA-HCD4/DQ8 mice. Total anti-gliadin IgAs were increased by 2.4-fold in untreated gluten-sensitized mice (ie, gavaged with gluten-food mixture only) compared with nonsensitized controls (Supplementary Figure 8). In mice receiving P(HEMA-*co*-SS) (HD and LD regimens) before gluten-food mixture challenge, the IgA levels were comparable to those observed in nonsensitized mice (Supplementary Figure 8). In the higher-level group, the IgA levels exhibited a similar pattern, although statistical significance was not reached (Supplementary Figure 7C). Since statistical significance was not achieved on all parameters (ie, permeability and mucosal immunity) in LD and higher-level groups, further investigations were performed at a gluten-to-polymer ratio of 1:2 wt/wt (HD).

Systemic immunity

The ability of oral P(HEMA-*co*-SS) to modulate systemic immune responses was examined using a splenocyte proliferation assay. Splenocytes from gluten-sensitized HLA-HCD4/DQ8 mice (subsequently gavaged with gluten-food mixture and/or P(HEMA-*co*-SS)) and from nonsensitized controls were harvested and incubated with

PT-gliadin. Negative controls from all groups were incubated with medium alone and positive controls with concanavalin A (5 $\mu\text{g}/\text{mL}$). Proliferation index of splenocytes incubated with medium alone was low and comparable in all groups. Concanavalin A-stimulated proliferation was robust and similar in all groups (controls, 17.2 ± 3.4 counts per minute; gluten, 16.7 ± 5.3 counts per minute; gluten + P[HEMA-*co*-SS], 19.4 ± 3 counts per minute). After incubation with PT-gliadin, untreated gluten-sensitized mice had increased thymidine incorporation compared with nonsensitized controls (Figure 4). However, splenocytes from gluten-sensitized mice that were treated with P(HEMA-*co*-SS) before the gluten-food mixture gavage exhibited a trend of reduced thymidine incorporation versus sensitized mice that had not received P(HEMA-*co*-SS) ($P = .068$). To further characterize the immunomodulatory effect of P(HEMA-*co*-SS), supernatants from splenocyte cultures stimulated with PT-gliadin were assayed for IL-10, TNF- α , and monocyte chemotactic protein 1 (MCP-1). Previous studies in HLA-HCD4/DQ8 mice have shown increases in the immunoregulatory cytokine IL-10.^{29, 30 and 31} Our results are in line with these studies showing enhanced IL-10 production after mucosal gluten sensitization (Figure 5A). Administration of P(HEMA-*co*-SS) before the gluten-food mixture challenge led to a more pronounced IL-10 secretion in vitro (Figure 5A). The proinflammatory cytokine TNF- α was increased in untreated gluten-sensitized mice compared with nonsensitized controls. Administration of P(HEMA-*co*-SS) before the gluten-food mixture challenge reduced TNF- α secretion by 55% (Figure 5B). In gluten-sensitized mice challenged with the gluten-containing mixture and treated with P(HEMA-*co*-SS), the TNF- α /IL-10 ratio dropped from 2.3 to 0.5 versus the control group.

Nonsensitized mice had low levels of MCP-1, a chemokine that prolongs lymphocyte survival (Figure 5C). Untreated gluten-sensitized mice exhibited higher levels of MCP-1 than controls and than gluten-sensitized mice treated with P(HEMA-*co*-SS).

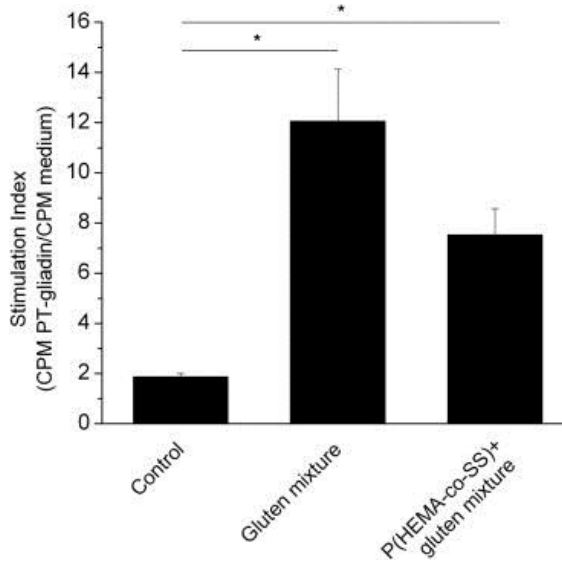


Figure 4. Administration of P(HEMA-*co*-SS) to gluten-sensitized mice challenged with gluten-food mixture tended to reduce splenocyte proliferation after incubation with PT-gliadin. Data represent the stimulation index from mean [³H]thymidine incorporation for triplicate cultures. Mean \pm SEM (n = 8–9/group), * $P \leq .001$. Controls: nonsensitized mice gavaged with BSA and starch; gluten mixture group: gluten-sensitized mice gavaged with gluten/BSA/starch; P(HEMA-*co*-SS) + gluten mixture group (treatment group): gluten-sensitized mice gavaged first with P(HEMA-*co*-SS) and subsequently with gluten/BSA/starch (HD).

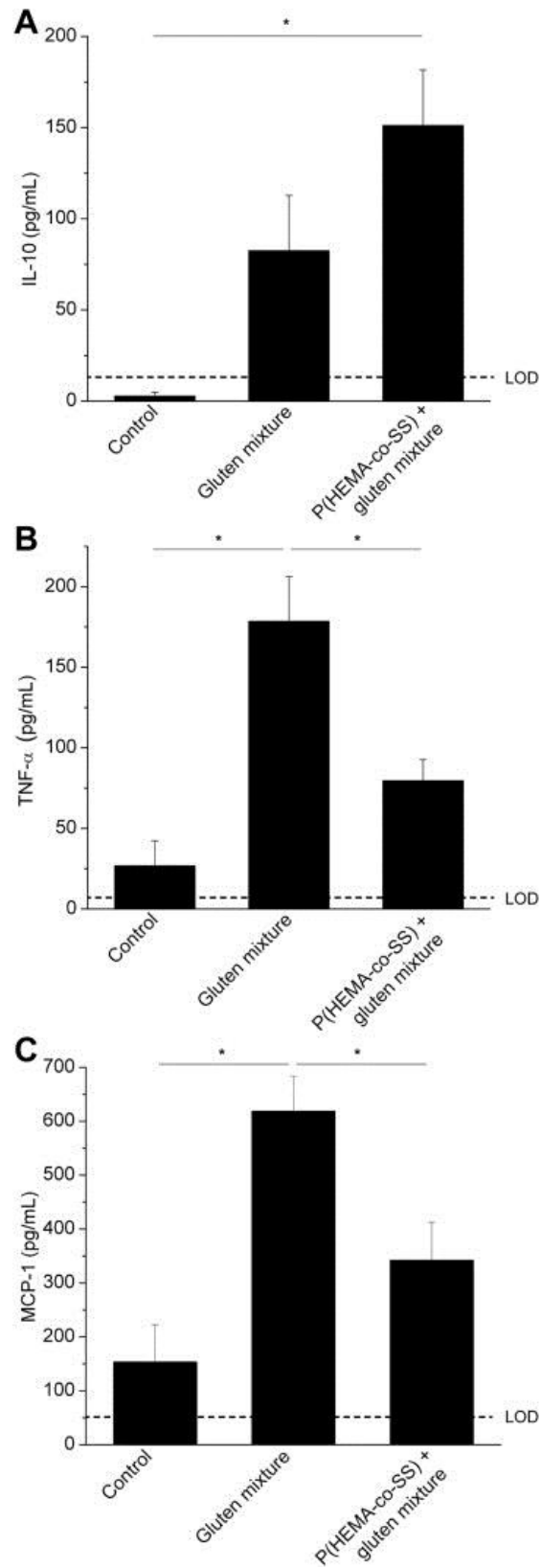


Figure 5. Modulation of cytokine production in splenocyte culture supernatants after PT-gliadin incubation: (A) IL-10, (B) TNF- α , and (C) MCP-1. IL-10 secretion was increased in gluten-sensitized mice receiving P(HEMA-co-SS) compared with controls. Administration of P(HEMA-co-SS) to gluten-sensitized mice led to lower TNF- α and MCP-1 levels in culture supernatants after PT stimulation. Controls: nonsensitized mice gavaged with BSA and starch; gluten mixture: gluten-sensitized mice gavaged with gluten/BSA/starch; P(HEMA-co-SS) + gluten mixture (treatment group): gluten-sensitized mice gavaged first with P(HEMA-co-SS) and subsequently with gluten/BSA/starch (HD). Mean \pm SEM (n = 10/group), * $P \leq .001$. In culture supernatants from the splenocytes incubated with medium alone, cytokines were below the limit of detection (LOD; *dashed lines*) (LOD_{IL-10} = 17.5 pg/mL; LOD_{TNF- α} = 7.3 pg/mL; LOD_{MCP-1} = 52.7 pg/mL).

P(HEMA-co-SS) Attenuates the Effects of Long-term Administration of Gluten in Gluten-Sensitized Mice

To further evaluate the therapeutic relevance of the polymeric binder, an in vivo assay was performed on gluten-sensitized HLA-HCD4/DQ8 mice after repeated oral challenge with gluten. HLA-HCD4/DQ8 mice gavaged with gluten twice a day, 3 times a week, for 3 weeks exhibited an increase in ⁵¹Cr-EDTA flux, intestinal tissue conductance, and total anti-gliadin IgA (Supplementary Figure 9) compared with negative controls. The gluten-induced effects on conductance and anti-gliadin IgA level were normalized in mice receiving P(HEMA-co-SS) before the gluten mixture. The same trend was observed for ⁵¹Cr-EDTA flux, although statistical significance was not achieved ($P = .058$). Interestingly, in the positive control group, long-term intake of gluten induced villous abnormalities and a decrease in villus-to-crypt ratio (from 5.96 ± 1.23 for the negative control group to 2.58 ± 0.43 for the gluten group; Figure 6A and B). This effect had not been observed in HCD4/DQ8 mice using the acute gluten exposure. Chronic gluten-

induced enteropathy was attenuated (Figure 6C) on administration of P(HEMA-*co*-SS) (villus-to-crypt ratio, 4.89 ± 1.51 ; $P < .05$), confirming that the polymer may exhibit some therapeutic benefits even after repeated dosing. At the end of the 3-week challenge (twice a day, 3 times a week), all 3 groups gained weight in a similar fashion (Supplementary Figure 10). Moreover, there was no difference in hemogram and biochemical parameters between the untreated and treated groups (Supplementary Table 1). Thus, no signs of polymer-related toxicity were noticed.

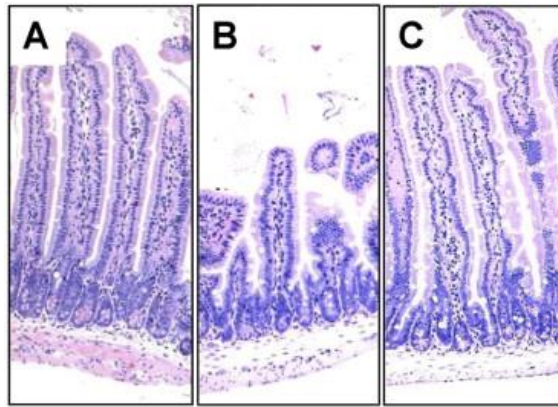


Figure 6. Administration of P(HEMA-*co*-SS) reduced the morphologic intestinal abnormalities induced by long-term administration of gluten. (A) Controls: nonsensitized mice gavaged with BSA and starch, (B) gluten mixture group: gluten-sensitized mice gavaged with wheat gluten (2 mg)/BSA (2 mg)/starch (2 mg), (C) P(HEMA-*co*-SS) + gluten mixture group (treatment group): gluten-sensitized mice gavaged first with P(HEMA-*co*-SS) (4 mg) and subsequently with wheat gluten (2 mg)/BSA (2 mg)/starch (2 mg). Challenges were performed twice a day, 3 times a week, for 3 weeks. Representative H&E-stained jejunum section of the various groups observed by optical microscopy

P(HEMA-co-SS) Affects Cytokine Release by Biopsy Specimens From Patients With Celiac Disease

The effect of P(HEMA-co-SS) on gliadin digestion was evaluated on mucosal biopsy specimens obtained from patients with celiac disease. Two series of experiments were conducted. First, the gliadin digest was generated in the presence or absence of P(HEMA-co-SS) to mimic a rapid complexation in the stomach (Figure 7). In the second case, P-gliadin was first produced and then admixed to P(HEMA-co-SS) to simulate a situation in which complexation would occur at a later stage when a substantial amount of gluten would have been digested (Supplementary Figure 11). In both cases, the digests were incubated with the isolated mucosa and the secretion of TNF- α and IL-10 was measured in the culture supernatant. IL-15 and IL-21 were tested, but the levels were below the limit of quantification in all groups. As shown in Figure 7 and Supplementary Figure 11, negative controls (nonstimulated biopsy specimens) released traces of TNF- α (<15.6 pg/mL) and IL-10 (<31.3 pg/mL). The addition of the P-gliadin in the medium increased the production of TNF- α and tended to increase IL-10. Biopsy specimens incubated with P(HEMA-co-SS) alone secreted traces of TNF- α , suggesting that P(HEMA-co-SS) applied to the intestinal mucosa is not proinflammatory in vitro. The incubation of the biopsy specimens with P-gliadin/P(HEMA-co-SS) mixture (digestion of gliadin before the addition of P(HEMA-co-SS); Supplementary Figure 11) tended to reduce TNF- α , whereas IL-10 concentrations remained unchanged. However, the effect of P(HEMA-co-SS) was more pronounced when intestinal biopsy specimens were incubated

with the digested gliadin/P(HEMA-*co*-SS) complex (Figure 7), showing that TNF- α levels were reduced to very low levels (<15.6 pg/mL).

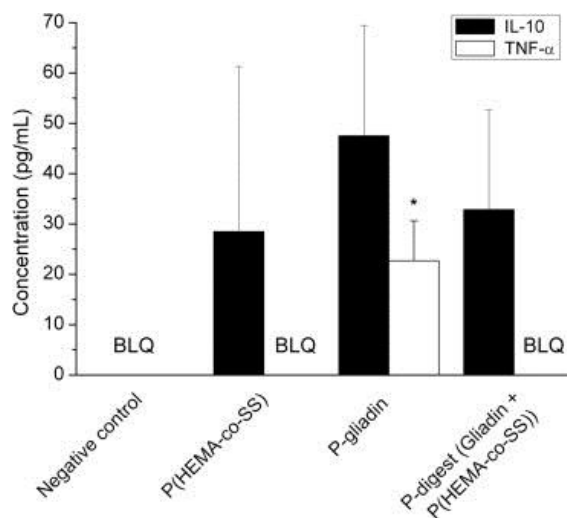


Figure 7. Incubation with P(HEMA-*co*-SS) abolished gliadin-induced TNF- α secretion (*white bars*) in culture supernatants of biopsy specimens from patients with celiac disease. P(HEMA-*co*-SS) alone or in P-digest did not affect gliadin-induced IL-10 secretion (*black bars*). The negative control corresponds to biopsy specimens incubated with medium only. Mean \pm SEM (n = 4), * $P \leq .05$ vs all other TNF- α groups. BLQ: below limit of quantification (<31.3 pg/mL for IL-10 and <15.6 pg/mL for TNF- α).

Discussion

In this paper, we report important findings regarding the *in vivo* biodistribution and therapeutic efficacy of P(HEMA-*co*-SS) when administered orally (single-dose and repeated administration) in a mixture containing whole wheat gluten and other food components to rodents. We also determined its effect on cytokine production by mucosal biopsy specimens of patients with celiac disease on gliadin stimulation. Previously, we have reported that P(HEMA-*co*-SS) hindered the digestion of isolated gliadin by

gastrointestinal enzymes, decreasing the formation of overall gliadin by-products.²⁸ Our present results show that P(HEMA-*co*-SS) hindered significantly the formation of major identified immunogenic peptides from wheat gluten (such as QLQPFQPQLPY and LQPQQPFQPQQPFQP)^{37 and 38} by gastrointestinal enzymes. P(HEMA-*co*-SS) also decreased the production of immunogenic peptides from barley. It is, however, unknown whether the degree of inhibition of peptide formation achieved *in vitro* can be directly correlated to the *in vivo* efficacy, because the mechanism of action of the binder might be more complex than initially expected.

Pharmacokinetic and biodistribution experiments showed that P(HEMA-*co*-SS) was essentially not absorbed systemically. Similarly to the oral enzymatic approach, P(HEMA-*co*-SS) reduces gluten toxicity. However, these 2 complementary strategies differ in their mechanism of action. The former cleaves the pathogenic peptides into less immunogenic smaller fragments, whereas the latter aims at protecting gluten from digestion by gastrointestinal enzymes, thereby excreting it with few toxic by-products.

We have formerly described that administration of P(HEMA-*co*-SS) with isolated gliadin to HLA-HCD4/DQ8 mice attenuated gliadin-induced changes in the intestinal barrier and reduced intraepithelial lymphocyte and macrophage cell counts.²⁸ Here we show that oral administration of P(HEMA-*co*-SS) to gluten-sensitized HLA-HCD4/DQ8 mice ameliorated mucosal and systemic consequences of an oral complex gluten-containing food mixture challenge. In mice, P(HEMA-*co*-SS) restored gluten-induced intestinal barrier dysfunction, reduced the levels of anti-gliadin IgA in intestinal washes,

tended to decrease the proliferation of splenocytes incubated with PT-gliadin, and shifted the immune response in vitro toward an anti-inflammatory profile. Our current results also suggest that P(HEMA-*co*-SS) has the capacity to modulate cytokine production in response to gliadin in mucosal biopsy specimens of patients with celiac disease. Repeated administration of P(HEMA-*co*-SS) to gluten-sensitized HLA-HCD4/DQ8 mice following long-term challenge with gluten revealed that the polymer exhibited some therapeutic benefits on the intestinal mucosa without affecting weight gain, hemogram, or biochemical parameters.

Innate and adaptive immune responses are involved in the pathogenesis of celiac disease. The intestinal epithelial uptake of gliadin has been extensively studied in the context of celiac disease.^{41, 42 and 43} Gliadin has been reported to increase intestinal permeability enabling its paracellular diffusion, thereby subsequently triggering an interaction with the mucosal immune system.^{41 and 42} Gliadin peptides can also translocate transcellularly by the transferrin receptor.⁴³ This translocation makes poorly digested gliadin available to the mucosa, leading to antigen presentation of HLA-DQ2/DQ8-bound gliadin peptides to CD4⁺ T cells and to the generation of effector cells.^{44 and 45} Increased availability of gliadin and its by-products in the mucosa and impairment of immunoregulatory mechanisms that control oral tolerance have been proposed as mechanisms that lead to autoimmune enteropathy in celiac disease.⁴⁶ In the present study, treatment of gluten-sensitized mice with P(HEMA-*co*-SS) reduced anti-gliadin IgAs in intestinal secretions. Oral P(HEMA-*co*-SS) also led to increased secretion of IL-10 and to decreased production of MCP-1 and TNF- α by splenocytes after in vitro stimulation with

PT-gliadin. The mechanism leading to the increase of IL-10 expression by splenocytes remains to be investigated. It can be hypothesized that the complexation of P(HEMA-*co*-SS) to gluten, in addition to hindering its degradation, may indirectly influence the immune response to gluten peptides. A recent work reported that the conjugation of mannoside units to BSA could increase IL-10 expression by CD4⁺ cell type 1 regulatory-like cells and induce oral tolerance in a mouse model of food allergy.⁴⁷

In human biopsy specimens obtained from patients with celiac disease that were incubated with gliadin, P(HEMA-*co*-SS) tended to reduce (Supplementary Figure 11) gliadin-induced TNF- α levels when applied with P-gliadin. However, when P(HEMA-*co*-SS) was incubated with gliadin before digestion, TNF- α secretion was abolished (Figure 7). In both sets of experiments, IL-10 concentration remained unchanged. However, a trend for increased IL-10 production was observed in human biopsy specimens in the presence of P(HEMA-*co*-SS), even without gliadin stimulation. This is intriguing in view of previous studies showing that recombinant IL-10 can suppress gliadin-dependent T-cell activation in mucosal T cells obtained from patients with celiac disease.⁴⁸ Clinical studies using the polymeric binder will be crucial to determine the therapeutic relevance of such *ex vivo* observations.

Polymeric binders are commonly used to sequester compounds (eg, bile salts, phosphate) in the gastrointestinal tract. They are designed not to be absorbed to minimize the risks of systemic toxicity.⁴⁹ P(HEMA-*co*-SS) has a high molecular weight (45–60 kilodaltons) and carries multiple negative charges. Furthermore, on complexation with

gliadin, particle sizes in the order of hundreds of nanometers are obtained.⁵⁰ Therefore, P(HEMA-*co*-SS) was not expected to be orally bioavailable. Our present in vivo data in both rats and mice support this hypothesis. Most of the administered P(HEMA-*co*-SS), whether given as a single dose or in a long-term fashion, was recovered in the feces after 3 days. There was some residual radioactivity found in the blood and in urine, which could be due to tritium exchange or urine contamination by the feces. The lack of absorption of P(HEMA-*co*-SS) supports the current and previously reported findings of no systemic toxicity as well as no alteration of several biochemical and cellular markers even with long-term administration of high doses.²⁸ Herein, the fact that P(HEMA-*co*-SS) absorption was not increased in gluten-sensitized mice, which are known to exhibit changes in intestinal permeability and low-grade inflammation, further supports the safety profile of the binder. Moreover, the direct action in the gastrointestinal tract, bypassing bioavailability issues, might be a tremendous advantage of this strategy.

In conclusion, these data strengthen the evidence in support of luminal gluten-polymeric binder as an effective adjunctive therapy to a GFD. P(HEMA-*co*-SS) was not systemically absorbed and blocked mucosal toxicity of gluten in mice, likely by decreasing the production of immunogenic peptides in the gastrointestinal tract and by limiting the absorption of gluten by-products. Taken in conjunction with the animal studies, the immunomodulatory effects suggested by our experiments in tissues from patients with celiac disease should encourage future work aiming at assessing the safety of P(HEMA-*co*-SS) after oral administration to healthy volunteers and progression into phase 1 clinical trials. The ultimate verdict on therapeutic efficacy and utilization

guidelines of P(HEMA-*co*-SS) will only come from clinical studies. Research on numerous adjuvant approaches to a GFD should be encouraged, because a combination of alternative therapies may be beneficial for patients with poorly controlled celiac disease.

Conflicts of interest: The authors disclose the following: J.-C. Leroux is an inventor of the patent on the use of polymeric binder in treating celiac disease. This patent has been licensed by the University of Montreal to BioLineRx. J.-C. Leroux has a consultancy agreement with BioLineRx. The remaining authors disclose no conflicts.

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Supplementary Methods

Effect of P(HEMA-co-SS) on Wheat Gliadin, Gluten and Barley Hordein Digestion

Isolation of hordein from barley was performed as previously reported.¹ Briefly, 10 g barley grains were milled and mixed with 32 mL 70% ethanol for 2 h. After centrifugation (30 min at 900 \times g) the supernatant was dialyzed against 0.01 M acetic acid and freeze-dried. The lyophilized product was dispersed in 0.01 M acetic acid, centrifuged and the supernatant freeze dried a second time to give the crude hordein.

Digestion

Stock solutions of the enzymes were prepared prior to use: i) pepsin (3480 U/mg) in HCl 0.02 M, 6 mg/mL, ii) trypsin (1310 U/mg) in Na₂HPO₄ buffer (pH 6.8, 20 mmol/L K₂HPO₄ and 2 mmol/L NaOH), 50 mg/mL, and iii) chymotrypsin (96 U/mg) in Na₂HPO₄ buffer (pH 6.8, 20 mmol/L K₂HPO₄ and 2 mmol/L NaOH) 50 mg/mL. α -Gliadin (supplied by Prof. Popineau, Institut National de Recherche Agronomique, Nantes; 7.5 mg/mL), gluten (15 mg/mL) or hordein (15 mg/mL) were suspended in HCl 0.02 M for 15 min at 37°C in the presence or absence of P(HEMA-co-SS) at different gliadin (or gluten)/P(HEMA-co-SS) mass ratios. In the calculation of the mass ratios, it was assumed that gluten contained 50% gliadin.² Pepsin was added to a final concentration of 0.024 mg/mL and incubated for 2 h, at 37°C under stirring. Thereafter, Na₂HPO₄ was added (0.12 M), and the pH adjusted to 6.8 by the addition of NaOH 1 M. The suspension was supplemented with trypsin to a final concentration of 0.375 mg/mL and incubated for 2 h,

at 37°C under stirring. Subsequently, chymotrypsin was added to the suspension to a final concentration of 0.375 mg/mL and incubated for 2 h, at 37°C under stirring. After completion of the digestion process, samples were heat-inactivated for 5 min at 95°C, freeze-dried and stored at -20°C until analysis. The freeze-dried α -gliadin and gluten digests were resuspended in ultrapure water and centrifuged (14,000 \times g) for 10 min. Supernatants were then further diluted 1 in 10 (v/v) with water containing 0.1% formic acid. Each dilution was injected at least in triplicate.

Analytical method

All samples were analyzed using an LC-system composed of a Rheos Allegro quaternary pump (Flux Instruments, Basel, Switzerland), column oven (hot dog 5090), C18-column (Hypersil Gold, 100 \times 1 mm, 1.9 μ m) and XCalibur control software (all obtained from Thermo Fisher Scientific Inc., San Jose, CA). Samples were injected at a column temperature of 35°C, at a volume of 10 μ L and a flow rate of 50 μ L/min using water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The gradient for wheat gliadin and gluten analysis consisted of 95% solvent A as initial value, 95–50% A in 1–30 min, 50–5% A in 30–35 min, 5% A in 35–40 min, 5–95% A in 40–41 min and 95% A in 41–50 min. The gradient for analysis of hordein samples was modified as follows: 95% solvent A as initial value, 95–50% A in 1–19 min, 50–5% A in 19–23 min, 5% A in 23–27 min, 5–95% A in 27–28 min, and 95% A in 28–31 min. The LC was directly connected to an LTQ XL linear quadrupole Ion Trap (Thermo Fisher Scientific Inc.). Ionization was conducted *via* electrospray ion (ESI)

source. Data acquisition was carried out by full MS, followed by MS²-fragmentation of the five most intense signals in an automated data dependent scan. Raw data were subjected to the Sequest search algorithm using Proteome Discoverer Software (Thermo Fisher Scientific Inc.). Detected peptides were searched against all sequences from the ExPASy Proteomics database (Swiss Institute of Bioinformatics, Lausanne, Switzerland) that match the following search terms: *triticum*, *aestivum*, wheat glutenin and gliadin for gluten and gliadin digests; and hordein, gluten, barley, *hordeum vulgare* for hordein digests. Exclusively identified peptides with a cross-correlation > 1.5 and a peptide probability > 30% were considered and reviewed in literature for T-cell stimulation. Quantitative analysis of peptides of interest was performed using LCquan Software (Thermo Fisher Scientific Inc.). The program integrated areas under the peak of individual peptides. The relative abundance (in %) in all samples was calculated setting the respective non-polymer treated sample to 100%. Calibration curve was performed by spiking gluten digest with increasing amounts of LGQQQPFPPQQPY. Linearity of MS quantification was verified by spiking a synthetic gluten peptide (PQPQLPYPQPQLP) into gluten- digest samples in a dose-dependent manner ($R^2 = 0.9945$).

Absorption Experiments

P(HEMA-co-SS) was purified by dialysis against water for 4 days and finally freeze-dried before use.

Study design

Male Sprague Dawley rats (250–280 g for single dose study and 410–430 g at gavage time for chronic dose study) were housed individually in metabolic cages. [³H]-P(HEMA-*co*-SS) was administered to 14 rats by gavage. Seven rats received a single dose of [³H]-P(HEMA-*co*-SS) (ca. 6.4 µCi/kg, ca. 150 mg/kg) and seven rats were treated with unlabeled P(HEMA-*co*-SS) in the drinking water at approximately 100 mg/kg/day for 21 days followed by a single dose of [³H]-P(HEMA-*co*-SS) (ca. 6.4 µCi/kg, ca. 150 mg/kg) by gavage on day 22. Total urines and feces were collected from 0 to 12, 12 to 24, 24 to 36, 36 to 48 and 48 to 72 h after administration of [³H]-P(HEMA-*co*-SS). Aliquots of blood were sampled at 30 min, 1, 2, 4, 8, 12, 24, 48 and 72 h by subclavian vein under isoflurane anesthesia. At sacrifice, exsanguination was performed followed by a saline flush before collecting organs (small intestine, colon, liver, kidneys and spleen).

Transgenic male mice (~25 g), 6 to 8 weeks old and expressing HLA-HCD4/DQ8 (HLA-DQA1*0301; HLA-DQB1*0302) genes in the absence of endogenous mouse class II genes were used.³ Mice (n = 4/group) were orally sensitized by gavaged with gluten (500 µg) and cholera toxin (CT, 25 µg) as mucosal adjuvant, once a week during 3 weeks. Controls (n = 4/group) received CT alone. The mice were housed individually in metabolic cages and received a single dose of [³H]-P(HEMA-*co*-SS) (ca. 22.4 µCi/kg, ca. 240 mg/kg) one week after the last sensitization. Urine and feces were collected from 0 to 12, 12 to 24, 24 to 36, 36 to 48 and 48 to 72 h after administration of [³H]-P(HEMA-*co*-

SS). Aliquots of blood were sampled at 72 h. At sacrifice, exsanguination was performed followed by a saline flush before collecting liver and kidneys.

Analytical method

Urines, feces and tissues were weighed and then homogenized. All homogenates were aliquoted (approximately 100–300 mg) and degraded using Solvable (Perkin Elmer, Waltham, MA) at 60°C and then bleached using hydrogen peroxide (30% v/v). Radioactivity was measured in Hionic Fluor (Perkin Elmer) using a Liquid Scintillation Analyser Tri-Carb 2100TR (Packard, Meriden, CT). Radioactivity in blood, urines, feces and tissues was expressed as a percentage of the administered dose. For blood, the percentage of the administered dose was calculated by multiplying the blood radioactivity per gram by $0.064 \times$ body weight in g for rats or $0.072 \times$ body weight in g for mice.⁴

In Vivo Experiments on Transgenic Mice

Effect of P(HEMA-co-SS) on Intestinal Permeability in Gluten-Sensitized Mice

Sections of tissues (jejunum) (5 cm) were removed and divided into two segments. Each segment was opened along the mesenteric border, rinsed and mounted in an Ussing chamber (exposed surface area 0.6 cm²). Tissues were bathed in oxygenated Krebs buffer containing 10 mM glucose (serosal side) or 10 mM mannitol (luminal side) at 37°C. The net active transport across the epithelium was measured *via* a short circuit current (I_{sc}) injected through the tissue under voltage clamp conditions. After a 15-min equilibration period, conductance (mS/cm²) was recorded. ⁵¹Cr-ethylene diamine tetra

acetic acid (EDTA) (Perkin Elmer, Waltham, MA) was used to probe paracellular permeability. ^{51}Cr -EDTA (6 $\mu\text{Ci}/\text{mL}$) was added to the luminal buffer once equilibrium was reached. Serosal samples (500 μL) were taken at 30-min intervals for 2 h and replaced with fresh buffer to maintain constant volume. The ^{51}Cr -EDTA was measured by scintillation counter (Beckman Coulter LS6500 Multi Purpose Scintillation Counter, Beckman, CA).

Effect of P(HEMA-co-SS) on Anti-Gliadin Antibodies in Gluten Sensitized-Mice

Anti-gliadin-immunoglobuline A (IgA) levels were determined in intestinal contents by ELISA. 96-well ELISA plates (Nunc-Immuno Plates Maxisorp, Roskilde, Denmark) were coated with 50 μL of phosphate buffered saline (PBS) including 5 μg of gliadin dissolved with 70% ethanol. 50 μL of intestinal wash supernatants were added in triplicate to the wells. Goat anti-mouse IgA-horseradish peroxidase (HRP) conjugate (α -chain specific) was then added as secondary antibodies (50 μL per well). Color was developed with 3,3',5,5'-tetramethylbenzidine substrate (Cedarlane Lab, ON), reaction stopped by HCl 1 N and optical densities (OD) were read at 450 nm using an ELISA plate reader (Bio-TEK Instruments Inc., Winooski, VT). The IgA OD were normalized by total protein concentration (Bio-Rad, Bradford protein assay reagent) per sample.

Effect of P(HEMA-co-SS) on Splenocyte Proliferation in Gluten Sensitized-Mice

Splenocytes were cultured in RPMI 10% fetal bovine serum (FBS) supplemented with either Con A, PT-gliadin (500 $\mu\text{g}/\text{mL}$ protein concentrations were measured with

bicinchoninic acid assay) or medium alone and incubated 72 h, 37°C, in 5% CO₂ humidified atmosphere. The cells were pulsed with 1 µCi/well [³H]-thymidine for additional 18 h. Cells were harvested on an automatic cell harvester and [³H]-thymidine uptake was measured by counting the radioactivity on filters with Beckman scintillation beta-counter. Results were expressed as stimulation index (SI) (mean cpm of triplicate culture containing Ag)/(mean of cpm of triplicate cells cultured with medium alone). The amounts of IL-10, MCP-1 and TNF-α in supernatants of cultured splenocytes were determined by ELISA using CBA kit (BD Bioscience ON) according to manufacturer's instructions.

Effect of P(HEMA-co-SS) in Gluten-Sensitized Mice After Chronic Administration

Mice were weighed daily throughout the study. Twenty-four hours following the final gluten challenge mice were anaesthetized and blood was collected *via* orbital bleed. Plasma samples were analyzed for biochemical parameters (albumin, alkaline phosphatase, alanine amino-transferase, creatinine, Na⁺, K⁺, Cl⁻ and urea). Hemoglobin levels were determined in blood samples using a hemoglobin assay kit (QuantiChrom, BioAssay Systems, Hayward, CA). White blood cells, red blood cells, and platelets were counted using a hemocytometer.

Histology

Cross-sections of the jejunum were collected, fixed in 10% formalin, embedded in paraffin, and stained with H&E for histological evaluation and measurement of villus-to-

crypt ratios using light microscopy. Twenty villus-to-crypt ratios were measured for each mouse in a blinded fashion.

Effect of P(HEMA-co-SS) in Biopsies from CD Patients

Endoscopic biopsies obtained from patients with confirmed CD were cultured in RPMI 1640 (BioWhittaker, Lonza, Belgium), supplemented with L-glutamine (2 mM), penicillin/streptomycin (100 UI penicillin/mL, 100 µg streptomycin/mL), 10% FBS and 0.001% protease inhibitor cocktail (Cytoskeleton, Denver, CO) and stimulated for 24 h with 100 µg of P-gliadin and/or 300 µg of P(HEMA-co-SS) or P-(gliadin/P(HEMA-co-SS)). When applied together, P-gliadin and P(HEMA-co-SS) were pre-incubated for 30 min in 37° C, 5% CO₂ and then added to the biopsies. The supernatants were collected and stored in -20°C until use. The levels of IL-10 and TNF-α were determined by ELISA (R&D Systems, Wiesbaden-Nordenstadt, Germany) according to the manufacturer's instructions. IL-15 and IL-21 were assayed using ELISA sets (BioLegend and eBioscience, San Diego, CA, respectively). The P-gliadin digestion was performed as previously reported.⁵ Beaded pepsin-agarose gel was tested for lipopolysaccharide contamination and shown to be below the limit of detection (LOD) of the assay. The gliadin/P(HEMA-co-SS) or P-gliadin/P(HEMA-co-SS) ratios were set at 1:3 (w/w).

Supplementary References

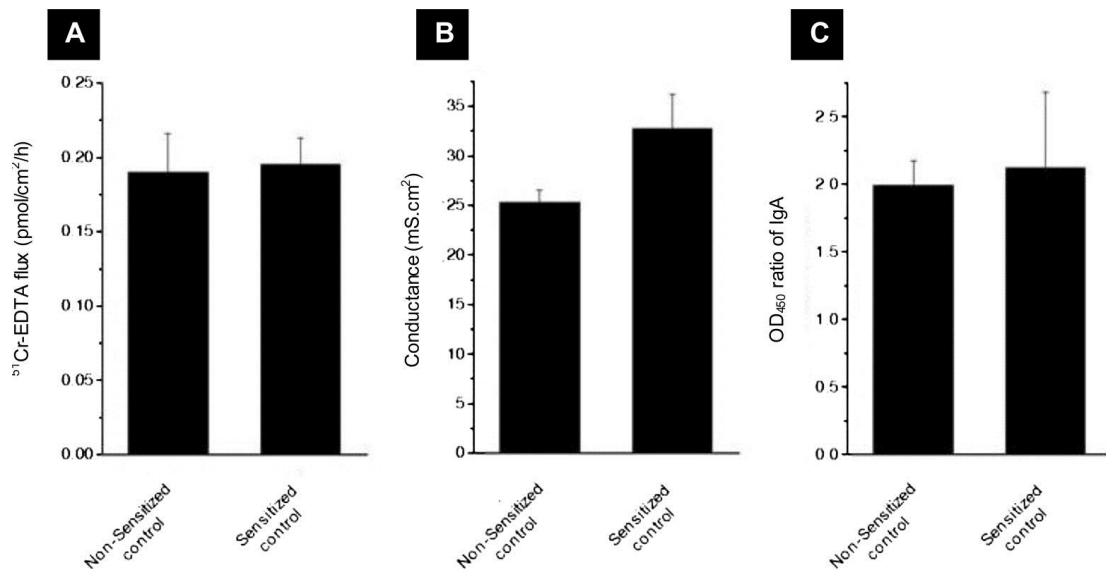
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Supplementary Figures

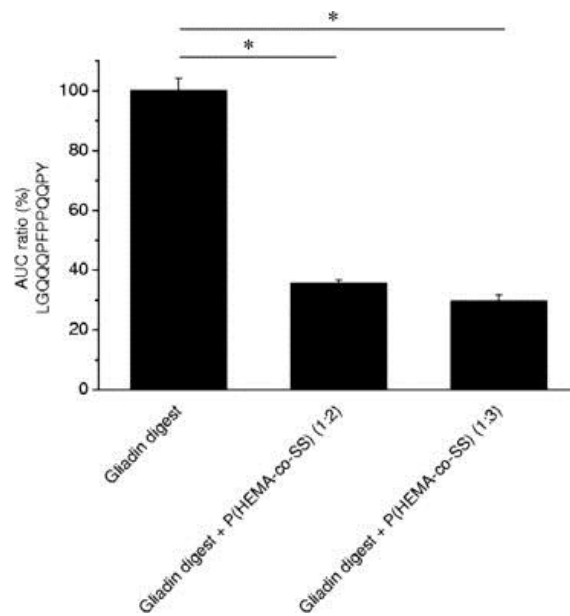
Supplementary Table 1. Biochemistry and hemogram data. Gluten-sensitized mice (n = 10/group) received gluten mixture (positive control group) or P(HEMA-co-SS) 5 min prior gluten mixture (treatment group) or only starch and albumin (negative control group). Gavages were performed twice a day, 3 times a week for 3 weeks

Parameter	Unit	Negative control group		Positive control group		Treatment group	
		Average	SD	Average	SD	Average	SD
Albumin	g/L	36.10	1.29	35.60	1.35	36.80	1.03
ALP	U/L	129.7	6.07	117.3	29.71	122.7	25.26
ALT	U/L	14.10	8.61	17.00	6.29	14.20	7.02
Creatinine	µmol/L	19.20	8.04	16.60	4.03	13.40	5.74
Na ⁺	mmol/L	146.4	4.00	149.6*	1.35	150.4*	1.78
K ⁺	mmol/L	4.84	0.39	5.13	0.44	4.95	0.24
Cl ⁻	mmol/L	104.8	3.40	108.8	3.52	106.9	3.54
Urea	mmol/L	8.04	1.30	7.76	0.94	7.84	1.21
Haemoglobin	g/100 mL	14.00	0.95	13.70	0.54	14.11	0.83
Red blood cells	10 ¹² /L	9.78	0.57	10.16	0.90	10.10	0.67
White blood cells	10 ⁹ /L	7.13	0.93	6.77	2.01	6.43	1.55
Platelets	10 ⁹ /L	379.3	48.43	369.4	42.82	361.4	49.75

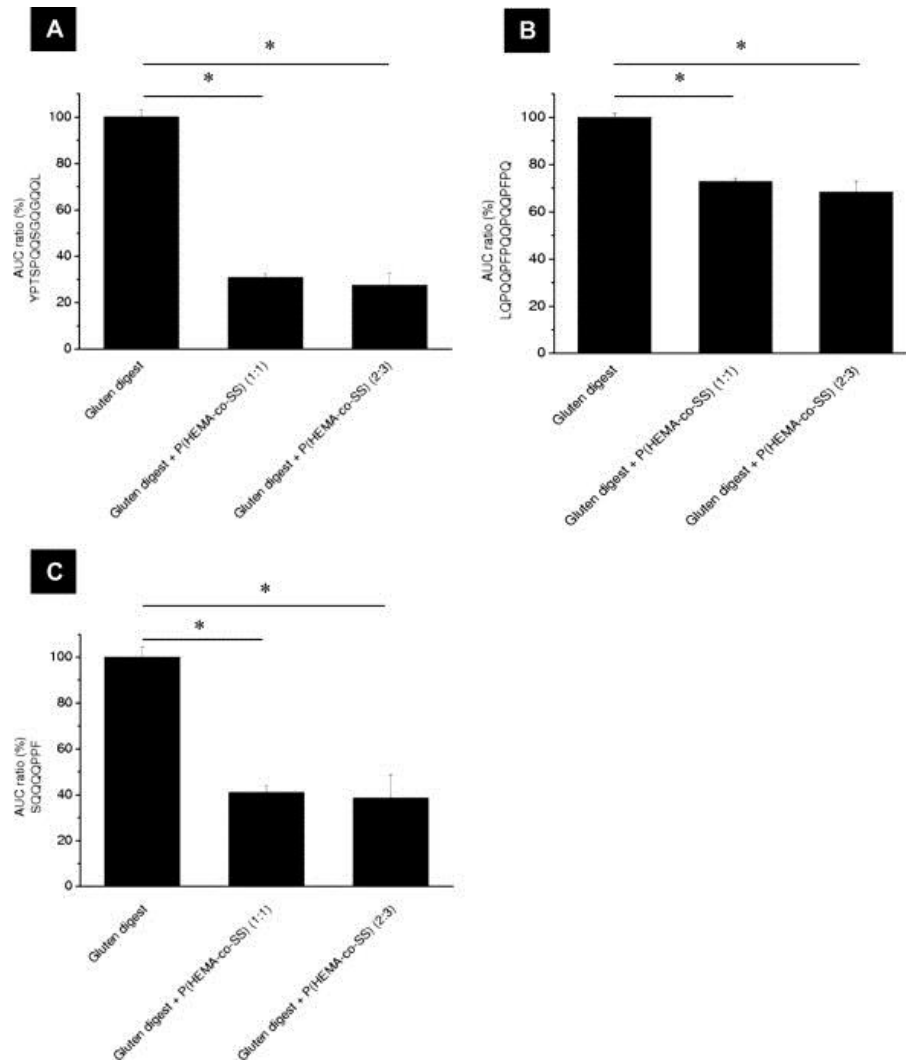
ALP: alkaline phosphatase, ALT: alanine amino-transferase. **P* < .05 vs. negative control group



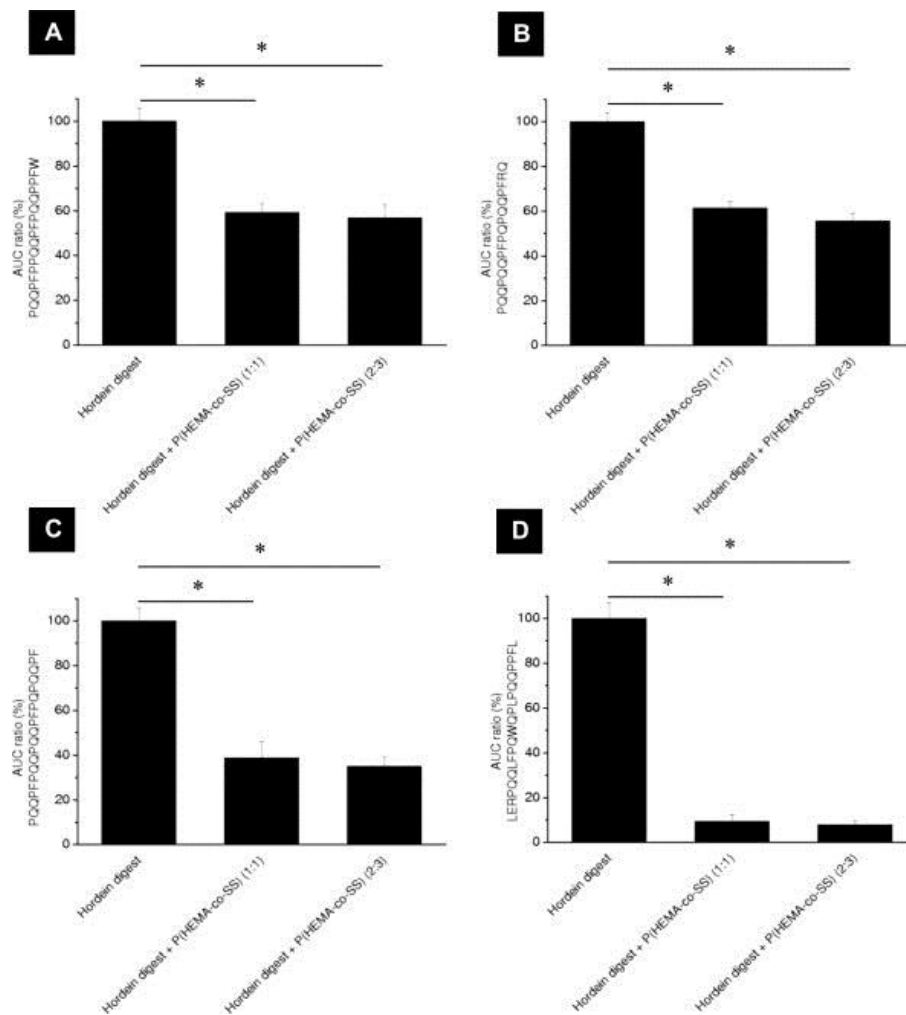
Supplementary Figure 1. (A) $^{51}\text{Cr-EDTA}$ flux (paracellular permeability), (B) intestinal tissue conductance and (C) anti-gliadin IgA in intestinal washes in non-sensitized (n = 12–14) vs. gliadin sensitized (n = 8–9) HCD4/DQ8 mice. Mean \pm SEM



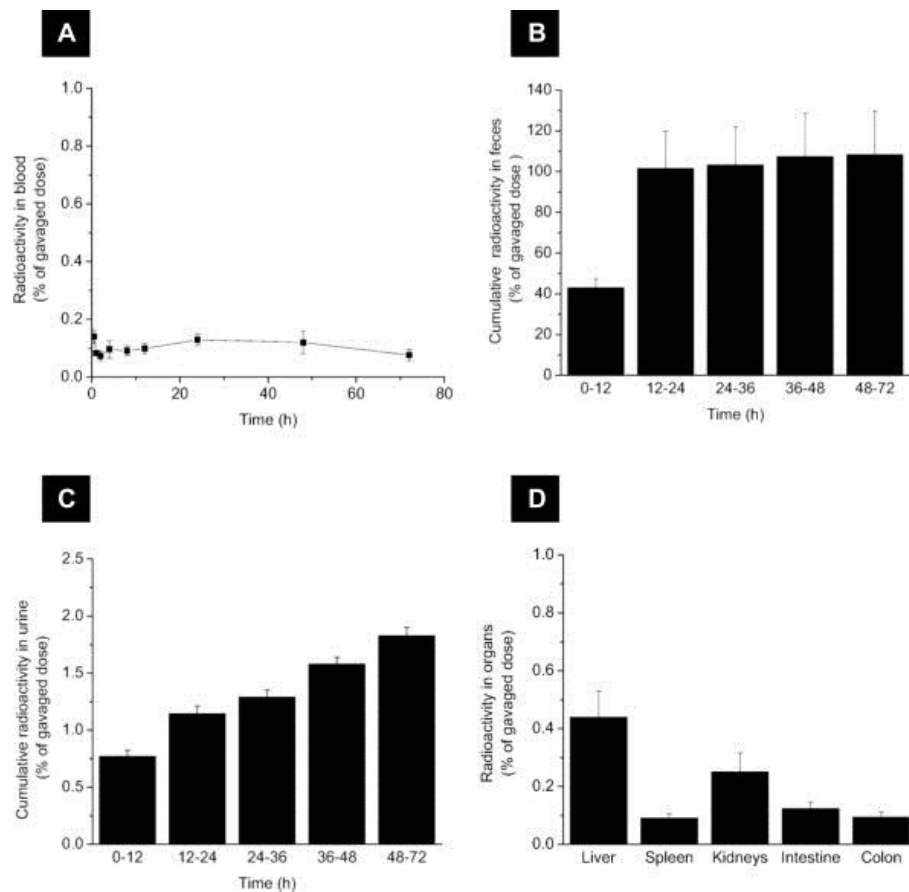
Supplementary Figure 2. P(HEMA-co-SS) decreases the formation of the 13-mer peptide, LGQQQPFPPQQPY from wheat gliadin. Digested gliadin samples were analyzed by LC-MS. Relative abundance was determined by comparison with the gliadin digest in the absence of P(HEMA-co-SS) (positive control). Mean \pm SEM displayed from 3 injections of 3 independent samples, $*P \leq 0.001$.



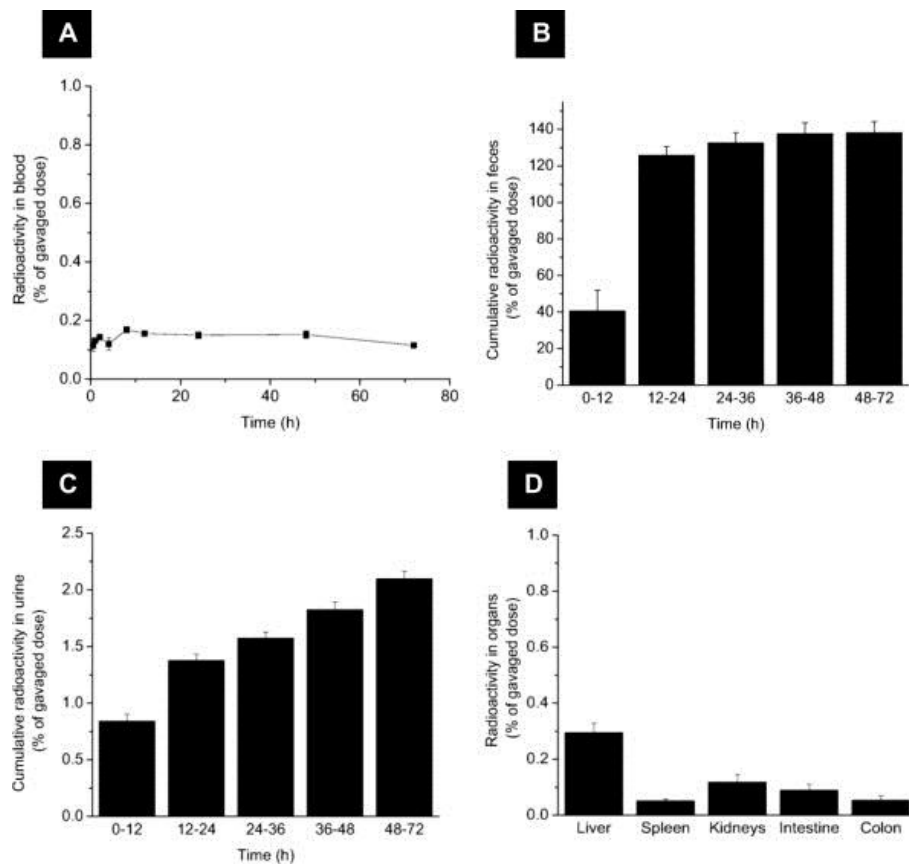
Supplementary Figure 3. P(HEMA-co-SS) decreases the formation of peptides YPTSPQQSGGQQL (A), LQPQQPFPQQPFPQ (B) and SQQQQPPF (C) from wheat gluten. Digested gluten samples were analyzed by LC-MS. Relative abundance was determined by comparison with the gluten digest in the absence of P(HEMA-co-SS). Mean \pm SEM displayed from 3 injections of 3 independent samples, $*P \leq 0.001$.



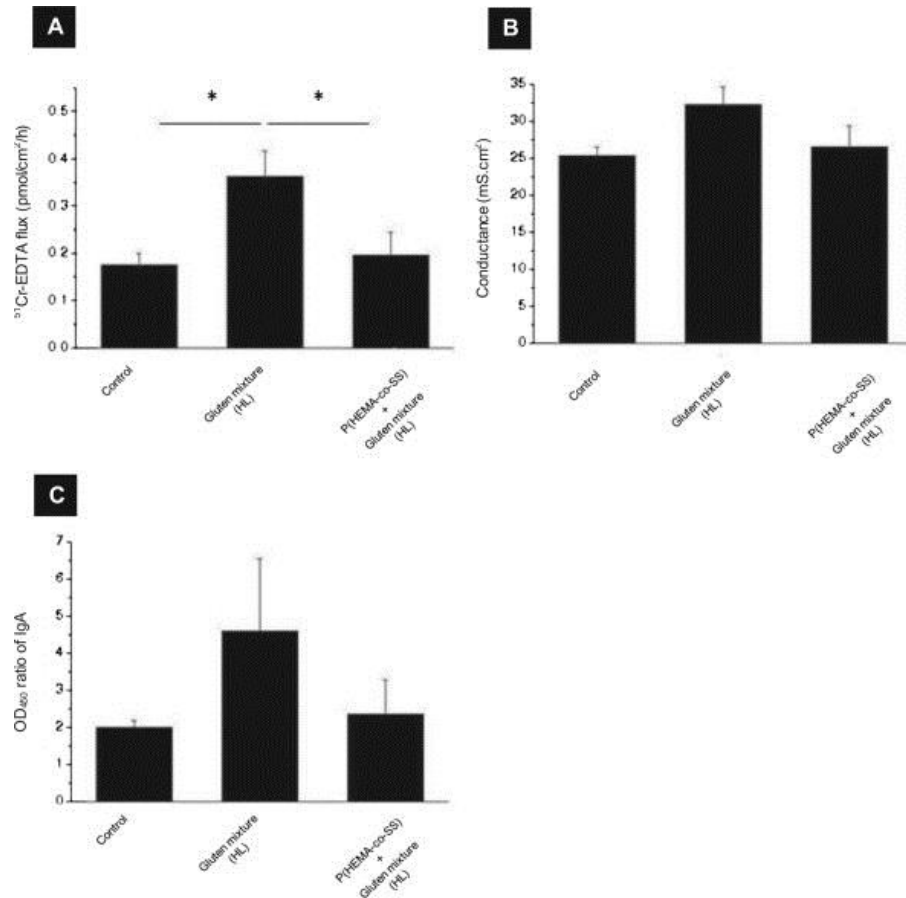
Supplementary Figure 4. P(HEMA-co-SS) decreases the formation of peptides PQQPFPPQQPFPPQPPFW (A), PQQPQQPFPPQPPFRQ (B), PQQPFPPQQPFPPQPPF (C), and LERPQQLFPQWQPLPQQPPFL (D) from barley hordein. Digested hordein samples were analyzed by LC-MS. Relative abundance was determined by comparison with the hordein digest in the absence of P(HEMA-co-SS) (positive control). Mean \pm SEM displayed from 3 injections of 3 independent samples, * $P \leq 0.005$.



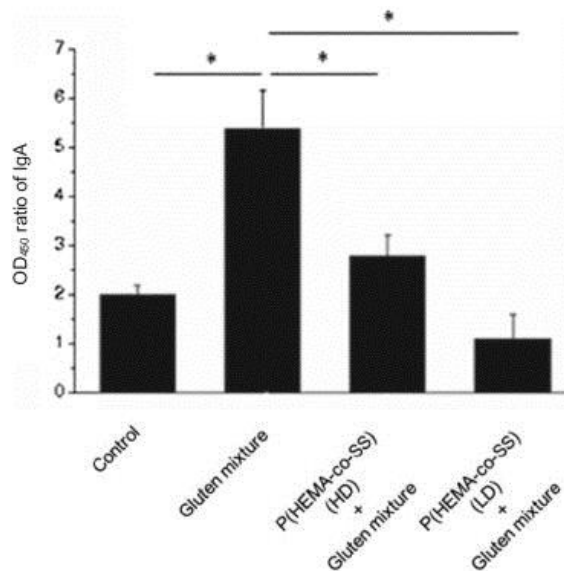
Supplementary Figure 5. P(HEMA-co-SS) is and mainly excreted in feces and poorly absorbed. Pharmacokinetics, excretion profiles and biodistribution of [³H]-P(HEMA-co-SS) in Sprague Dawley rats after single oral dosing (150 mg/kg, 6.4 μCi/kg). (A) Blood concentrations, (B) cumulative dose excreted in the feces, (C) cumulative dose excreted in urine, (D) organ distribution. Mean ± SEM (n = 7)



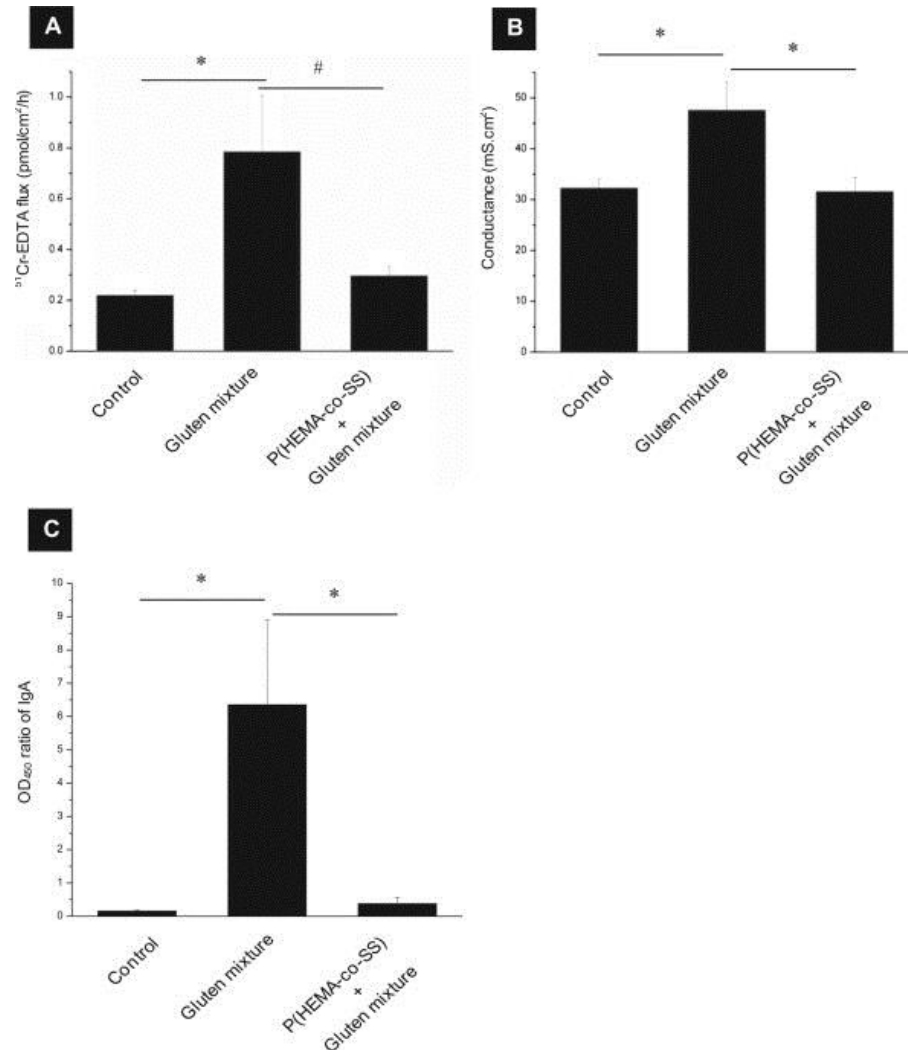
Supplementary Figure 6. P(HEMA-co-SS) is and mainly excreted in feces and poorly absorbed. Pharmacokinetics, excretion profiles and biodistribution of [³H]-P(HEMA-co-SS) in Sprague Dawley rats, after a polymer exposure for 21 days (100 mg/kg/day) and a single dose of [³H]-P(HEMA-co-SS) (150 mg/kg, 6.4 μCi/kg) at day 22. (A) Blood concentrations, (B) cumulative dose excreted in the feces, (C) cumulative dose excreted in urine, (D) organ distribution. Mean ± SEM (n = 7).



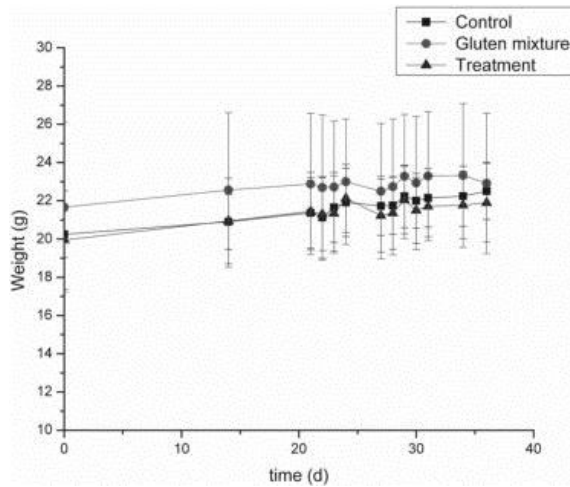
Supplementary Figure 7. (A) ⁵¹Cr-EDTA flux (paracellular permeability), (B) intestinal tissue conductance, (C) anti-gliadin IgA in intestinal washes. Controls: non-sensitized mice gavaged with BSA and starch (n = 12–14); gluten mixture (HL) (gluten mixture with a higher level of gluten, 4 mg) group: gluten-sensitized mice gavaged with wheat gluten (4 mg)/BSA (2 mg)/starch (2 mg) (n = 8–9); P(HEMA-*co*-SS) + gluten mixture (HL) group (treatment group): gluten-sensitized mice gavaged first with P(HEMA-*co*-SS) (4 mg) and subsequently with wheat gluten (4 mg)/BSA (2 mg)/starch (2 mg) (gluten/polymer 1:1 w/w) (n = 8–9). Mean ± SEM **P* ≤ 0.02.



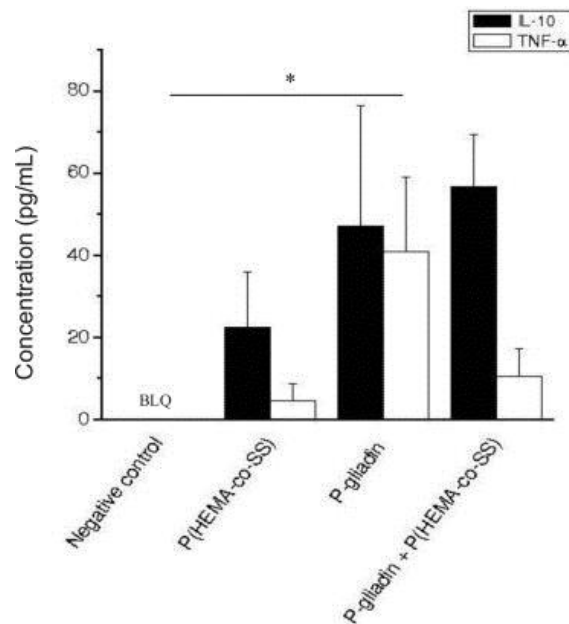
Supplementary Figure 8. Administration of P(HEMA-co-SS) to gluten-sensitized HCD4/DQ8 mice challenged with gluten-food mixture decreased IgA levels. Gluten sensitization and gluten-food mixture challenge to HCD4/DQ8 mice increased OD levels for anti-gliadin IgA in intestinal washes. In gluten-sensitized mice, administration of P(HEMA-co-SS) returned IgA levels to control values. The control group was used as reference (100% optical density at 450 nm, OD₄₅₀). Mean ± SEM, * $P \leq 0.001$. Controls: non-sensitized mice gavaged with BSA and starch (n = 14); gluten mixture group: gluten-sensitized mice gavaged with gluten/BSA/starch (n = 10); P(HEMA-co-SS) (HD) + gluten mixture group (treatment group): gluten-sensitized mice gavaged first with P(HEMA-co-SS) and subsequently with gluten/BSA/starch (gluten/polymer 1:2 w/w) (n = 10); P(HEMA-co-SS) (LD) + gluten mixture group (second treatment group): gluten-sensitized mice gavaged first with P(HEMA-co-SS) and subsequently with gluten/BSA/starch (gluten/polymer 2:1 w/w) (n = 6).



Supplementary Figure 9. Effect of treatment following a chronic exposure to gluten: (A) ^{51}Cr -EDTA flux (paracellular permeability), (B) intestinal tissue conductance, (C) anti-gliadin IgA in intestinal washes. Controls: non-sensitized mice gavaged with BSA and starch; gluten mixture group: gluten-sensitized mice gavaged with wheat gluten (2 mg)/BSA (2 mg)/starch (2 mg); P(HEMA-co-SS) + gluten mixture group (treatment group): gluten-sensitized mice gavaged first with P(HEMA-co-SS) (4 mg) and subsequently with wheat gluten (2 mg)/BSA (2 mg)/starch (2 mg). Challenges were performed twice a day, 3 times a week for 3 weeks. Mean \pm SEM, (n = 9–10), * $P \leq 0.02$; # $P = 0.0585$)



Supplementary Figure 10. P(HEMA-co-SS) did not affect the evolution of the weight gain *in vivo* of HLA-DQ8 transgenic mice after a 3 week challenge. Controls: non-sensitized mice gavaged with BSA and starch; gluten mixture group: gluten-sensitized mice gavaged with wheat gluten (2 mg)/BSA (2 mg)/starch (2 mg); Treatment group: gluten-sensitized mice gavaged first with P(HEMA-co-SS) (4 mg) and subsequently with wheat gluten (2 mg)/BSA (2 mg)/starch (2 mg). Challenges were performed twice a day, 3 times a week for 3 weeks (n = 10). Mean \pm SD.



Supplementary Figure 11. Production of TNF- α (white bars) and IL-10 (black bars) in culture supernatants from biopsies of CD patients after incubation with P(HEMA-co-SS) alone or with P-gliadin +/- P(HEMA-co-SS). Negative control corresponds to biopsies incubated with medium only. Mean \pm SEM (n = 5), * $P \leq 0.05$. BLQ: below limit of quantification (<31.3 pg/mL for IL-10 and <15.6 pg/mL for TNF- α).

CHAPTER 6

**NOVEL ROLE OF THE SERINE PROTEASE INHIBITOR ELAFIN IN
GLUTEN-RELATED DISORDERS.**

Novel role of the serine protease inhibitor elafin in gluten-related disorders.

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Preface: The work in this manuscript was performed from Sept 2010 until Oct 2013. I am a primary co-author of the manuscript along with M. Wiepjes. The manuscript was written by my supervisor, E. Verdu and myself. *In vivo* (mouse) experiments were designed by my supervisor, M. Weipjes, and myself, and conducted by M. Weipjes and myself. Data was analyzed by M. Weipjes and myself. Patient biopsies were provided by I. Pinto-Sanchez, E. Smecuol and J.C. Bai and patient information collected by I. Pinto-Sanchez. J.P Motta assisted with Elafin staining in human biopsies. Deamidation assays were designed by J.C Leroux and performed by J.D. Schultz. Technical assistance for Ussing chamber experiments was provided by J. Jury. J.M Natividad provided assistance for mouse ZO-1 immunofluorescence staining. D. Sinclair assisted with *in vivo* work. Recombinant *L. lactis* strains were developed by L. Bermudez-Humaran and P. Langella

and produced for this study by Perrine Rousset and Rebeca Martin-Rosique. Protease activity was measured with assistance from J.P Motta and N. Vergnolle. J.P. Motta, J.C. Leroux, J.A. Murray, J.C. Bai, N. Vergnolle, and P. Langella contributed scientific input and critically appraised the manuscript.

Summary and significance: The need for adjuvant therapies to the gluten free diet (GFD) is emphasized by the incomplete mucosal and symptomatic recovery of celiac patients, as well as the difficulty in adhering to strict gluten exclusion. Elafin is a human protease inhibitor that has previously been shown to have barrier-protecting and anti-inflammatory properties in models of colitis, but its expression and therapeutic value in celiac disease (CD) is unknown. Therefore, this article investigated the small intestinal expression of elafin in CD patients and the therapeutic potential of elafin using *in vitro* assays and gluten-sensitized NOD/DQ8 mice. Elafin was found to be decreased in small intestinal biopsies obtained from CD patients compared to control patients. Moreover, elafin inhibited the deamidation of an immunogenic gliadin peptide, an important step in CD pathogenesis. Finally, when administered to gluten-sensitive NOD/DQ8 mice, elafin protected against gluten-induced barrier dysfunction and inflammation. These findings provide the first description of elafin expression in patients with CD and provide experimental evidence to support its therapeutic value for treating gluten-related disorders. Moreover, the findings in this article demonstrated a novel mechanisms (inhibition of gliadin deamidation) through which elafin may exert its therapeutic effects.

Title: Novel role of the serine protease inhibitor elafin in gluten-related disorders.

Short title: Elafin and celiac disease

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Keywords: Gluten-sensitivity, elafin, celiac disease, proteolytic therapy

Abstract

OBJECTIVES: Elafin, an endogenous serine protease inhibitor, modulates colonic inflammation. We investigated the role of elafin in celiac disease (CD) using human small intestinal tissues and *in vitro* assays of gliadin deamidation. We also investigated the potential beneficial effects of elafin in a mouse model of gluten sensitivity.

METHODS: Epithelial elafin expression in the small intestine of patients with active CD, treated CD, and controls without CD was determined by immunofluorescence. Interaction of elafin with human tissue transglutaminase-2 (TG-2) was investigated *in vitro*. The 33-mer peptide, a highly immunogenic gliadin peptide, was incubated with TG-2 and elafin at different concentrations. The degree of deamidation of the 33-mer peptide was analyzed by liquid chromatography-mass spectrometry. Elafin was delivered to the intestine of gluten-sensitive mice using a recombinant *Lactococcus lactis* vector. Small intestinal barrier function, inflammation, proteolytic activity, and zonula occludens-1 (ZO-1) expression were assessed.

RESULTS: Elafin expression in the small intestinal epithelium was lower in patients with active CD compared with control patients. *In vitro*, elafin significantly slowed the kinetics of the deamidation of the 33-mer peptide to its more immunogenic form. Treatment of gluten-sensitive mice with elafin delivered by the *L. lactis* vector normalized inflammation, improved permeability, and maintained ZO-1 expression.

CONCLUSIONS: The decreased elafin expression in the small intestine of patients with active CD, the reduction of 33-mer peptide deamidation by elafin, coupled to the barrier enhancing and anti-inflammatory effects observed in gluten-sensitive mice, suggest that this molecule may have pathophysiological and therapeutic importance in gluten-related disorders.

Introduction

Celiac disease (CD) is triggered by the ingestion of gluten in genetically susceptible individuals. Gluten has been proposed to contribute to health issues other than CD (1-4). Currently, there are no pharmacological treatments available for patients with CD or other gluten-related disorders. A life-long adherence to a gluten-free diet (GFD) is required after the diagnosis of CD is confirmed. However, a GFD is associated with high nonadherence rates (5,6). Gluten is a common ingredient in many food items, making complete removal of gluten from the diet challenging (7,8). Although effective in inducing clinical remission of CD, following a strict GFD may have a negative impact on lifestyle and quality of life (9-11).

Elafin is a human serine protease inhibitor that has potent inhibitory capacity against various forms of elastases, as well as proteinase-3 (12-14). Elafin is expressed throughout the epithelium of the gastrointestinal tract, and its expression and induction is decreased in patients with inflammatory bowel disease (15-17). However, it is unknown whether elafin expression is altered and whether it has a role in chronic inflammatory disorders of the proximal small intestine, such as CD.

Elafin has been identified as a substrate for the cross-linking activity of tissue transglutaminase (TG-2) (18,19). The discovery that TG-2 has an important role in CD by introducing negatively charged residues in gluten peptides through deamidation, thereby increasing binding affinity to HLA-DQ2/DQ8 molecules, is one of the recent milestones in CD pathogenesis (20). Given the importance of TG-2 to the pathogenesis of CD, its inhibition is an attractive therapeutic target. TG-2, however, is found systemically and has

important functions in a number of other biological processes including wound healing, cell migration, cell death, and inflammation (21). Whether the inhibition of TG-2 is reversible and local to the small intestine are important considerations for a CD-targeted therapy. The ability of elafin to inhibit deamidation of gliadin peptides by TG-2 has not yet been tested. The small intestinal expression profile of this immunomodulatory protease inhibitor is unknown in CD. Thus, we first investigated the expression of elafin in patients with active CD and 1 year after a GFD. Patients undergoing gastroduodenal endoscopy, in whom CD was excluded, were included as controls. The potential interaction of elafin and human TG-2, an enzyme with an established role in CD pathogenesis, was tested using 33-mer peptide deamidation assays. Finally, the anti-inflammatory and barrier-enhancing properties of elafin in the proximal small intestine were investigated in an animal model of gluten sensitivity.

Methods

Elafin expression in human small intestinal mucosa

Human small intestinal biopsies used in this study were randomly selected from a series of subjects participating in previous studies (22,23). The baseline clinical characteristics of subjects are shown in Table 1. The CD diagnosis was based on the concomitant presence of histological damage in the first and second duodenal portions (Marsh IIIa or greater) and a positive CD serology (IgA anti-TG-2; Inova, San Diego, CA). Histology was investigated in biopsy specimens of patients at the time of the diagnosis ($n=10$), in the same patients after 1 year of a GFD ($n=9$), and in patients with

dyspepsia, peptic ulcer, gastroesophageal reflux disease, or irritable bowel syndrome, where the diagnosis of CD was excluded ($n=11$).

Table 1. Patient population

Characteristics	NC Controls (n=11)	Active CD (n=10)	1 yr GFD (n=9)
Age (mean+/- SD)	48 (+/-15)	40 (+/-18)	47 (+/-15)
Gender (F/M)	10/1	9/1	8/1
IgA TG-2; cut-off 20 U/mL)	11/11 negative	10/10 positive	9/9 negative
Biopsy (Marsh's modified)	11/11 Marsh 0	10/10 positive (>IIIa)	7/9 Marsh 0; 1/9 Marsh I; 1/9 Marsh II

CD, celiac disease; F, female; GFD, gluten-free diet; IgA, immunoglobulin A; M, male; NC, non-celiac; TG-2, tissue transglutaminase

Five-micrometer sections were deparaffinized and treated with a buffer solution for heat-induced epitope retrieval (pH 6.0). Slides were incubated overnight at 4 °C with anti-elafin (Santa Cruz Biotechnology, Montrouge, France) and anti-cytokeratin 18 (CK18; Santa Cruz Biotechnology) antibodies. Anti-CK18 was used as a marker for epithelial cells. Slides were mounted and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) fluorescent mounting medium (Invitrogen, Burlington, ON, Canada) and analyzed on confocal microscope Zeiss LSM710 (Carl Zeiss, Toronto, ON, Canada). For relative quantification by immunofluorescence staining, four different fields of acquisitions for one patient were taken with the same microscope settings in a blinded

manner. We have converted images from mucosal epithelial marker (CK18) to a binary mask in order to obtain a final image representing only the specific staining of elafin in the mucosa. Elafin fluorescence intensity was then quantified in this image using the Image J software (National Institutes of Health, Bethesda, MD) and was reported as arbitrary unit per surface of tissue. Each plot represents the mean fluorescence intensity (MFI) of four different acquisition fields of the same patient. Data were represented as fold increase of signal intensity quantified in control patients.

Inhibition of deamidation of 33-mer peptide by elafin

The 33-mer peptide (25 μ M, alpha2-gliadin, 33mer (aa 56-88), Zedira, Darmstadt, Germany, diluted in 50 mM phosphate buffer, pH 7.5) was incubated with 1 μ M TG-2 (0.59 U/mg, purity >90 %, Zedira) at 37 °C in 100 mM Tris/HCl buffer, pH 7.6, with 10 mM CaCl₂. The TG-2-catalyzed deamidation of 33-mer served as a control reaction in the assay. In the competitive reaction, 25, 50, and 100 μ M elafin (human, recombinant, purity >90%, Sigma-Aldrich, St. Louis, MO) was mixed with the peptide before starting the enzymatic reaction. A negative control with insulin (human, recombinant, purity >98%, Sigma-Aldrich) and a positive control with tridegin (recombinant, purity >95%, Zedira) were prepared each in a 1:1 molar ratio with the 33-mer peptide. The reaction was inactivated at different time points (0, 5, 15, 30, 60 min) by the addition of 25 mM iodoacetamide. All reactions were performed in triplicate. The analysis of the samples was performed using a liquid chromatography system consisting of Rheos Allegroquaternary pump, C18-column (Hypersil Gold, 100 \times 1 mm, 1.9 μ m), and

XCalibur control software (Thermo Fisher Scientific, San Jose, CA). Samples were injected (10 μ L) with a flow rate of 50 μ L/min, 95 % water (+0.1 % v/v formic acid) as an initial value for 1 min, followed by a linear gradient of ACN (+0.1 % v/v formic acid) over 19 min (95 – 5 % v/v). The liquid chromatography was directly connected to an LTQ XL linear quadrupole Ion Trap (Thermo Fisher Scientific) equipped with an electrospray ion source. Data were acquired by full mass spectrometry. Given that deamidation yields in a mass increase of 1 Da only, the untreated and deamidated peptide overlap in the mass spectrum. Therefore, the centroid mass determination was used for the quantification of deamidation (Eq. 1).

$$m_c = \frac{\sum_{i=0}^m m_i I_i}{\sum I_i} \quad (1)$$

where m_i and I_i are the masses and intensities of individual signals, respectively (24). The total deamidation degree was determined by calculating the difference of centroid masses of TG-2-reacted and untreated peptide.

Animal model of gluten sensitivity

Eight- to 10-week-old male NOD AB^o DQ8 (NOD/DQ8) transgenic mice were used for experiments (25). Mice were weaned and maintained on a low-fat (4.4%), GFD, purchased from Harlan Laboratories (Indianapolis, IN) and bred in a conventional, specific pathogen-free colony at McMaster University. All experiments were conducted with approval from the McMaster University Animal Care Committee.

Mice were orally sensitized with a peptic-tryptic digest of gliadin (PT-gliadin), prepared as described previously (26). Mice were gavaged with 500 µg of PT-gliadin plus 25 µg of cholera toxin (Sigma-Aldrich) once per week for 3 weeks (27). Nonsensitized controls were gavaged with 25 µg cholera toxin only. After sensitization, mice were challenged with gliadin (2 mg/mouse) dissolved in 0.02 M acetic acid by intragastric gavage once a week for 2 weeks.

Mucosal delivery of elafin

We chose a *L. lactis* vector as mucosal delivery system for elafin, as this approach was extensively validated in previous mouse models of colitis (17). Briefly, an elafin-producing recombinant strain of *L. lactis* was used as a bacterial vector to deliver elafin to the intestine. *L. lactis* carrying the empty plasmid vector (*Ll*-WT) was used as a wild-type (WT) control (17). All bacteria were suspended in PBS containing 15% glycerol. Twenty-four hours after oral sensitization with PT-gliadin and cholera toxin, NOD/DQ8 mice were treated with *Ll*-WT, *Ll*-E (10^9 colony forming units), or PBS–glycerol by oral gavage, daily for 15 days. Mice received gliadin challenges (2 mg/mouse) on days 7 and 15 of *L. lactis* treatment.

Measurement of small intestinal permeability

Twenty-four hours following the final gliadin challenge, *in vitro* intestinal permeability was assessed by the Ussing chamber technique, as previously described (World Precision Instruments, Sarasota, FL) (27-29). Two sections of jejunum from each

mouse were assessed. Tissue conductance (the passive permeability to ions) and mucosal-to-serosal flux of the paracellular probe $^{51}\text{Cr-EDTA}$ were used to assess barrier function and paracellular permeability, as previously described (30). $^{51}\text{Cr-EDTA}$ flux was calculated as the average over a 2-h period and expressed as %hotsample/h/cm².

Evaluation of small intestinal inflammation

Cross-sections of the proximal small intestine were fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin for histologic evaluation by light microscopy (Olympus, Richmond Hill, ON, Canada), as previously described (27). Paraffin sections were used to preserve tissue morphology and orientation. Immunostaining for CD3⁺ cells was performed on paraffin sections to detect the presence of intraepithelial lymphocytes (IELs) in sections of the proximal small intestine, as described previously (29,31). Slides were viewed in a blinded manner by light microscopy under $\times 20$ magnification. IELs per 20 enterocytes in five randomly chosen villus tips were counted according to the previously described methods and expressed as IEL/100 enterocytes (29).

ZO-1 expression in small intestine

Staining for the tight-junction protein zonula occludens-1 (ZO-1) was performed on jejunal sections. Sections were incubated for 2 h at room temperature with an anti-ZO-1 (primary) antibody (Invitrogen), washed, and secondary antibody was applied for 2 h at room temperature. Slides were then mounted and coverslipped with Prolong Gold Anti-

fade with DAPI (Invitrogen), and staining was assessed in a blinded manner by using a fluorescence microscope (Olympus). A total of 10 images were obtained for each slide, and the MFI of five identically sized regions of interest was determined for each image using the Image J Software (National Institutes of Health). Each region of interest was centered on the epithelial border. The MFI for the negatively stained sections was then subtracted from that of positively stained sections.

Trypsin-like and elastase-like activities in the small intestine

A section of the jejunum from nonsensitized and sensitized NOD/DQ8 mice ($n=5$) was collected using a 4-mm biopsy punch and transferred into a 48-well plate (BD Biosciences, Mississauga ON, Canada) containing RPMI 1640 enriched with 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM L-glutamine. Wells were supplemented with 100 µg/ml of one the following gliadin peptides: 19-mer (p31-49), 20-mer (p120-139), and 33-mer (p56-89; ProImmune, Sarasota, FL). Media alone were used for negative controls. Plates were incubated for 3 h at 37 °C with 95% O₂ and 5% CO₂. After incubation, the tissue section was removed and frozen at -80 °C. Supernatants were collected and stored at -80 °C. Trypsin and elastase-like activity was measured in organ culture supernatant, biopsies, as well as intestinal samples from nonsensitized and gliadin-sensitized mice, as previously described (32).

Statistical analysis

Statistical analysis was conducted using the GraphPad Prism software (version 4, 2004, GraphPad Software Inc., San Diego, CA). Statistical significance was determined by one-way analysis of variance followed by Bonferroni's multiple comparison tests. Significance was reported for P values <0.05 at a 95% confidence level. All data are presented as mean \pm s.e.m.

Results

Elafin expression is reduced in the small intestine of patients with active CD

We tested whether elafin expression is altered in small intestinal biopsies of patients with CD. Biopsies were collected from the duodenum and jejunum of patients with active CD (on a gluten-containing diet), treated CD patients (on a GFD for at least 1 year), and control patients (gastrointestinal complaints in which CD has been excluded). Biopsies were then stained for elafin expression by immunofluorescence. Elafin expression in the epithelium, as determined by co-staining with CK18, was lower in patients with active CD compared with control patients (Figure 1a). This reduction was confirmed by determining the MFI of elafin expression in areas of tissue staining with the CK18 marker. Elafin intensity was significantly lower in active CD patients compared with control patients (Figure 1b). Elafin levels in treated CD were intermediate between untreated CD and controls but were not statistically different from either.

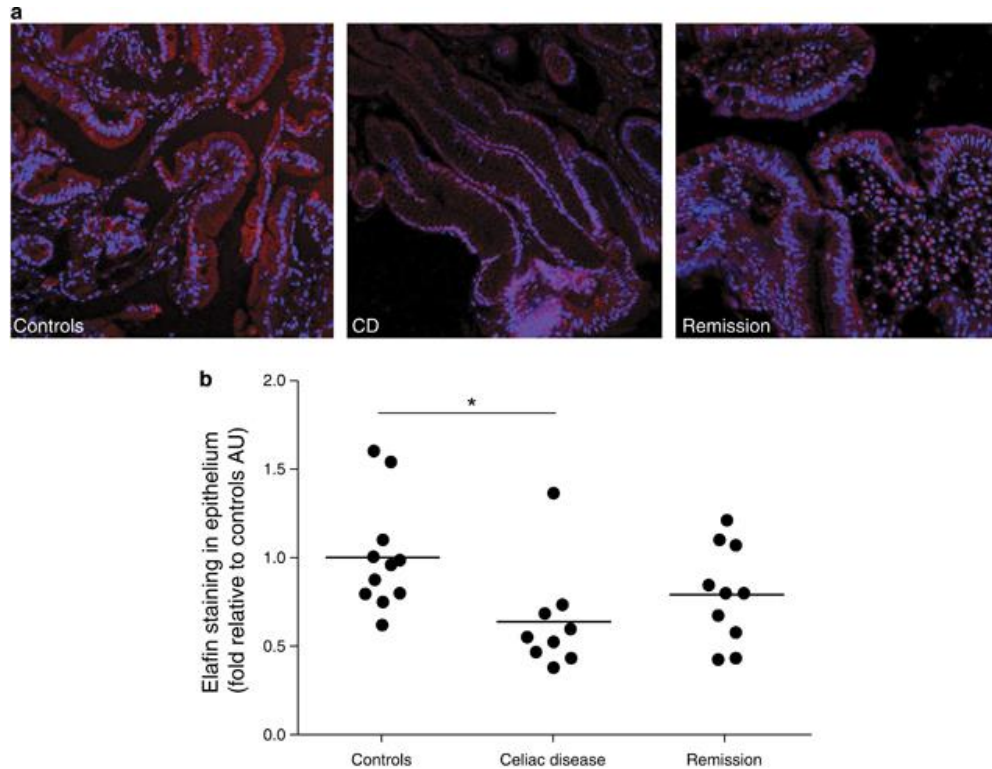


Figure 1. Decreased elafin expression in patients with active celiac disease (CD). Biopsies were obtained from patients in whom CD was excluded (controls), patients with treated CD (1 year gluten-free diet (GFD); remission), and patients with active CD and were stained for elafin (red) expression. (a) Representative immunofluorescence figures are shown. Nuclei labeled with 4'6-diamidino-2-phenylindole (DAPI; blue). (b) Quantification of fluorescence intensity, expressed as a fold increase to control patients. Each dot represents the mean fluorescent value of four different areas of one patient, and the line represents the mean of each group. $*P < 0.05$.

Inhibition of deamidation of 33-mer peptide in vitro

Elafin has been identified as a substrate for TG-2 (19). Using a competitive enzymatic assay, we therefore tested elafin's inhibiting effect on the deamidation of 33-mer gliadin peptide, which served as a model immunogenic substrate. The addition of elafin significantly slowed the kinetics of the deamidation of the 33-mer peptide to its

more immunogenic form (Figure 2). The inhibiting effect of elafin was found to be dose and time-dependent, but relatively modest compared with tridegin, the positive control (33). When used at a concentration that was 4-fold higher than tridegin, the deamidation of the 33-mer peptide was approximately 50% less effective after 30 min of incubation. As expected, the negative protein control, insulin, did not show any inhibition of TG-2 activity (Figure 2).

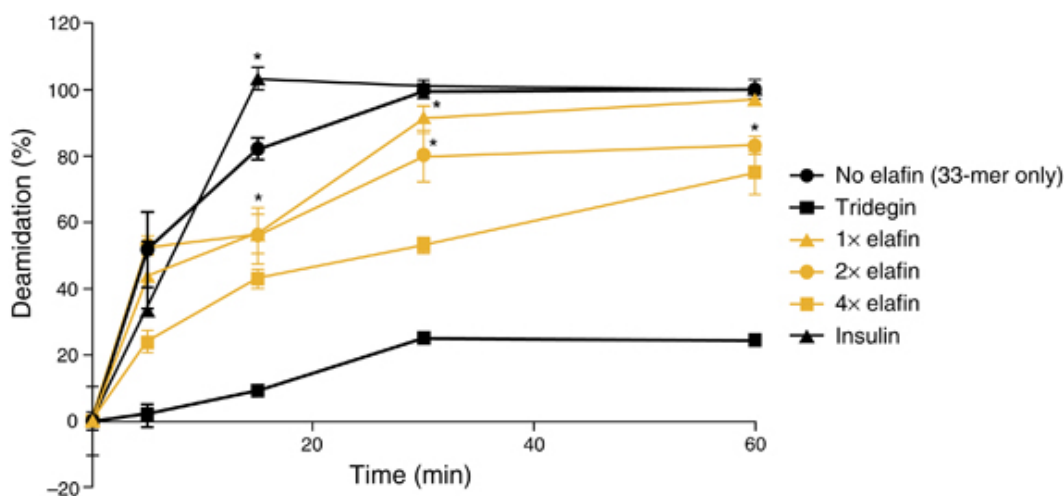


Figure 2. Deamidation of 33-mer in the competitive reactions. 33-mer peptide (25 μ M) was incubated with tissue transglutaminase-2 (TG-2; 1 μ M) that served as a control reaction. For the competitive reactions, 25 (\times 1), 50 (\times 2), or 100 μ M (\times 4) elafin was added. Tridegin and insulin were used as positive and negative controls, respectively. The degree of deamidation was analyzed by liquid chromatography-mass spectrometry (LC-MS). Data are represented as mean \pm s.e.m. ($n=3$). * $P<0.05$ vs. control reaction (no elafin); all data points of the enzymatic reaction with 100 μ M (\times 4) elafin and tridegin are statistically significant ($P<0.02$) vs. control reaction (no elafin).

Ll-elafin reduces gliadin-induced IEL infiltration in gliadin-sensitized NOD/DQ8 mice

We then tested the therapeutic potential of elafin in gliadin-sensitized NOD/DQ8 mice, a mouse model of gluten sensitivity, using *L. lactis* to deliver elafin (*Ll-E*) to the

small intestine. In agreement with previous findings, (5,27) sensitization and acute gliadin challenge increased IELs in the jejunum (Figure 3). *Ll*-E treatment normalized IEL counts, whereas *Ll*-WT had no effect (Figure 3).

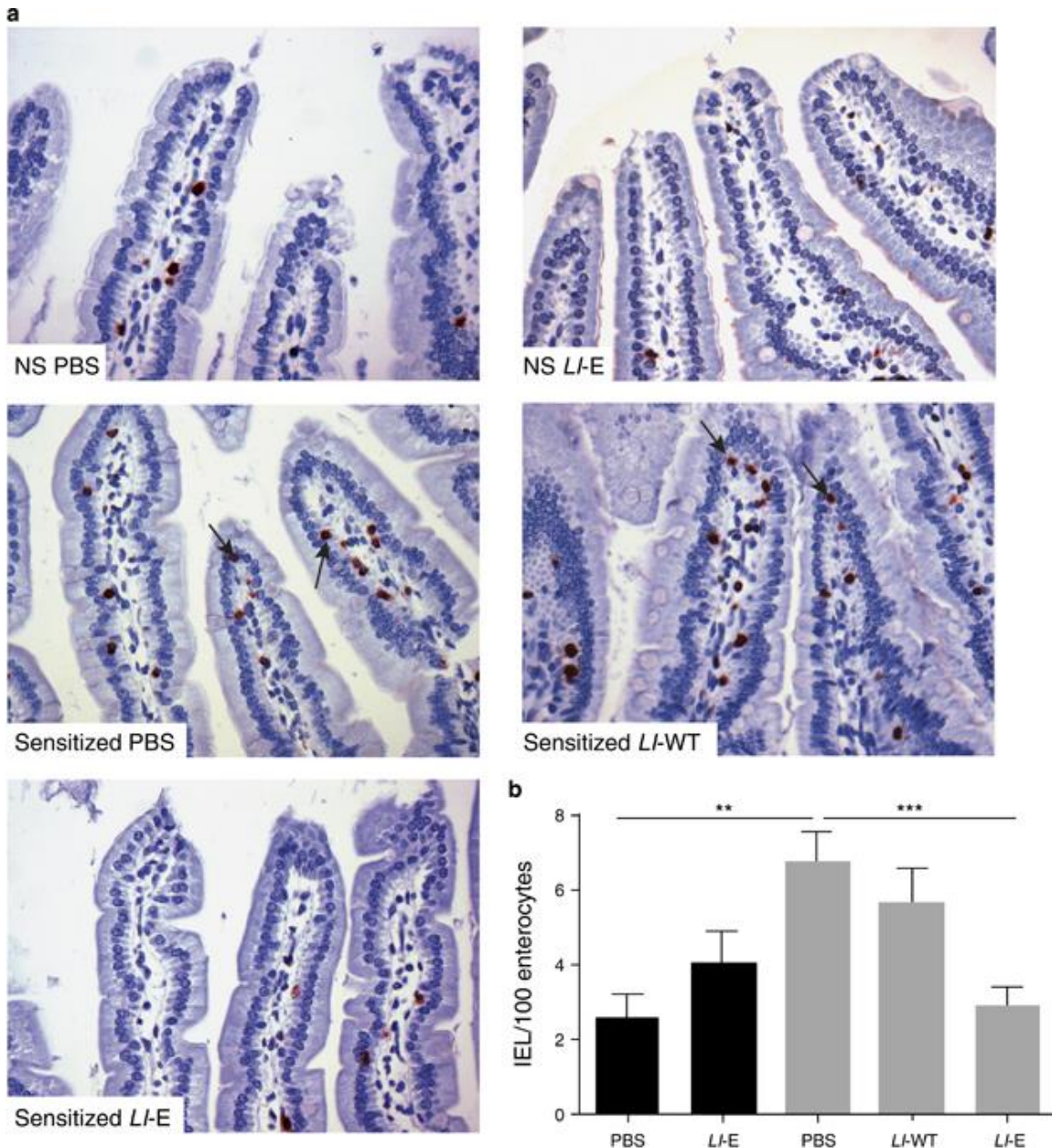


Figure 3. *L. lactis*-expressing elafin (*Ll-E*) treatment attenuates gliadin-induced intraepithelial lymphocytosis in sensitized NOD/DQ8 mice. **(a)** CD3⁺-stained sections of the proximal small intestine. Original magnification, × 20. Black arrows indicate intraepithelial lymphocytes (IELs). **(b)** Quantification of CD3⁺ cells in villi tips, expressed as IEL per 100 enterocytes ($n=8-12$ per group). Black bars represent nonsensitized animals, gray bars represent sensitized animals. Data are represented as mean±s.e.m. ** $P<0.01$, *** $P<0.001$. Nonsensitized (NS); *L. lactis* wild type (*Ll-WT*); *L. lactis*-expressing elafin (*Ll-E*)

Ll-elafin reduces intestinal barrier dysfunction and preserves ZO-1 distribution following gliadin challenge in sensitized NOD/DQ8 mice.

Gliadin sensitization and acute gliadin challenge increased small intestinal paracellular permeability, as indicated by increased *in vitro* ⁵¹Cr-EDTA flux in NOD/DQ8 mice (Figure 4a). Gliadin challenge in sensitized mice also increased tissue conductance (Figure 4b). Sensitized mice treated with *Ll-E* had lower ⁵¹Cr-EDTA flux and tissue conductance compared with sensitized animals receiving either PBS–glycerol or *Ll-WT* (Figure 4a and b).

We investigated a previously unexplored effect of elafin on ZO-1 distribution in the small intestine of gluten-sensitive mice. Staining for ZO-1 was decreased in gliadin-sensitized mice compared with controls. The expression of ZO-1 was patchy, with most of the expression being limited to the villus base and crypts. The distribution of ZO-1 normalized in sensitized mice treated with *Ll-E* (Figure 4c). We confirmed this reduction in ZO-1 staining by determining the MFI (Figure 4d). Sensitized animals treated with PBS–glycerol had significantly lower ZO-1 MFI than nonsensitized controls. Treatment of sensitized animals with *Ll-E* normalized ZO-1 MFI (Figure 4d).

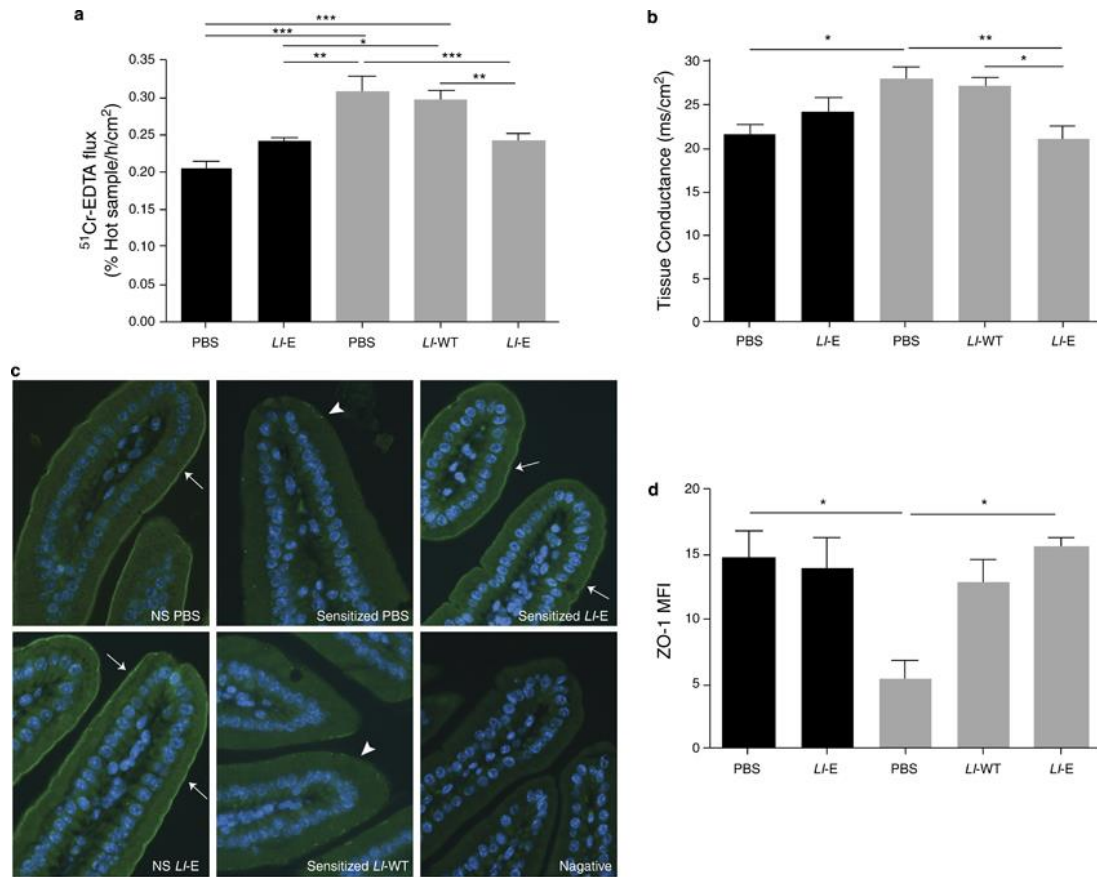


Figure 4. *L. lactis*-expressing elafin (*Ll-E*) therapy protects NOD/DQ8 mice from gliadin-induced increases in paracellular permeability and preserves zonula occludens-1 (ZO-1) distribution. Sections of small intestine were mounted in Ussing chambers and (a) ^{51}Cr -EDTA flux (% hot sample/h/cm²; $n=8-11$ per group); and (b) tissue conductance (mS/cm²; $n=4-7$ per group) was measured 24 h after the final gliadin challenge. (c) Sections of the proximal small intestine were stained for ZO-1 (green) expression. Nuclei labeled with 4'6-diamidino-2-phenylindole (DAPI; blue). Original magnification, $\times 20$. White arrows indicate strong immunofluorescence at the apical junctional complex; arrowheads indicate patchy expression. (d) Mean fluorescence intensity (MFI) of ZO-1 staining in proximal small intestinal sections was determined ($n=3-4$ per group). MFI was corrected for background fluorescence. Black bars represent nonsensitized animals, gray bars represent sensitized animals. Data are represented as mean \pm s.e.m. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. *L. lactis* wild type (*Ll-WT*); *L. lactis*-expressing elafin (*Ll-E*).

Gliadin challenge does not affect small intestinal trypsin-like or elastase-like proteolytic activity

To investigate the potential mechanisms of action of elafin, we measured trypsin- and elastase-like activity in small intestinal tissues from sensitized and nonsensitized NOD/DQ8 mice treated with PBS, *LI*-WT, or *LI*-E. The levels of trypsin-like proteases were similar in sensitized and nonsensitized mice, and they were not affected by elafin treatment (Figure 5a). Elastase-like activity was undetectable (data not shown). Proteolytic activity was also tested in small intestinal tissue from nonsensitized and sensitized mice stimulated *in vitro* with the gliadin peptides 33-mer, 20-mer, and 19-mer. Elastase-like activity was undetectable (data not shown) and trypsin-like activity was not significantly affected in either the small intestinal biopsies or culture supernatant following *in vitro* stimulation with the gliadin fragments (Figure 5b and c).

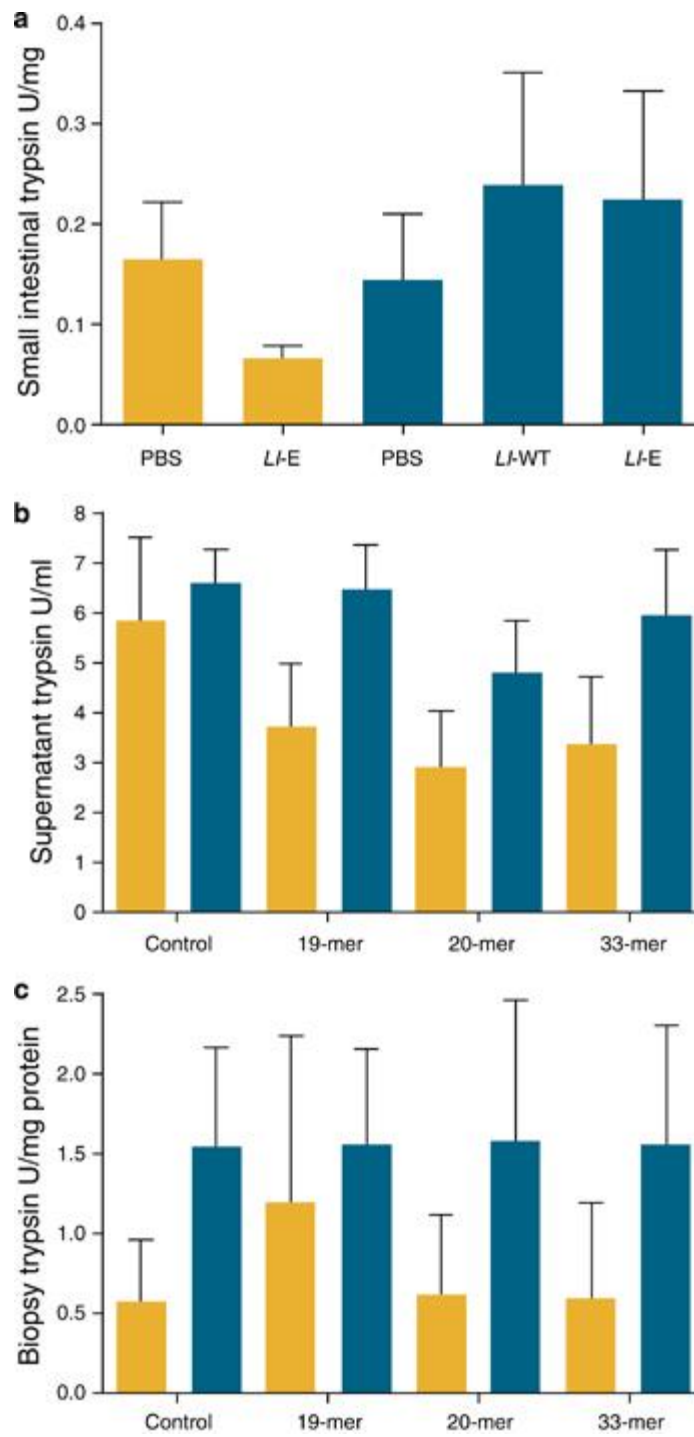


Figure 5. Gliadin sensitization and challenge do not alter trypsin-like activity in NOD/DQ8 mice *in vivo* or *in vitro*. **(a)** Sections of proximal small intestine were collected 24 h after final gliadin challenge. Levels of trypsin-like proteases were quantified in tissue homogenates and expressed as U/mg protein ($n=8-11$ per group). **(b,c)** Proximal small intestinal biopsies were collected from sensitized and nonsensitized NOD-DQ8 mice and stimulated for 3 h *in vitro* with gliadin peptides. Trypsin-like activity was quantified in the **(b)** culture supernatant and **(c)** biopsies and expressed as U/ml and U/mg protein, respectively. Yellow bars represent nonsensitized animals, blue bars represent sensitized animals. Data are represented as mean \pm s.e.m. *L. lactis* wild type (*Ll-WT*); *L. lactis*-expressing elafin (*Ll-E*).

Discussion

CD is one of the most common gastrointestinal diseases, affecting about 1% of the Caucasian population, and its incidence has substantially risen in the past two decades (34,35). The only treatment is a life-long adherence to a GFD, which is costly and difficult to follow, leading to high nonadherence rates (6). In CD, gluten-derived peptides are recognized by HLA-DQ2 or HLA-DQ8 heterodimers on antigen-presenting cells, which trigger a proinflammatory gluten-specific T-cell response (36). Through the process of deamidation, TG-2 activity has an important role by introducing negatively charged residues on gluten peptides. This enhances recognition of these gluten peptides by the HLA-DQ2 or -DQ8 molecules, which are efficiently recognized by T cells (36,37). However, the factors that drive TG-2 activation in patients with CD are not completely understood (37,38).

Elafin is a potent endogenous human serine protease inhibitor, and its levels have been found to be reduced in the colon of patients with inflammatory bowel disease (IBD) (15,17). In this study, we show that patients with active CD also have reduced elafin

expression within the small intestinal epithelium compared with patients in whom CD was excluded. After 1 year of GFD, elafin expression was somewhat higher than in active CD patients, but was not statistically different from nonceliac controls. The reduced elafin expression in patients with active CD may reflect the presence of enteropathy, and its loss may contribute to enhanced inflammation in CD. In fact, expression of other enzymes in the small intestine, such as cytochrome P450, CYP3A, was found to correlate with the degree of enteropathy, raising the possibility of using these markers as an indication of villous health (39,40). Reduced expression of elafin in patients with active CD may reflect activity of inflammation or mucosal damage in the small intestine, similar to what has been described in the colon of patients with IBD (15-17).

We then hypothesized that elafin may have a role in gluten-related disorders and tested the interaction of elafin with human TG-2, the enzyme that catalyzes the deamidation of gluten peptides enhancing T-cell recognition (36). Indeed, elafin has recently been identified as a substrate for the cross-linking activity of tissue transglutaminase (19,41,42). Our *in vitro* results demonstrate that the addition of elafin inhibited the deamidation of the digestion-resistant 33-mer gliadin peptide, which is one of the potential triggers of the adaptive immune response in CD (43). The comparison of elafin to tridegin, a known transglutaminase inhibitor, revealed that elafin moderately inhibits TG-2. These data support that elafin interacts with TG-2, raising the possibility that in addition to correlating with mucosal damage a reduction of elafin in active CD could have a pathogenic role. TG-2 has important roles in tissue homeostasis and wound healing (21,44). Thus, the moderate inhibition and small intestinal delivery of elafin could

constitute an advantage over systemic, complete, and irreversible inhibition, particularly when combined with other potential beneficial effects of elafin. Our *in vitro* results point at an exciting novel role and specific mechanism of action of elafin in CD that should be further explored.

Elafin has been shown to have anti-inflammatory and barrier-modulating properties in models of respiratory, cardiovascular, and colonic inflammation (17,32,41,45). We explored whether elafin administration affected these parameters in the small intestine using a model of gluten sensitivity. We used a validated delivery method that exploits a food-grade strain of *L. lactis*, to ensure administration of elafin to the gastrointestinal tract in mice (17,32). Although the quantification of elafin delivered to the intestine was not determined, using GFP-tagged *Ll*-WT and *Ll*-E, we have previously demonstrated effective delivery of *L. lactis* and elafin to the gastrointestinal tract. Elafin was found to cluster around *L. lactis*, but was also detected at sites remote from the GFP-tagged *L. lactis*, suggesting that elafin is secreted in the mucosa *in situ* (17). We found that *Ll*-E reduced intraepithelial lymphocytosis and restored barrier function in gliadin-sensitized mice. We showed that treatment with *Ll*-E normalized ⁵¹Cr-EDTA flux and conductance in the small intestine, suggesting that *Ll*-E protects the paracellular pathway in the small intestine. Furthermore, increased permeability after gliadin challenge was accompanied by a reduction in ZO-1 protein expression, and *Ll*-E therapy preserved ZO-1 protein distribution in sensitized mice. Overall, our results suggest that there are beneficial effects of *Ll*-E in this model of gluten-induced small intestinal dysfunction that involve immunomodulatory effects and regulation of barrier and tight-junction function.

This is consistent with previous reports on the effect of elafin in the colon using animal models of colitis (14,17,32). Proteolytic imbalances have been described in patients with irritable bowel syndrome, IBD, and in animal models of experimental colitis (17,32). However, we found no differences in trypsin or elastase-like activity between gliadin-sensitized and control mice. In addition, treatment with *Ll-E* had no effect on small intestinal protease levels. Our findings highlight a difference in the mechanisms of action of elafin in gluten intolerance compared with colitis, which does not involve correction of elastolytic or trypsin activity imbalance in the small intestine.

Although the NOD/DQ8 mouse model does not develop severe atrophy that is characteristic of classical CD, sensitization and challenge induce moderate inflammation (27). It will be important to test the effect of elafin, as well as other potential therapies for CD, in a variety of models of gluten sensitivity that express different pathological characteristics and underlying mechanisms, such as IL-15-driven models of CD (46).

In conclusion, the reduced small intestinal elafin expression in patients with active CD, coupled to the finding that elafin may compete with gliadin peptides and reduce their deamidation, raise the possibility that loss of this protease inhibitor contributes to CD pathogenesis. The results in the animal model of gluten sensitivity suggest that mucosal delivery of elafin restores small intestinal barrier function and inflammation, likely through preservation of tight-junction function and anti-inflammatory effects in the small intestine. Owing to the combination of specific and nonspecific beneficial effects of elafin in our study, we propose that its replacement could have potential as adjuvant therapy in gluten-related disorders.

Study highlight

What is current knowledge?

- The only treatment for celiac disease (CD) is a life-long adherence to a gluten-free diet (GFD)
- A strict GFD is costly and difficult to follow, leading to high non-adherence rates and increased co-morbidities, highlighting the need for alternative treatments.
- Elafin is an anti-inflammatory human serine protease inhibitor that has potent inhibitory activity against murine neutrophil elastase and proteinase-3
- Elafin is decreased in IBD patients and prevents inflammation in murine models of experimental colitis
- The role of elafin in gluten-related disorders is unknown.

What is new here?

- Elafin expression is significantly decreased in patients with active CD, compared to patients without CD.
- *In vitro* elafin reduces the deamidation of an immunogenic gliadin peptide, an important step in the autoimmune process of CD.
- In an animal model of gluten sensitivity, elafin delivery at the mucosal level by a food grade *L. lactis* vector prevented gluten-induced barrier dysfunction and inflammation.
- The identification of a molecule (elafin) that is altered in the small intestine of active CD patients and that reduces deamidation of gliadin peptide, and that has

barrier-enhancing effects in the small intestine, will help increase understanding of the pathophysiology of CD and promote development of new supportive treatments for gluten-related disorders.

Conflict of Interest

Guarantor of the article: Elena F. Verdu, MD, PhD.

Specific author contributions: H.J.G. and M.W. contributed equally to the manuscript. We acknowledge the technical help of Xianxi Huang and Yikang Deng with molecular analysis and of Jean-Jacques Gratadoux for the preparation of bacterial cultures.

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Potential competing interests: E.F.V. holds a Canada Research Chair.

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CHAPTER 7

DISCUSSION

7.1 Summary

The ability to maintain a constant internal environment, or homeostasis, is a key feature of host physiology. This is particularly important in the gastrointestinal tract, where the body is faced with a large antigenic load that includes dietary and microbial antigens. Dysfunctional interactions between dietary factors, microbes, and the host can lead to a breakdown in homeostasis and promote inflammatory responses to normally innocuous luminal antigens, such as commensal bacteria and dietary proteins.

The best example of an inflammatory and autoimmune process caused by a dietary antigen is celiac disease (CD), which is triggered by the ingestion of gluten, the water insoluble protein fraction in wheat, rye and barley. However, the environmental and host factors involved in disease onset and progression are not fully understood. Furthermore, the current treatment for CD entails the strict adherence to a gluten free diet (GFD), which is difficult and expensive to maintain and ineffective at completely resolving symptoms and intestinal damage in many patients. **The overall aim of my thesis was to address some of the unknown factors regarding the pathogenesis of CD. For this I set out to characterize a humanized mouse model of gluten sensitivity and investigate factors that contribute to gluten-induced inflammation in order to develop novel potential therapeutic strategies for this condition.** CD often associates with autoimmune co-morbidities such as type 1 diabetes (T1D). One common factor described in CD and T1D is a dysfunctional intestinal barrier (Vaarala et al., 2008). Although gluten, or the consequent inflammation, has been implicated in altered intestinal permeability in CD, it is unknown whether this mechanism also plays a role in T1D

progression in patients who also have CD. In **chapter 3** of this thesis I demonstrated that gluten sensitization in NOD/DQ8 mice induced barrier dysfunction with a moderate degree of enteropathy. However, gluten-induced barrier dysfunction was not sufficient to induce insulinitis, a precursor of T1D. Instead, partial depletion of Tregs plus the functional and immune abnormalities triggered by gluten sensitization were required to induce severe insulitis (Figure 7.1). The results suggested that a gut-pancreas axis may be an important adjuvant step in the development of immune responses leading to insulitis, and that this may relate to a gluten-induced leaky intestinal barrier when combined with systemic immunoregulatory deficiency.

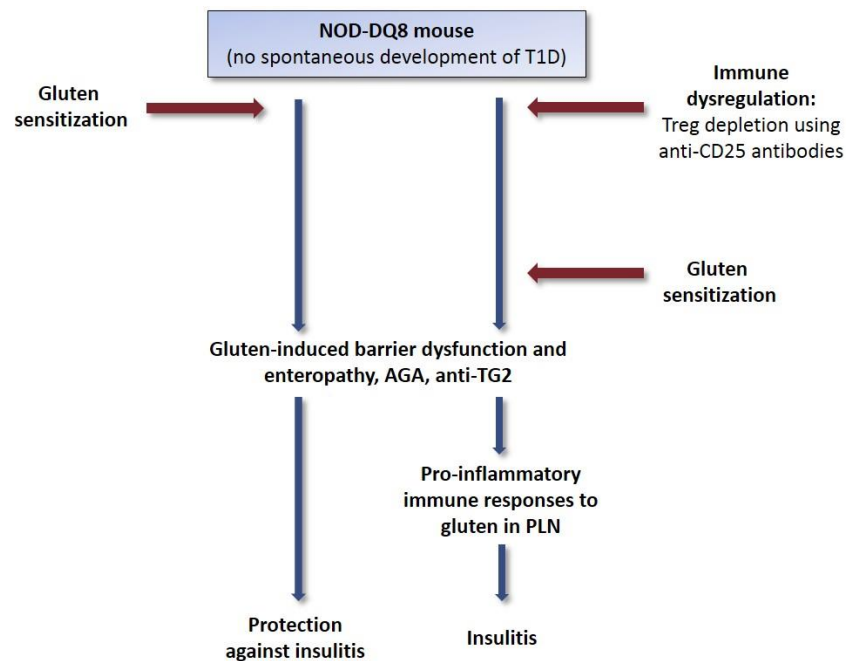


Figure 7.1 Gluten-sensitive NOD/DQ8 mice develop gluten-induced barrier dysfunction, moderate enteropathy, anti-gliadin antibodies (AGA) and anti-tissue transglutaminase (TG-2) antibodies, but are protected from insulitis and type 1 diabetes. A prior immune dysregulation plus gluten sensitization induces the development of insulitis.

The intestinal microbiota has recently been suggested as a key environmental modulator of health and disease, but its role in CD development is unknown. The results presented in **chapter 4** of this thesis demonstrated that the microbiota modulated host responses to gluten in NOD/DQ8 mice (Figure 7.2; Figure 7.3; Figure 7.4). Specifically, I showed that mice maintained in a germ-free environment and mice colonized with a diverse microbiota that harbours opportunistic bacteria, including Proteobacteria, developed heightened gluten-induced immune responses compared to mice colonized with a clean, benign microbiota. Supplementation of the clean microbiota with *E. coli* led to more severe gluten-induced pathology. Furthermore, disruption of the colonization process at birth by antibiotic treatment led to increased Proteobacteria and increased the severity of gluten-induced immune responses. Together, these results provide an in depth characterization of a humanized animal model for studying gluten-induced intestinal and extra-intestinal immune responses, and their modulation by environmental factors, such as the microbiota. It also represents a useful preclinical tool to test potential adjuvant therapies to the GFD.

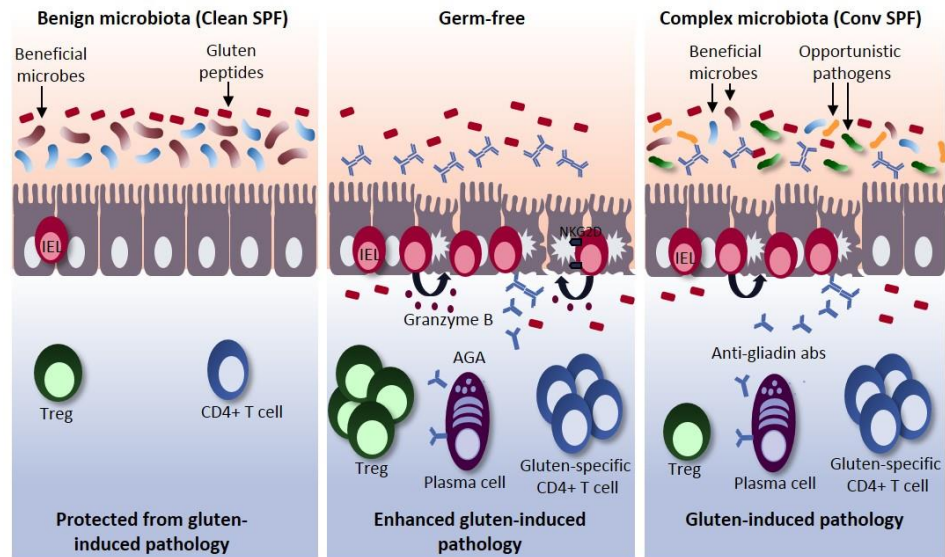


Figure 7.2 Colonization with a benign microbiota protects from gluten-induced pathology. Mice harbouring a benign microbiota (clean specific pathogen free; SPF) are protected from gluten-induced pathology compared to mice colonized with a more complex conventional (conv) SPF microbiota or germ-free mice.

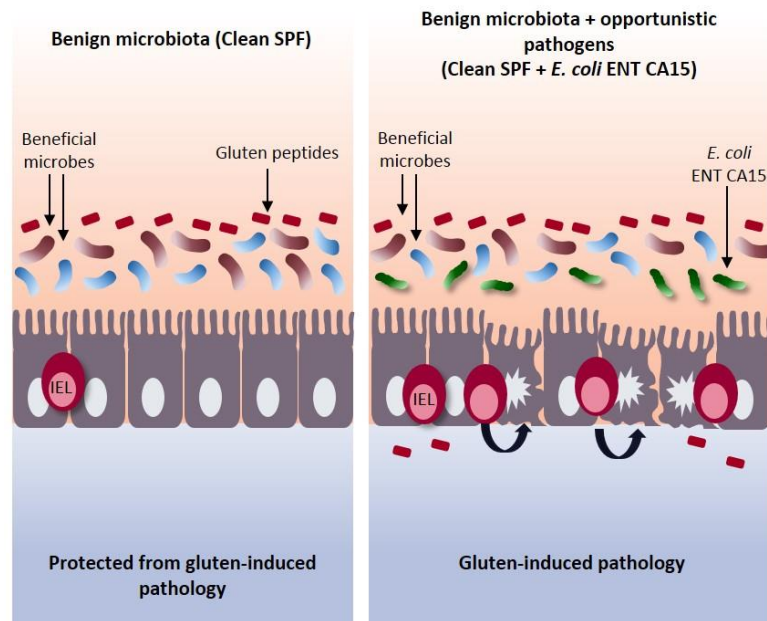


Figure 7.3 Supplementation of a benign microbiota with an opportunistic pathogen results in gluten-induced pathology. Supplementation of clean specific pathogen free (SPF) mice with an opportunistic pathogen (*E. coli* ENT CA15) induces gluten-induced pathology.

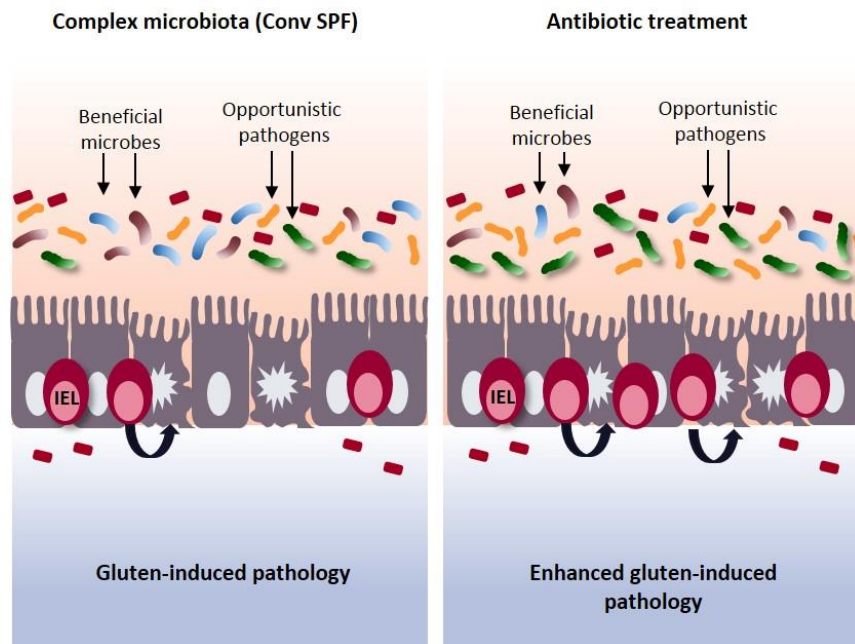


Figure 7.4 Perinatal antibiotic treatment enhances gluten induced pathology. Treatment of conventional (conv) specific pathogen free (SPF) mice with antibiotics increases the abundance of opportunistic pathogens and enhances gluten-induced pathology.

In the last part of this thesis, I utilized transgenic DQ8 mice to investigate the therapeutic potential of two novel therapies that target key pathophysiological processes in CD. The results presented in **chapter 5** demonstrated that a gluten binding polymer, P(HEMA-*co*-SS, inhibited the *in vitro* digestion of gliadin to immunogenic gliadin peptides. Administration of P(HEMA-*co*-SS to gluten-sensitive HLA/DQ8 mice reduced short-term and long-term gluten-induced barrier dysfunction and inflammation. In **chapter 6** of this thesis, I demonstrated that elafin, a protease inhibitor with barrier protecting and anti-inflammatory properties in the colon, was decreased in the small intestine of patients with active CD and *in vitro*, it also inhibited the deamidation of gliadin peptides, a key step in the pathogenesis of CD. Using NOD/DQ8 mice, I showed

that administration of elafin prevented gluten-induced barrier dysfunction and intraepithelial lymphocytosis.

Results for each individual chapter have been discussed separately within each manuscript. In this section, the interplay between studies and their significance will be discussed. This section will also highlight the potential limitations of this work as well as future directions.

7.2 Host-environment interactions play a critical role in host responses to gluten

Most inflammatory diseases follow a similar pathophysiological model that includes genetics, host responses (immune responses and/or barrier function) and environmental factors (diet, microbiota, and/or infections). Likewise, CD requires the presence of genetic susceptibility genes, and is dependent on the cooperation of innate and adaptive immune responses that are triggered by gluten. The intestinal microbiota has been proposed as an environmental factor that contributes to disease development. How these factors interact to promote CD development is not understood, but is critical for the development of preventative or therapeutic strategies (Figure 7.5).

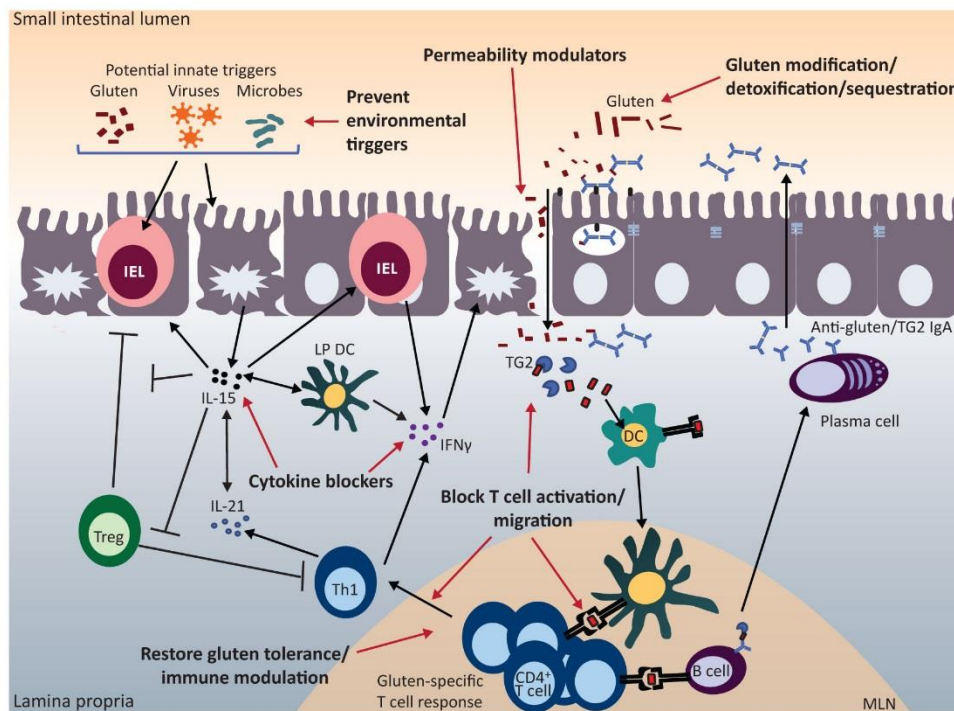


Figure 7.5 Preventative and therapeutic strategies for CD. A complete understanding of CD pathogenesis will enable the development of preventative and therapeutic strategies for CD, such as the prevention of environmental triggers (i.e. microbes, timing of gluten introduction), modulation of intestinal permeability, gluten detoxification, modification, or sequestration (i.e. hydrolysis of gluten peptides, gluten-binding polymer), blocking T cell activation or migration (i.e. inhibition of TG-2, HLA-DQ8, or mucosal homing markers), cytokine inhibition (i.e. blocking IL-15), or restoration of gluten tolerance (i.e. vaccination).

The presence of the HLA/DQ2 or DQ8 gene is required for the development of CD. About 30% of the general population carries the HLA/DQ2 or DQ8 gene, and most are exposed to gluten, but only 1% of the population will develop CD. This suggests that both gluten and the presence of HLA/DQ2 or DQ8 are required for disease development, but that other factors must also be involved. Similar to the clinical situation, the mere presence of the human DQ8 gene in NOD mice was not sufficient for the spontaneous

development of gluten-induced pathology. In order for moderate enteropathy to develop, tolerance to gluten had to be broken with the use of a mucosal adjuvant, such as cholera toxin. In other transgenic models, overexpression of IL-15 in the presence of the DQ8 transgene has been used to break tolerance to gluten (DePaolo et al., 2011) or ovalbumin (Korneychuk et al., 2014). Together, these results emphasize the need for additional environmental or immune factors in disease development and support the multifactorial basis of CD. While the development of a model that spontaneously shows gluten-induced villus atrophy is desirable, such a model may not be optimal for translating all clinical situations. For instance, a spontaneous model caused by transgenic overexpression of cytokines may not be ideal for testing therapeutic interventions, beyond blocking the cytokines that are driving the pathology. On the other hand, the spectrum of gluten sensitivity is very broad, and models that exhibit mild to moderate enteropathy may be better suited, than those that exhibit total atrophy, to investigate unknown environmental factors that promote progression from potential to active CD. As with IBD, where a variety of experimental colitis models exist (Maloy and Powrie, 2011), it is likely that a multitude of different models will be useful in the study of CD and the gluten sensitivity spectrum. Ultimately, animal models only serve to investigate novel pathways of potential clinical significance, and provide proof of concept for new theories and therapies that will need to be confirmed in the clinical scenario.

The increasing incidence of chronic inflammatory and autoimmune diseases, such as CD and T1D, and their association implicates the contribution of common environmental factors in disease development. The microbiota is one factor that has been

proposed to influence susceptibility to chronic inflammatory diseases. Indeed, clinical studies have shown a link between altered microbial composition and allergy, obesity, IBD, T1D, and CD (Belkaid and Hand, 2014; Maslowski and Mackay, 2011; Thorburn et al., 2014). However, causation has been difficult to prove. Studies exploring the potential role of the microbiota in CD have only emerged in the last eight years. The association between factors influencing early microbial colonization and increased CD risk, the altered microbial composition in at-risk children, and the microbial modulation of gluten-induced immune responses *in vitro* suggests a potential primary role of the microbiota in CD pathogenesis. In **chapter 4** of this thesis I therefore utilized gluten-sensitive NOD/DQ8 mice to explore whether microbial colonization influences host responses to gluten. I demonstrated that NOD/DQ8 mice maintained in a clean SPF environment, free from any opportunistic pathogens, are protected from gluten-induced pathology, even when sensitized to gluten using an adjuvant. Thus, even in the presence of susceptibility genes, gluten, and an inflammatory stimulus, tolerance to gluten was maintained when the gut harboured a benign microbiota. However, the presence of opportunistic pathogens or the absence of bacteria led to gluten-induced pathology in gliadin-sensitized NOD/DQ8 mice, suggesting that interactions between the environment, genetics, and immune responses can positively or negatively influence gluten sensitivity.

The findings of enhanced host responses to gluten in germ-free and antibiotic treated mice support the “hygiene hypothesis” or the more recently coined “old friends hypothesis”, which suggests that the reduced exposure to particular microbes, which have co-evolved with humans, is responsible for the increasing incidence of inflammatory

diseases (Rook, 2012). Microbes have co-evolved with human hosts and are critical for immune regulation and maintaining intestinal homeostasis, and a loss of these microbes due to changes in lifestyle (diet, drugs, and/or hygiene) may have profound effects on health and disease (Thorburn et al., 2014; Willing et al., 2011). On the other hand, I also demonstrated that the presence of opportunistic pathogens (*E. coli* ENT CA15) enhanced host responses to gluten. It is unlikely, however, that the presence of a single bacterial species is responsible for triggering CD. Under certain conditions, such as acute inflammation or dietary changes, particular opportunistic bacterial populations may disrupt the balance between homeostasis and inflammation, and increase susceptibility to disease in genetically susceptible hosts. The findings in **chapter 4** suggest that there is a delicate balance between homeostasis and pathogenicity and a disruption of this balance, due to the lack of commensal stimulation or the presence of certain microbes, can contribute to its breakdown. Recent changes in lifestyle, including dietary changes or increased antibiotic use, may be contributing to shifts in the microbial composition, disrupting the balance between regulatory and inflammatory mediators and may explain the increasing incidence of inflammatory and autoimmune diseases (Belkaid and Hand, 2014). One clear example relates to the increasing incidence of CD and T1D, two conditions that also have a clear clinical association (Cohn et al., 2014). While gluten and, or barrier dysfunction have been suggested to play a pathogenic role in T1D, it is unclear why only some individuals with CD develop pancreatic autoimmunity. In line with the concepts previously raised, the role of additional immune or environmental factors has been raised (Dunne et al., 2014). Indeed, in **chapter 3**, I demonstrated that both an

immune dysregulation plus gluten-induced barrier dysfunction was required to induce insulinitis in NOD/ DQ8 mice. Whether the abnormal translocation of gut microbiota played a role in these findings remains to be determined. However, others have hypothesized that increased uptake of gut bacteria may drive a pro-inflammatory immune response that promotes T1D in a host at risk (Patrick et al., 2013). Thus, future studies using gnotobiotic NOD/DQ8 mice can be used to explore the interplay or synergistic effect between gluten, the microbiota, and host factors on the development of extra-intestinal CD co-morbidities, including T1D.

The findings in **chapter 4** also demonstrated that the composition of an SPF microbiota influenced immune responses to dietary antigens. This is a very important consideration when comparing findings across studies and across different animal facilities, where housing and microbial conditions likely vary considerably. One clear example of this is the presence of segmented filamentous bacteria (SFB) in mice from Taconic (which correlate with high levels of small intestinal Th17 cells) and the absence of SFB in mice from Jackson Laboratory (which correlate with low levels of small intestinal Th17 cells) (Ivanov et al., 2009; Ivanov et al., 2008). These differences in microbial “SPF” composition influence immune responses as well as disease development (Kriegel et al., 2011). This highlights the importance of designing experiments using gnotobiotic technology where the microbial composition can be regulated and host-microbial interactions carefully studied.

7.3 Adjuvant therapies for celiac disease

The only current treatment for CD is the strict adherence to a GFD. Following a GFD, however, is very difficult due to frequent gluten contamination (Collin et al., 2004), the high cost of gluten-free foods, and the low availability of gluten-free alternatives in certain parts of the world (Zarkadas et al., 2013). Poor adherence rates (Hall et al., 2009) and inadvertent gluten consumption can have important clinical implications, as incomplete clinical and mucosal recovery have been reported in CD, even after following a GFD for up to 5 years (Midhagen and Hallert, 2003; Rubio-Tapia et al., 2010). Non-adherence to a GFD can also increase the risk of serious side effects, including malignancy (Rubio-Tapia et al., 2013). Furthermore, in a recent survey, CD patients reported having a very high treatment burden, which was greater than that reported for patients with T1D, gastroesophageal reflux disease, and hypertension, and similar to what was reported for patients with end-stage renal disease (Shah et al., 2014). It was also demonstrated that more than 40% of CD patients are unsatisfied with the GFD (Aziz et al., 2011). High treatment burden, coupled with incomplete mucosal healing and clinical recovery emphasize the need for non-dietary adjuvant therapies.

In **chapters 5 and 6** of this thesis I explored two different potential adjuvant therapies for CD using NOD/DQ8 and HLA/DQ8 mice: elafin and P(HEMA-*co*-SS). While these studies on P(HEMA-*co*-SS) and elafin are in phase I and preclinical development respectively, they provide exciting new therapeutic options. However, given the complexity and the spectrum of CD, a single alternative treatment is not likely to provide full protection from gluten-induced pathology and symptoms in patients

consuming a gluten-containing diet. More likely, therapies will be used to support the GFD. Experimental CD treatments have begun focusing on the ability of treatments to relieve ongoing symptoms and mucosal damage in patients who are adhering to a gluten-free diet (Wang et al., 2014). Very small amounts of gluten can trigger symptoms and intestinal damage in many patients (Catassi et al., 2007), and inadvertent gluten consumption is thought to contribute to persistent symptoms and intestinal damage. In our studies, I demonstrated that elafin and P(HEMA-co-SS) were effective in inhibiting gluten-induced responses following a low-to-moderate gluten challenge. Elafin and P(HEMA-co-SS) may therefore be appropriate for patients who are already adhering to a GFD, but require protection against intentional or unintentional consumption of small amounts of gluten. Alternatively, emerging therapies may require a combination of several therapeutic options. For example, the gluten-binding polymer may be combined with therapies that target different areas of CD pathogenesis, such as modulators of T cell activation or permeability modulators. On the other hand, the combination of therapies that target the same area of CD pathogenesis, but with opposite mechanisms of action would not likely be beneficial. For example, the combination of gluten-binding polymers, which aim to sequester and prevent gluten digestion, with oral proteases (ALV003), which increase the digestion of gluten, would not be advisable. Future studies are needed to determine whether combinations of treatments, if any, will be more effective than single treatments for alternatives to the GFD. It is an exciting time in CD research and it is expected that in the next years, one or more therapeutic alternatives will be in the market to help manage this disease.

7.4 Limitations and future directions

One of the biggest obstacles in CD research has been the lack of an animal model that fully represents CD. The models I described in this thesis could be criticized for the need of microbial adjuvant as well as the development of moderate rather than total atrophic enteropathy. Like many chronic inflammatory diseases, CD is heterogeneous in its pathogenesis and clinical presentation and has a complex spectrum of disease associations. With this in mind, I characterized transgenic NOD/DQ8 mice in order to study extra-intestinal co-morbidities, environmental modulators of the disease, and potential adjuvant therapies. Nonetheless, the findings in **chapter 3**, in conjunction with previous results (DePaolo et al., 2011), demonstrated that the development of intraepithelial lymphocytosis and gliadin-specific T and B cell responses in gliadin-sensitized NOD/DQ8 mice can develop independently of severe intestinal damage, a presentation of CD that is becoming more common (Fasano and Catassi, 2001; Green, 2005; Mäki et al., 1988; Pare et al., 1988). The model has also proven useful to investigate additional potential factors in CD pathogenesis, which will be critical for the development of novel preventative and therapeutic strategies.

The results from **chapter 4** demonstrated for the first time that the microbiota modulates host responses to gluten *in vivo*. However, the precise mechanisms through which the absence of a microbiota or differences in the composition of the microbiota can influence host responses to gluten still need to be deciphered. An important consideration is that germ-free and colonized mice differ in a number of immune and physiological responses (Sommer and Bäckhed, 2013). Therefore although the germ-free condition

represent a valuable experimental tool, it will never correspond to a clinical scenario. However, the germ-free mouse allows for specific gnotobiotic colonizations and investigation of host responses to bacteria of interest. Increased susceptibility to inflammation and antigen-sensitization in germ-free conditions has been reported in several different animal models (Herbst et al., 2011; Maslowski et al., 2009; Stefka et al., 2014), but the exact mechanisms leading to heightened gluten-induced responses in the absence of bacteria in NOD/DQ8 mice is unclear. Bacteria isolated from the gut also possess proteolytic activity against gluten and the immunogenic 33-mer peptide (Caminero et al., 2014). Differences in the microbial composition could therefore lead to differences in the generation of toxic or immunogenic gluten peptides in the gut, a concept currently being explored in the lab. Future studies should also address whether differences in host responses to gluten are due to the presence or absence of specific bacterial species or to changes in the overall balance between anti-inflammatory and pro-inflammatory bacteria or mediators. Finally, there may be a “window of opportunity” early in life during which host-microbial interactions are critical for development of proper immune and physiological responses (Cahenzli et al., 2013; Olszak et al., 2012; Sudo et al., 1997). In **chapter 4**, I demonstrated that perinatal antibiotic use was associated with increased gluten-induced pathology. Future studies in NOD/DQ8 mice should address whether age, duration, or the timing of altered microbial composition affects host responses to gluten later in life. These studies will have important translational relevance and may provide experimental evidence to support the clinical

association between factors affecting early microbial colonization and increased susceptibility to inflammatory diseases such as CD.

I demonstrated that both elafin and P(HEMA-*co*-SS) exerted therapeutic effects in animal models of gluten sensitization and challenge. The effectiveness of P(HEMA-*co*-SS) was initially tested in HLA/DQ8 mice. In our lab, we have observed that in response to gluten sensitization and challenge, NOD/DQ8 mice develop more severe gluten-induced pathology compared to HLA/DQ8 mice. After publishing the results in HLA/DQ8 mice, which are included in this thesis, the effectiveness of P(HEMA-*co*-SS), now called BL-7010, was tested in NOD/DQ8 mice, where we observed a reduction in gluten-induced pathology in BL-7010-treated mice (McCarville et al., 2014). The models utilized in this thesis are transgenic mice that do not develop total gluten-induced villus atrophy, which is a potential limitation when translating these findings to the clinical situation. Although there is no single animal model that fully represents the spectrum of CD, additional studies should be conducted using models that display other features of CD, such as mice that over express IL-15. Given the additional role of TG-2 in maintaining tissue homeostasis and in wound healing, the ability of elafin to inhibit TG-2 should also be tested *in vivo*. However, the regulation and activation of murine TG-2 and the role of TG-2 in gluten-induced responses in transgenic mice is not clear, as T cells isolated from gluten-immunized HLA.DQ8 mice respond to both native and deamidated gluten peptides (Black et al., 2002; Senger et al., 2005). Finally, the quantity of gluten being consumed is another important factor that needs to be considered when translating the findings from animal models to the clinical setting. Further, whether there is a

threshold for the safe amount or frequency of gluten that can be consumed during elafin and P(HEMA-co-SS) treatment, still needs to be determined. Finally, whether treatment with elafin or P(HEMA-co-SS) can protect against extra-intestinal inflammation or co-morbidities associated with GRD should also be investigated.

The findings in **chapter 4** also raise the possibility of microbial modulation as a potential adjuvant therapy for CD. I found that increased abundance of Proteobacteria, including *Escherichia* and *Helicobacter*, was associated with more severe gluten-induced enteropathy, and the supplementation of a benign microbiota with an enteroadherent strain of *E. coli* increased gluten-induced pathology. Increased bacteria belonging to the Enterobacteriaceae family have also been reported in children at risk for CD, suggesting that imbalances in the microbiota may contribute to the loss of gluten tolerance in genetically susceptible individuals. Future studies should test whether the targeted reduction of opportunistic pathogens, such as *Escherichia* spp, prevents the loss of gluten tolerance in genetically susceptible hosts.

7.5 Conclusions

The work in this thesis provides insight into the environmental and host factors that contribute to the development of gluten sensitivity. Furthermore, the work presented here demonstrates the potential of two novel therapeutic strategies. The continued use of different animal models with transgenic DQ2 or 8 expression, cytokine overexpression, and, or epithelial abnormalities will be critical for delineating novel pathogenic mechanisms in CD. I am convinced that collectively, all of these approaches will provide

additional avenues through which further preventative and therapeutic strategies can be explored. The studies presented in this thesis have significant clinical implications given the increasing prevalence of CD and other GRD. Moreover, the lessons learned from studying CD pathogenesis, a disease for which the dietary trigger and major susceptibility genes have been identified, can be applied to other chronic inflammatory diseases that are not as well understood. Perhaps the quest for the “Holy Grail” of a perfect model for CD will never be fulfilled, but will continue to guide us in the search for more insight and better understanding of CD and its complex spectrum.

APPENDIX I

SUPPLEMENTARY TABLES

Table S1 Summary of microbial alterations in CD patients

<i>Study</i>	<i>Subjects</i>	<i>Method/sample</i>	<i>Findings relative to controls</i>	<i>Findings relative to treated celiacs</i>
(Forsberg <i>et al.</i> , 2004)	ACD children (n=55) TCD children (n=53) Challenged CD (n=3) Ctrls children (n= 78)	Electron microscopy (duodenal biopsy)	CD* ↑ Rod-shaped bacteria adhering to mucosa.	
(Tjellström <i>et al.</i> , 2005)	ACD children (n=36) TCD children (n=47) Ctrls children (n=42)	GC-MS (fecal sample)	CD ↑ Total SCFA ↑ Acetic acid ↑ <i>i</i> -Butyric acid ↑ <i>i</i> -Valeric acid TCD ↑ <i>n</i> -Valeric acid ↑ Propionic acid	
(Collado <i>et al.</i> , 2007)	ACD children (n=26) Ctrls children (n=23)	Culture (fecal samples)	ACD ↑ <i>Bacteroides</i> ↑ <i>Staphylococcus</i> ↑ <i>Clostridium</i>	
		FISH (fecal sample)	ACD ↑ <i>Bacteroides/ Prevotella</i> ↑ <i>Clostridium histolyticum</i> ↑ <i>E. rectale- C. coccoides</i> ↑ <i>Atopobium</i> ↑ sulfate-reducing bacteria	
(Sanz <i>et al.</i> , 2007)	ACD children (n=10) Ctrls children (n=10)	DGGE (fecal sample)	ACD ↑ overall diversity ↓ <i>Lactobacillus casei</i> ↑ <i>Lactobacillus curvatus</i> ↑ <i>Leuconostoc mesenteroides</i> , <i>Leuconostoc carnosum</i> ↓ Bifidobacterium diversity ↓ <i>B. adolescentis</i>	
(Nadal <i>et al.</i> , 2007)	ACD children (n=20) TCD children (n=10) Ctrls children (n=8)	FISH (duodenal biopsy)	ACD ↑ Total bacteria ↑ gram negative bacteria ↑ <i>Bacteroides-Prevotella, E. coli</i> CD ↓ Ratio <i>Lactobacillus + Bifidobacterium</i> to <i>Bacteroides-Prevotella + E. coli</i> TCD ↓ Total bacteria ↓ gram positive bacteria ↓ gram positive bacteria ↓ <i>Atopobium</i> ↓ <i>Bifidobacterium</i> ↓ <i>Lactobacillus</i> group ↓ <i>Eubacterium rectale, Clostridium coccoides</i> ↓ <i>Clostridium histolyticum</i> ↓ <i>Clostridium lituseburense</i> ↓ <i>F. prausnitzii</i>	ACD ↑ gram positive bacteria ↑ Total bacteria ↑ gram negative bacteria ↑ <i>Bacteroides-Prevotella, E. coli</i> ↑ <i>Bifidobacterium</i> ↑ <i>Atopobium</i> ↑ <i>Lactobacillus</i> group ↑ <i>Eubacterium rectale Clostridium coccoides</i> ↑ <i>Clostridium lituseburense</i> ↑ sulfate reducing bacteria
CD	ACD children (n=10) TCD children (n=10)	Cultured fecal samples, <i>Entero-</i>	↑ virulence genes in virulent <i>E. coli</i> clones	

	Ctrl children (n=11)	<i>bacteriaceae</i> clones isolated for virulence	(P fimbriae, capsule k5, haemolysin)	
(Collado et al., 2008)	ACD children (n=30) TCD children (n=18) Ctrl children (n=30)	PCR (fecal sample)	CD ↓ <i>Bifidobacterium</i> counts ACD ↑ <i>B. adolescentis</i> counts ↓ <i>B. longum</i> counts TCD ↑ <i>B. adolescentis</i> prevalence ↑ <i>B. dentium</i> prevalence ↓ <i>B. breve</i> counts ↓ <i>B. bifidum</i> counts ↓ <i>B. longum</i> counts	ACD ↓ <i>B. adolescentis</i> prevalence ↑ <i>B. breve</i> counts ↑ <i>B. longum</i> counts ↑ <i>B. bifidum</i> counts
		PCR (duodenal biopsy)	CD ↓ <i>B. catenulatum</i> prevalence ACD ↓ <i>Bifidobacterium</i> counts ↓ <i>B. longum</i> counts ↑ <i>B. lactis</i> counts TCD ↓ <i>B. lactis</i> prevalence	ACD ↑ <i>B. lactis</i> prevalence ↑ <i>B. breve</i> counts
(Collado et al., 2009)	ACD children (n=25-30) TCD children (n=8-18) Ctrl children (n= 8-30)	PCR (fecal sample)	CD ↑ <i>E. coli</i> prevalence ↑ Bacterial counts ↓ <i>Bifidobacterium</i> counts ↑ <i>Bacteroides</i> counts ↑ <i>C. leptum</i> counts ACD ↑ <i>Staphylococcus</i> prevalence, counts ↑ <i>E. coli</i> counts TCR ↑ <i>Lactobacillus</i> counts	ACD ↑ <i>Staphylococcus</i> counts
		PCR (duodenal biopsy)	CD ↑ <i>Bacteroides</i> counts ↑ <i>C. leptum</i> counts ACD ↓ <i>Bifidobacterium</i> counts ↑ <i>Lactobacillus</i> counts ↑ <i>E. coli</i> counts ↑ <i>Staphylococcus</i> counts TCR ↓ <i>C. coccoides</i> prevalence ↓ <i>Lactobacillus</i> prevalence	ACD ↑ <i>Staphylococcus</i> counts ↑ <i>Lactobacillus</i> counts ↑ <i>E. coli</i> counts ↑ <i>Lactobacillus</i> prevalence ↑ <i>Akkermansia mucinophilia</i> prevalence
(De Palma et al., 2010b)	ACD children (n=24) TCD children (n=18) Ctrl children (n=20)	FISH (fecal sample)	CD ↓ IgA coated bacteria ↓ Ratio of gram+/gram- bacteria ACD ↓ Gram positive bacteria ↓ <i>Bifidobacterium</i> ↓ <i>C. histolyticum</i> ↓ <i>C. lituseburensis</i> ↓ <i>F. prausnitzii</i> ↑ <i>Bacteroides- Prevotella</i> ↓ IgA coated <i>Bacteroides</i> , <i>Prevotella</i> TCD ↓ IgG coated bacteria	ACD ↑ IgG coated bacteria ↑ IgM coated bacteria

			↓ IgM coated bacteria	
(Schippa et al., 2010)	ACD children (n=20) TCD children (n=20) Ctrls children (n=10)	TTGE (duodenal biopsy)	CD ↑ Interindividual similarity ACD ↑ Diversity	ACD ↑ diversity
		PCR 16s rDNA (duodenal biopsy)	CD ↑ <i>B. vulgatus</i> prevalence ↑ <i>E. coli</i> prevalence	ACD ↓ <i>B. vulgatus</i> prevalence ↓ <i>C. coccoides</i> prevalence
(Sánchez et al., 2010)	ACD children (n=20) TCD children (n=12) Ctrls children (n=8)	DGGE/PCR (duodenal biopsy)	CD ↓ <i>Bacteroides</i> diversity ↓ <i>B. distonis</i> ↓ <i>B. fragilis/B. thetaiotaomicron</i> ↓ <i>B. uniformis</i> ↓ <i>B. ovatus</i> ↑ Bifidobacterium diversity ACD ↑ <i>B. dorei</i> ↑ <i>B. adolescentis</i> ↑ <i>B. animalis</i> subsp <i>lactis</i> ↓ LAB diversity TCD ↓ <i>B. vulgatus</i> ↑ <i>B. coprocola</i> ↑ <i>Weissella</i> spp ↑ <i>L. fermentum</i>	ACD ↓ <i>B. ovatus</i> ↑ <i>B. vulgatus</i> ↑ <i>B. dorei</i> ↓ <i>B. coprocola</i> ↑ <i>B. adolescentis</i> ↑ <i>B. animalis</i> subsp <i>lactis</i> ↓ LAB diversity ↓ <i>Weissella</i> spp ↓ <i>L. fermentum</i> ↓ <i>L. reuteri</i> ↓ <i>Pediococcus pentosaceus</i>
(Di Cagno et al., 2011)	TCD children (n=19) Ctrls children (n=15)	DGGE/PCR (duodenal biopsy) Culture/PCR (fecal sample)	TCD ↑ <i>Eubacteria</i> diversity ↓ Lactobacilli counts ↓ Enterococci counts ↓ Bifidobacteria counts ↑ <i>Bacteroides</i> , <i>Porphyromonas</i> , <i>Prevotella</i> ↑ Staphylococci/ micrococci ↓ Total anaerobes	
		GC-MS/SPMC (fecal sample)	TCD ↓ Total SCFA ↓ Butyric acid ↑ Isobutyric acid ↓ Propanoic acid ↑ Acetic acid	
(Sanchez et al., 2012)	ACD children (n=20) TCD children (n=18) Ctrls children (n=20)	Culture/PCR (fecal sample)	CD ↑ <i>B. fragilis</i> ↓ <i>B. ovatus</i> ACD ↑ <i>Parabacteroides distasonis</i> ↓ <i>B. finegoldii</i> ↑ <i>B. fragilis</i> carrying virulence genes	ACD ↑ <i>Parabacteroides distasonis</i>
(Nistal et al., 2012)	ACD adults (n=10) TCD adults (n=11) Ctrls adults (n=11)	GC-MS (fecal sample)	CD ↑ Total SFCA ↑ Acetic acid ↑ Propionic acid ↑ Butyric acid TCD ↑ Valeric acid	
		DGGE/PCR (fecal sample)	ACD ↑ <i>B. bifidum</i> TCD ↓ Lactobacilli diversity ↓ <i>L. sakei</i>	ACD ↑ Lactobacilli diversity ↑ <i>L. sakei</i> ↑ Bifidobacteria diversity

			↓ Bifidobacteria diversity ↓ <i>Bifidobacterium</i> sp	↑ <i>B. catenulatum</i>
(<i>Sánchez et al., 2012</i>)	ACD children (n=20) TCD children (n=20) Ctrls children (n=20)	Culture/PCR (fecal sample)	CD ↑ <i>Staphylococcus epidermidis</i> ↑ <i>S. haemolyticus</i> ↓ <i>Enterococcus faecium</i> ↑ <i>S. epidermidis</i> with <i>mecA</i> and <i>altE</i> virulence ACD ↓ <i>S. aureus</i> ↑ <i>Staphylococcus</i> diversity TCD ↓ <i>S. warneri</i>	ACD ↓ <i>S. aureus</i> ↑ <i>S. warneri</i> ↑ <i>Staphylococcus</i> diversity
(<i>de Meij et al., 2013</i>)	ACD children (n=21) Ctrls children (n=21)	PCR/IS-pro (duodenal biopsy)	No differences	
(<i>Sánchez et al., 2013</i>)	ACD children (n=32) TCD children (n=17) Ctrls children (n=8)	Culture/16s rRNA sequencing (duodenal biopsy)	CD ↓ <i>Streptococcus mutans</i> group ↓ <i>Streptococcus anginosus</i> group ACD ↓ Firmicutes ↑ Staphylococcaceae ↑ <i>Staphylococcus epidermidis</i> ↑ <i>Staphylococcus pasteurii</i> ↓ Streptococcaceae ↑ Proteobacteria ↑ Enterobacteriaceae ↑ <i>Klebsiella oxytoca</i> TCR ↑ <i>Streptococcus mitis</i> group	ACD ↑ Actinobacteria ↑ Actinomycetaceae ↑ <i>Actinomyces odontolyticus</i> ↓ Firmicutes ↑ Staphylococcaceae ↑ <i>Staphylococcus epidermidis</i> ↑ <i>Staphylococcus pasteurii</i> ↓ Streptococcaceae ↓ <i>Streptococcus mitis</i> group ↑ Proteobacteria ↑ Enterobacteriaceae
(<i>Cheng et al., 2013</i>)	ACD children (n=10) Ctrls children (n=10)	16s rRNA sequencing (duodenal biopsy)	ACD ↑ <i>Prevotella melaninogenica</i> , <i>Haemophilus</i> , <i>Serratia</i> ↓ <i>Prevotella oralis</i> , <i>Proteus</i> , <i>Clostridium stercorarium</i> , <i>Ruminococcus bromii</i> , <i>Papillibacter cinnamivorans</i>	
(<i>Tjellström et al., 2013</i>)	ACD children (n=53) TCD children <1yr (n=74) TCD children >1yr (n=25) Ctrls children (n=54)	GC-MS (fecal sample)	ACD ↑ Acetic acid ↑ <i>i</i> -Butyric acid ↑ <i>i</i> -Valeric acid ↑ Total SCFA ↑ fermentation index [†] TCR <1yr ↑ Acetic acid ↑ Propionic acid ↑ <i>i</i> -Butyric acid ↑ <i>i</i> -Valeric acid ↑ <i>n</i> -Valeric acid ↑ Total SCFA ↑ fermentation index	

ACD, active celiac disease; TCD, treated CD; Ctrl, non-celiac controls; FISH, fluorescent in situ hybridization; LAB, lactic acid bacteria; SCFA, short chain fatty acid; GC-MS/SPMC - Gas chromatography-mass spectrometry/ solid-phase microextraction

* CD, includes active and treated CD

† Fermentation index (amount of acetic acid minus propionic acid and *n*-butyric acid, together divided by the total amount of SCFAs)

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