GLUTEN-MEDIATED T CELL ACTIVATION BY MHC CLASS II-EXPRESSING EPITHELIUM

A NEW MODEL TO INVESTIGATE THE ROLE OF INTESTINAL EPITHELIAL CELLS IN GLUTEN-SPECIFIC CD4$^+$ T CELL RESPONSES

By

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LAY ABSTRACT

Celiac disease is one of the most common food sensitivities, affecting approximately 1 in 100 people worldwide, including Canada. It occurs in people with specific genes (DQ2 and/or DQ8) when they eat gluten-containing foods such as wheat, barley, and rye. In people with celiac disease the immune system overreacts to gluten, damaging the lining of the upper gut, which we call "epithelium. This lining of cells constitutes the first barrier between the external world and our body, allowing in healthy conditions for nutrients to be absorbed but blocking the passage of gut microbes, some of which can cause disease or worsen gut inflammation. In patients with celiac disease, gluten crosses the epithelium into the gut tissue, where it activates specific cells of the immune system called "T cells". Recently, there has been growing interest in whether the gut lining itself plays a role in triggering this immune response in celiac disease, though this has not yet been proven. If proven, this would suggest that the gut lining is responsible for directing the harmful immune response to gluten and should be considered a target site when developing therapies to prevent or treat celiac disease. This concept has been difficult to prove because we do not have a model to investigate this question. Such a model would require a gut lining that carries the genes linked to celiac disease. My thesis describes the development of such a model, made of a gut lining from a mouse genetically modified to carry human celiac disease genes. Using this model, I found that when the gut lining was exposed to certain molecules present in celiac patients (cytokines), it switched on other molecules that ultimately activated T cells. Additionally, I demonstrated that certain microbes, such as *Pseudomonas aeruginosa*, which are present in higher numbers in the upper gut of patients

with celiac disease, can break down gluten into fragments that further activate T cells. The results validated the use of this model to understand what other co-factors can tip the balance in a person with celiac genes to remain healthy or develop inflammation. In summary, I demonstrated that the gut lining expressing celiac genes actively participates in the activation of immune cells that drive intestinal damage in celiac disease. This new model is a novel tool to continue to identify additional co-factors that predispose patients to celiac disease, as well as to screen for novel therapies for celiac disease. This is important, as the only currently available treatment is a strict lifelong gluten-free diet, which has many limitations, including frequent contamination and celiac disease reactivation.

ABSTRACT

Celiac disease is an autoimmune enteropathy driven by the ingestion of gluten in genetically predisposed individuals carrying HLA-DQ2 and/or -DQ8 genes. Currently, the only available treatment is a strict, life-long, gluten-free diet (GFD), which is very restrictive and not always effective, highlighting the need for alternative therapies. Celiac disease requires activation of both the innate (intraepithelial lymphocytes or IELs) and adaptive (lamina propria $CD4^+$ T cells) arms of the immune system. Activation of these two pathways leads to the destruction of IEC and villous atrophy. Thus, IEC damage is a hallmark of CeD. However, IECs are not only the target of tissue damage; they also actively participate in CeD pathogenesis by translocating gluten peptides, expressing stress-induced markers, and releasing TG2 into the gut lumen to generate TG2-gluten complexes. Although IECs are known to express MHC, their role in gluten-dependent T cell activation has never been proven, partly because of the lack of an appropriate *in vitro* epithelial model expressing human MHC class II. This thesis aims to address this gap by developing a humanized organoid monolayer expressing the CeD risk gene HLA-DQ2.5, to investigate the interaction between IEC-gluten-T cells. The expression of epithelial MHC class II was evaluated in active and treated CeD patients, as well as in gluten-immunized and control (non-immunized; NI) DR3-DQ2.5 transgenic mice that express only CeD-associated MHC class II (HLA-DQ2.5). Active CeD patients and gluten-immunized DR3-DQ2.5 mice demonstrated higher expression of epithelial MHC class II compared with their treated and NI counterparts. Organoid monolayers developed from these mice and were treated with or without IFN-γ. Organoid monolayers derived from gluten-immunized DR3-DQ2.5 mice

showed higher expression of MHC class II compared with NI mice, and this expression was upregulated by IFN-γ treatment. The functional consequences of MHC class II expression were determined by co-culturing organoid monolayers with $CD4^+$ T cells in the presence of gluten and zein (a non-gluten protein). In the co-culture, gluten, but not zein, enhanced $CD4^+$ T cell proliferation, activation, and release of cytokines, including IL-2, IFN-γ, and IL-15, in the co-culture supernatants. Bacteria have recently emerged as modulators of inflammation in patients with CeD. It has been shown that opportunistic pathogens, including *Pseudomonas aeruginos*a, partially metabolize gluten into more immunogenic peptides. As such, the role of bacterially modified gluten in modulating the T cell response was assessed using the *in vitro* co-culture system I described. For this, monolayers were treated with the gluten pre-digested, or not, by elastase-producing *P. aeruginosa* or its *lasB* mutant. Gluten metabolized by *P. aeruginosa*, but not by the *lasB* mutant, significantly increased $CD4^+$ T cell responses. In conclusion, MHC class II-expressing organoid monolayers are a functional model that can promote T cell responses under certain conditions. The model described in this thesis reveals a new immunomodulatory role for IECs in activating $CD4^+$ T cells through MHC class II. This mechanism may serve to localize and further increase injury to the epithelium caused by gluten-specific $CD4^+$ T cells in CeD. Therefore, therapeutics directed at IECs may offer a novel approach for modulating both adaptive and innate immunity in CeD, providing an alternative or adjuvant therapy to the current GFD treatment.

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"Life is not about waiting for the storm to pass. It is about learning to dance in the rain"- Vivien Greene

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LIST OF ABBREVIATIONS

Ab Antibody

Ag Antigen

AMP Antimicrobial peptides

ANOVA Analysis of variance

ASF Altered Schaedler flora

APCs Antigen-presenting cells

ATIs Amylase trypsin inhibitor

BSA Bovine serum albumin

BMP Bone morphogenetic protein

CeD Celiac disease

CD Cluster of differentiation

CIITA Class II major histocompatibility complex transactivator

CLIP Class II-associated invariant-chain peptide

CLR C-type lectin receptor

CTV CellTrace Violet

CT Cholera toxin

CXCR1 C-X-C motif chemokine receptor 1

DAPI 4′,6-diamidino-2-phenylindole

DAMPs Damage-associated molecular patterns

DCs Dendritic cells

DGP Deamidated gluten peptides

DMEM/F12 Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12

DTT Dithiothreitol

EDTA Ethylenediaminetetra-acetic acid

ELISA Enzyme linked immunosorbent assay

EGF Epidermal growth factor

ER Endoplasmic reticulum

ESCs Embryonic stem cells

FACS Fluorescence activated cell sorting

FBS Fetal bovine serum

FoxP3 Forkhead box P3

FSC-A Forward scatter area

FSC-H Forward scatter height

GALT Gut-associated lymphoid tissue

GI Gastrointestinal tract

GFD Gluten-free diet

GvHD Graft-vs-host disease

H&E Hematoxylin and eosin

hCD4 Human CD4

hPSCs Human pluripotent stem cells

HIOs Human intestinal organoids

HEPES 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid

HLA Human leukocyte antigen

HRP Horseradish peroxidase

IBD Inflammatory bowel disease

IEC Intestinal epithelial cells

IEL Intraepithelial lymphocyte

IE-CTL Intraepithelial cytotoxic T lymphocytes

ILC Innate lymphoid cells

ILF Isolated lymphoid follicle

ILVs Intraluminal vesicles

IFN Interferon

IRF1 Interferon regulatory factor 1

IL Interleukin

Ig Immunoglobulin

IgA/G Immunoglobulin A/G

i.p. Intraperitoneal

ICOS-L Inducible co-stimulatory ligand

Ii MHC class II invariant chain

ISCs Intestinal stem cells

M cells Microfold cells

MAMPs Microbial-associated molecular patterns

MHC Major histocompatibility complex

MIIC MHC class II compartment

MICA/B MHC class I-related chain A/B

MLN Mesenteric lymph nodes

MMPs Metalloproteases

MVB multi-vesicular bodies

mTG Microbial transglutaminase

NK cells Natural killer cells

NKG2D Natural killer group 2D

NOD/DQ8 non-obese diabeticDQ8

NOD nucleotide-binding oligomerization domain-containing protein

NLR nod-like receptor

NI non-immunized

OD Optical density

PAR Protease activated receptors

PBS Phosphate buffered saline

PBS-T Phosphate buffered saline-Tween 20

PD-L1 Programmed death ligand 1

RIG-I retinoic acid-inducible gene-1

RLR retinoic acid-inducible gene-1-like receptor

PRRs Pattern recognition receptors

PT-gliadin Pepsin-trypsin digested gliadin

PP Peyer's patches

P. aeruginosa *Pseudomonas aeruginosa*

RCD Refractory celiac disease

RNA Ribonucleic acid

RPMI Roswell Park Memorial Institute medium

SEM Standard error of the mean

sIgA Secretory IgA

SPF Specific pathogen free

SSC-A Side scatter area

TA cells Transit-amplifying cells

T-bet T-box transcription factor TBX21

TCR T-cell receptor

TCCs T cell clones

TG2 Tissue transglutaminase 2

TGF Transforming growth factor

Th T helper

TLR Toll-like receptors

TNF Tumour necrosis factor

Tregs Regulatory T cells

Vh/Cd Villus height-to-crypt depth

WT Wild type

α Alpha

β Beta

γ Gamma

δ Delta

μ Micro

μm Micrometre

mg Milligram

h Hour

ºC Degrees Celsius

DECLARATIION OF ACADEMIC ACHIEVEMENT

I, Sara Rahmani, declare that this thesis titled "A new model to investigate the role of intestinal epithelial cells in gluten specific $CD4^+$ T cell response" was written by me. The experiments presented therein were all designed, and performed, and the data collected and analyzed by me. Here, I confirm that:

- Antibody measurements (anti-gliadin and anti-TG2) in the serum and intestinal content of DR3-DQ2.5 mice, as well as quantification of villous height to crypt depth ratios, quantification of $CD3^+$ IELs counts, and nanostring gene expression measurements and analysis in the small intestine tissue of DR3-DQ2.5 mice, were performed by Alex V. Clarizio.

- Chapter 3, 4 and 5 are adopted from my recently published paper (Rahmani et al., 2024) titled "Gluten dependent activation of $CD4^+$ T cells by MHC class II-expressing epithelium, *Gastroenterology*, 2024", in which I am the primary author.

CHAPTER 1 INTRODUCTION

INTRODUCTION

1.1 Structure of the small intestine

The small intestine (SI) is the proximal part of the gastrointestinal tract (GI), which is responsible for the digestion and absorption of nutrients, except for complex fibers that are mainly metabolized by microbes in the colon (Rescigno 2011). The structure of the SI facilitates the maximum absorption of nutrients. The walls of the SI consist of four layers: (1) an inner mucosa facing the lumen; (2) a layer known as the submucosa; (3) layers of smooth muscle known as the *muscularis propia or externa*; and (4) a covering of connective tissue referred to as the serosa (Gourevitch 2005)(Figure 1.1). The mucosa (inner lining) of the SI consists of finger-like extensions known as "villi" that project into the lumen to increase its surface area to enhance nutrient absorption. Additional surface area is added by tubular invaginations of the surface, referred to as crypts, which extend down into connective tissues. The submucosal layer is composed of connective tissue with blood vessels and the submucosal plexus, which is one of the two major nerve networks of the enteric nervous system that helps coordinate secretion and absorption as well as visceral perception. The outer wall of the SI, the *muscularis externa*, consists of two layers of smooth muscle: an inner circular layer and an outer longitudinal layer. Contraction of the inner circular layer decreases the diameter of the lumen, while contraction of the longitudinal layer shortens the SI tube to mechanically mix the foodstuffs and push them forward down the SI length. The second network of the enteric nervous system, the myenteric plexus, resides between these two muscle layers, and regulates and coordinates the motor activity of the muscularis externa. The outer covering of the SI, the serosa, is a

connective tissue membrane that is a continuation of the peritoneal membrane lining of the abdominal cavity. The peritoneum creates sheets of mesentery that hold SI in place so that they do not tangle as they move.

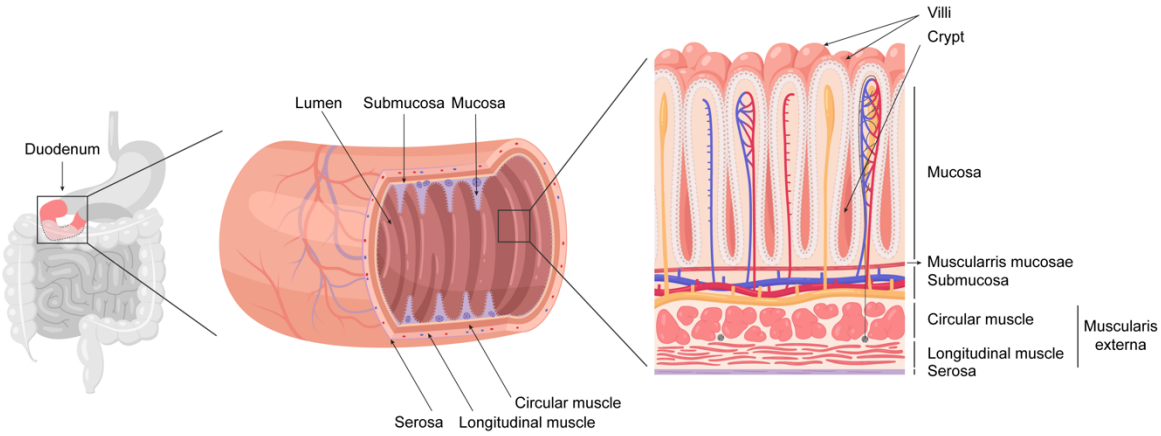


Figure 1.1 Overview of small intestine anatomy. The small intestine comprises the duodenum, jejunum, and ileum. Depicted here is the most proximal section, the duodenum, and the site of celiac disease (CeD). The transversal cut shows multiple layers, which, from the outermost to the innermost are the serosa and muscularis propria composed of a longitudinal and a circular muscle layer, followed by the submucosa, mucosa, and the lumen.

1.2 The gut mucosal immune system

The mucosal surface of the GI tract is vulnerable to infection because it contains a complex community of microbes that are constantly exposed to dietary and other environmental antigens. Homeostasis within the gut is governed by the gut mucosal immune system, which comprises the gut-associated lymphoid tissue (GALT), mesenteric lymph nodes (MLNs), lamina propria, and epithelium. GALT is organized into Peyer's patches (PPs) and isolated lymphoid follicles (ILFs) (Mowat & Agace, 2014). PPs are

mainly scattered in the submucosa of the distal part of the small intestine (ileum), as compared to the proximal sections (duodenum and jejunum). Many different cell types, including T lymphocytes, B lymphocytes (majority IgA-producing B cells), dendritic cells (DCs), and macrophages, reside within PPs, which are vital for the initiation of the immune response (Mowat, 2003a)(Van Wijk & Cheroutre, 2010). PPs are covered by specialized follicle-associated epithelium (FAE), which contain microfold (M) cells, a specialized type of intestinal epithelial cells (IECs) that mediate the delivery of luminal antigens across the mucosa to the LP (Mörbe et al., 2021). ILFs are present in the lamina propria of the small and large intestines.

The gut mucosal immune system can be functionally classified into inductive and effector sites. Inductive sites are where immune responses, such as antigen sampling and processing are initiated, as well as where the activated antigen-specific B and T cells reside (Parham 2021)(Mowat and Agace, 2014)(P. Smith et al., 2020). The main inductive sites for gut mucosal immune responses are the GALT and mesenteric lymph nodes (MLNs) in the small and large intestines (Pabst and Mowat 2012). MLNs are lymph nodes that drain the GI tract and are essential sites for immune cell development. Through M cells, antigens are passed to APCs, such as DCs that activate naïve cluster differentiation (CD) 4 T cells, CD8 T cells, and B cells within the inductive sites, and initiate an immune response (Pabst &Mowat, 2012). In contrast, the effector sites are compartments where the effector cells are located and are composed of epithelia and the underlying LP (Mowat & Agace, 2014). Effector cells are immune cells from lymphoid tissues; they initiate downstream inflammatory and immune responses when activated (Mowat & Agace 2014). Together,

these compartments play a role in both innate and adaptive immune responses to protect the gut microenvironment from invading pathogens while maintaining intestinal homeostasis and tolerance towards innocuous antigens (Figure 1.2). These immune mechanisms, coupled with physical barriers such as mucosal and skin surfaces, are essential for preventing pathogens from the environment to more easily enter the body and cause severe infection. In the following sections, we discuss in more detail the two arms of the immune response: the immediate innate immune responses and specific and long-lasting adaptive immune responses.

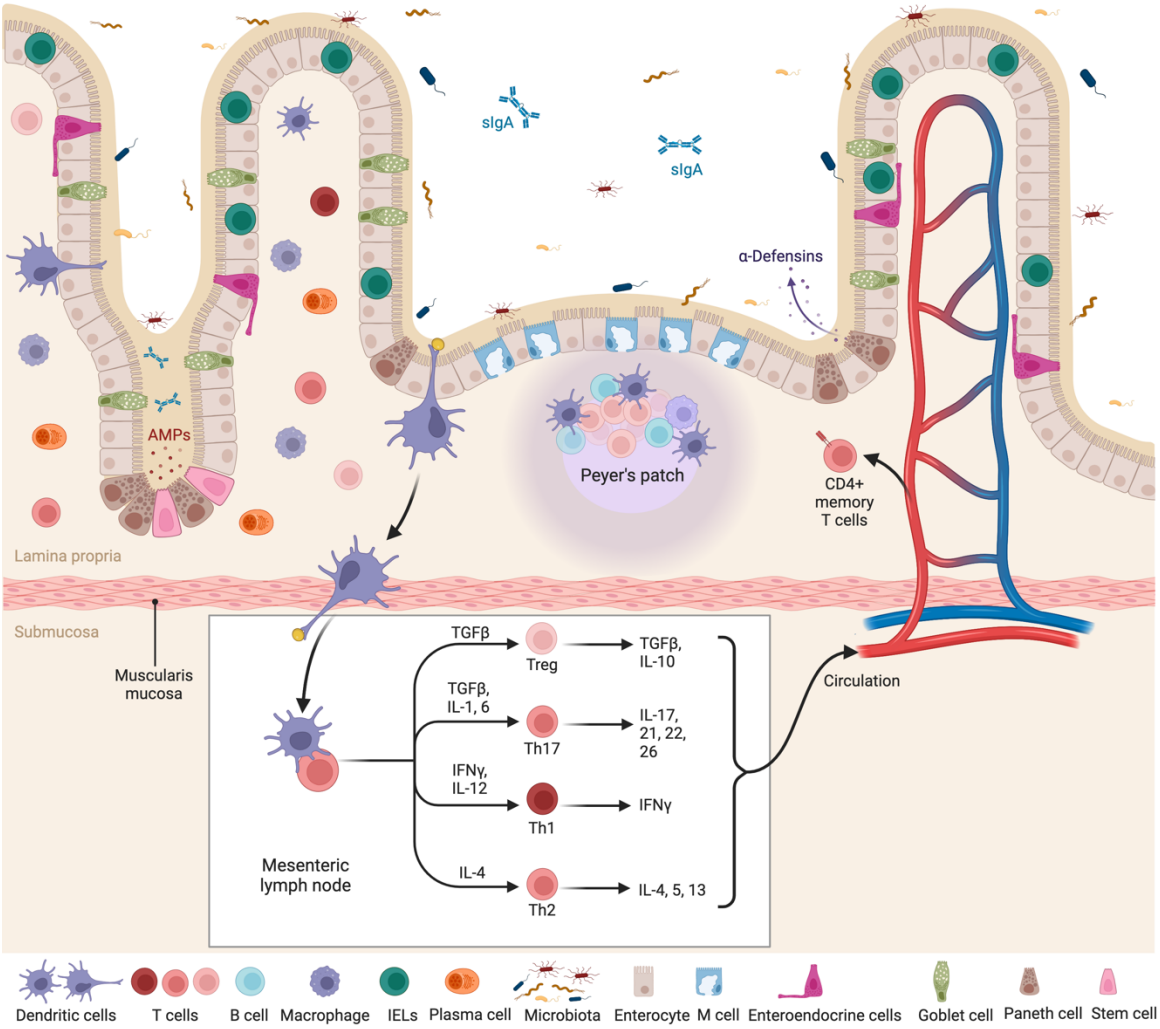


Figure 1.2 Overview of the small intestine immune system. The mucosal system comprises effector sites, such as the lamina propria and epithelial cells, as well as inductive sites, including Peyer's patches and mesenteric lymph nodes, which play crucial roles in the immune responses of the small intestine. The intestinal epithelial cells, which form a single layer, and the molecules they secrete, as well as the intraepithelial lymphocytes (IELs), which primarily consist of activated T cells, the microbiota, and other immune cells, all collaborate to protect the host and maintain intestinal homeostasis. sIgA: secretory immunoglobulin A; AMPs: antimicrobial peptides. Image created with BioRender.com

1.2.1 The gut innate immune response

The innate immune system represents the immediate first line of defense that acts when an infection occurs, leading to a more specific downstream adaptive immune response (Parham, 2021)(Murphy & Weaver, 2016)(Bedoui et al., 2016). The cell types capable of inducing an innate immune response in the gut are the intestinal epithelial cells (IECs), myeloid and lymphoid lineage cells. IECs are non-classical immune cells with some innate immune functions, such as upregulation of stress-induced ligands during infections that signal IEL populations (Parham, 2021). The myeloid cells with innate immune functions include granulocytes (eosinophils, basophils, and neutrophils), macrophages, DCs, and mast cells. Lymphoid lineage cells include intraepithelial lymphocytes (IELs) and innate lymphoid cells (ILCs) (Peter Parham 2021)(Murphy and Weaver 2016).

The proximity of the gut mucosal immune system to the community of microbes that inhabit the gut (microbiota) and dietary antigens ingested with food, makes its innate immune cells central to homeostasis. These cells are equipped with an extensive array of receptors known as pattern recognition receptors (PRRs) that detect different ligands on pathogens or damaged or dying cells, such as microbial-associated molecular patterns (MAMPs) and damage-associated molecular patterns (DAMPs), respectively. There are different types of PRRs, including toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene-1 (RIG-1)-like receptors (RLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), absent in melanoma (AIM)-like

receptors (ALR) (Muñoz-Wolf & Lavelle, 2016), and protease-activated receptors (PARs) (Shpacovitch et al., 2007)(Shpacovitch et al., 2008).

1.2.1.1 *Intraepithelial lymphocytes (IELs)*

IELs are a group of heterogeneous, non-circulating tissue-resident lymphocytes interspersed between IECs that have innate-like characteristics and perform surveillance roles *(Jabri & Sollid, 2017)*(Cheroutre, et al., 2011). Most IELs express TCR, respond to antigens when presented by APCs, and are referred to as intraepithelial T cells (IETs) (Swamy et al., 2015)*(Sheridan & Lefrançois, 2010)*. In contrast, some IELs lack antigen receptors, respond to infection, stress, and damage in a TCR-independent fashion, and are classified as ILCs (Bedoui et al. 2016). In this thesis, I refer to IETs as IELs.

IELs are antigen-experienced cells that do not express activation markers such as CD25, which are characteristics of systemic T cells in the periphery. IELs can be categorized into two subtypes based on the antigens they recognize and the activation pathways they follow. The first type of IELs is referred to as "natural" IELs, consisting of TCR$\gamma\delta^+$ and TCR$\alpha\beta^+$ T cells that develop in the thymus. Upon encountering a strong TCR signal, these IELs adopt an activated phenotype, undergoing self-antigen-induced differentiation in the thymus. Subsequently, they are trafficked directly to the intestine and co-express CD8$\alpha\alpha$. The primary role of natural IELs is to maintain the integrity of the intestinal barrier and regulate the local immune balance by releasing cytokines that modulate the barrier function (Komano et al., 1995)(Guy-Grand et al., 1998)(Walker et al., 2013) and epithelial growth factors (Powrie et al., 1993)(Yang et al., 2004). However, the

exact functions and ligands of TCRγδ have yet to be determined. Natural IELs represent the earliest antigen-experienced T cells that populate the intestine before birth (Latthe et al., 1994). Although the population of natural IELs is consistent throughout life, it constitutes a smaller proportion of IELs in older age when the induced IEL population increases.

The second type of IELs is known as "induced" IELs, which are comprised of $CD4^+$ or $CD8\alpha\beta^+$ $TCR\alpha\beta^+$ T cells (Cheroutre et al., 2011). Initially, induced IELs develop as naïve cells in the thymus and adopt their activated phenotype in peripheral locations, such as MLNs, upon exposure to peripheral antigens, and subsequently traffic to the intestine (Iwata et al. 2004). Following activation, IELs express CD8αα homodimers, particularly in the small intestine (Madakamutil et al., 2004)(Denning et al., 2007). CD8αα can be co-expressed with both CD4 and CD8αβ molecules on $TCR\alpha\beta^+$ IELs, although a significant portion primarily expresses CD8αα alone. $CD4^+$ IELs constitute a minority of the overall IEL population, particularly in the small intestine (Mosley et al., 1990), whereas $TCR\gamma\delta^+$ cells expressing CD8 comprise a large proportion, up to 60%, of the small intestine IELs (Bonneville et al., 1988)(Goodman & Lefranfois, 1988).

IELs can express either activating or inhibitory natural killer (NK)-like receptors, and as such, they can play either pathogenic or beneficial roles. The main role of IELs is to support the integrity of the intestinal epithelium. Studies have shown that natural $TCR\gamma\delta^+$ IELs have demonstrated protective functions in the gut by repairing the epithelium (Guy-Grand et al., 1998)(Komano et al., 1995) and play a role in resistance against certain infections (Dharakul et al. 1991)(Müller, Bühler-Jungo, and Mueller 2000)(Chardès et al.

1994)(Cuff et al. 1993). Induced $CD4^+$ IELs can upregulate CD8αα and adopt a cytotoxic yet regulatory phenotype. It has been shown that induced $CD4^+CD8\alpha\alpha^+$ IELs can express Th1-induced intestinal inflammation in an IL-10 dependent manner (Das et al. 2003). The pro-inflammatory role of natural $TCR\gamma\delta^+$ IELs has been revealed in mouse studies, where $TCR\gamma\delta^+$ IELs induce intestinal inflammation (Simpson et al. 1997)(Mizoguchi et al. 2003). Therefore, IELs are essential for identifying and destroying infected IECs and mediating the restoration of mucosal tissue following inflammatory damage. They play a fundamental role in connecting innate and adaptive immune responses.

1.2.1.2 *Innate lymphoid cells (ILCs)*

ILCs serve as initial effectors against damage and infection and mediate immune responses analogous to T helper (Th) cells. ILCs are classified into three subsets: ILC1, ILC2, and ILC3. ILCs are present in low numbers in the blood, and their preference is to primarily reside on mucosal surfaces, including the gut, lungs, and skin. The distribution of different subsets across these surfaces is also different, with ILC3 being the most dominant class in the gut. Although ILCs are like Th cells in terms of phenotype and function, they do not express T-cell antigen-specific receptors (TCR). Thus, ILCs respond promptly to tissue infection or damage (faster than T cells) but cannot directly provide antigen-specific memory responses. ILC1 have functional and phenotypic similarities to NK cells and the intestine; mainly IEC is home to ILC1, which releases a significant amount of Interferon (IFN)-γ. They are essential for resistance to infection and are associated with chronic inflammation; for example, they are present in the intestines of patients with

inflammatory bowel disease (IBD), where a large amount of IFN-γ is produced (Spits, Bernink, and Lanier 2016). ILC2 are prompt producers of T helper type 2 (Th2) cytokines, such as IL-4, IL13, and IL-5, upon infection of the intestine by helminths (Von Moltke et al. 2016). Also, ILC2 cells play an important role in enhancing allergic responses (Halim et al. 2014). The presence of intestinal microbiota induces a high density of ILC3, regarded as an innate counterpart of Th17 cells, and leads to a substantial release of IL-17 and IL22 (Qiu et al. 2013). ILC3 are essential for inducing tolerance towards dietary antigens and commensal bacteria by activating regulatory T (Treg) cells. In addition, ILC3 express major histocompatibility complex (MHC) class II, and in the intestine, these expressions allow ILC3 to process and present microbial peptides to $CD4^+$ T cells and activate adaptive immune responses (Hepworth et al. 2013). It was found to promote the release of IL-22 by ILC3 promotes IECs to release serum amyloid A, which in turn activates Th17 and the production of IL-17. Despite increasing functional characterization of ILCs subtypes in many inflammatory conditions, there is limited understanding of their direct contribution in CeD. Emerging data show some ILC alterations in association with CeD and in refractory CeD, but a role in disease induction will require further basic and translational investigation (Uhde et al. 2020)(Yu et al. 2021).

1.2.2 Adaptive immune response

The adaptive immune response is the second line of defence, which involves an antigen-specific cell response. The adaptive immune response organizes a memory response and activates a long-lasting and specialized cellular response upon re-exposure to

the antigen or pathogen, whereas the innate immune response remains constant and broad during re-exposure (Abbas et al., 2014) (Murphy & Weaver 2016). The main cellular components of the adaptive immune response are white blood cells called lymphocytes, namely T- and B-lymphocytes, which offer cell-mediated and humoral immunity, respectively (Murphy & Weaver 2016)(Wucherpfennig et al. 2010). In the gut, T- and B-cells reside within lymphoid follicles of the GALT or MLN in B- and T- cell zones in the naïve state. Upon encountering antigens in lamina propria, professional APCs such as DCs take up this antigen and traffic them to ILF or MLNs, where they present the processed antigen bound to major histocompatibility complex (MHC) to prime naïve T cells (Smith et al., 2020). Following this, T cells undergo proliferation and differentiation into effector antigen-specific T cell clones (TCCs) and move to lamina propria (Rossjohn et al. 2015)(La Gruta et al. 2018), where they release cytokines to mediate the maturation of B cells into antibody-producing plasma cells, which release antibodies (Lindeman et al. 2021)(Høydahl et al. 2019)(Chatzileontiadou et al. 2021).

T lymphocytes originate from lymphoid stem cells in the bone marrow and migrate into the thymus. T cells then pass through several stages of maturation in the thymus, where they proliferate and generate functional T cell receptor (TCR) capable of recognizing a broad range of pathogenic peptides (Murphy and Weaver 2016). These naïve mature T cells then migrate to secondary lymphoid organs, including the spleen, lymph nodes, and mucosal lymphoid tissues, to protect the host from harmful antigens that the host might encounter (Peter Parham 2021) (Murphy and Weaver 2016). The TCR in T cells plays a crucial role in responding to foreign antigens and initiating intracellular signaling pathways

that are essential for T cell activation and functions. TCR are composed of disulfide-linked membrane-bound heterodimeric protein that express α and β chains (αβ TCRs) or γ and δ chains (γδ TCRs) (Murphy and Weaver 2016)(P. Smith, Blumberg, and MacDonald 2020). Typically, αβ TCRs are expressed by cluster of differentiation (CD)4 $^{+}$ T cells, while $CD8^{+}$ T cells express αβ TCRs or γδ TCRs. Of note, some subsets of γδ T cells do not express the combination of CD4 and CD8. T lymphocytes are generally categorized into classical αβ TCRs expressing $CD4^{+}$ and $CD8^{+}$ T cells, as well as innate immunity-like T cells namely, γδ T cells and natural killer (NK) T cells. T cells expressing the CD8 are cytotoxic T cells that mediate the killing of infected or damaged cells (Peter Parham 2021)(Murphy and Weaver 2016), whereas T cells expressing the CD4 co-receptor are referred to as T helper(h) cells, because they mediate the activation of effector cells by other cells (Peter Parham 2021)(Murphy and Weaver 2016).

As mentioned above, T cells are composed of a diverse array of TCR to recognize a variety of peptide antigens, for example, αβ TCRs recognize peptides bound MHC molecules (in humans referred to as human leukocyte antigen or HLA). Recognition of pathogenic antigens by TCR primes naïve T cells and activates the intracellular signaling pathways required for T cell activation and effector functions (Rossjohn et al. 2015). CD4 and CD8 serve as co-receptors for TCRs, to enhance the affinity of TCR for MHC class II and MHC class I molecules, respectively. MHC class I molecules, present on all nucleated cells, display intracellular antigens to $CD8^{+}$ T cells, facilitating a cytotoxic response. On the other hand, MHC class II molecules, however, are restricted to professional antigen-presenting cells (APCs) and engage $CD4^{+}$ T cells to present extracellular antigens, initiating

a helper T cell response. Additionally, $CD4^+$ and CD8 T^+ cells can be further classified into naïve, effector, and memory subtypes, depending on the type of antigen they get expose to and their activation status. This thesis focuses mainly on T cells, specifically cluster of differentiation (CD)4^+ T cells, which are discussed in detail in the following sections.

1.2.2.1 *$CD4^+$ T cells*

Naïve $CD4^+$ T cells that have completed the maturation process in the thymus migrate through the bloodstream into secondary lymphoid organs and then continue to recirculate between the blood and secondary lymphoid tissue (Peter Parham 2021). Recirculation continues until the initial encounter with the cognate antigen, called priming. When naïve $CD4^+$ T cells recognize a foreign antigen presented by MHC class II molecules on professional APC, they become activated and clonally expanded. In addition, depending on the polarizing cytokinin in the. $CD4^+$ cell environment during activation, they differentiate into a specific T cell effector lineage. The different lineages can be distinguished based on their unique transcriptional and cytokine profiles (Murphy and Weaver 2016)(Abbas, Lichtman, and Pillai 2014). Five main $CD4^+$ effector T cell subsets were defined with their respective effector cytokines and transcription factors as follow: Th1 (IFN-γ, tumor necrosis factor-α (TNF)-α, and T-box transcription factor TBX21 (T-bet), Th2 (IL-4, IL-5, IL-13, and GATA3), Th17 (IL-17A, IL-17F, IL-22, and ROR-γt), follicular helper T cells (IL-4, IL21, and BCl-6), and peripheral regulatory T cells (IL-10, Transforming growth factor (TGF)-β, and Forkhead box P3 (FoxP3)) (X. Zhu and Zhu 2020)(J. Zhu et al., 2010)(Murphy and Weaver 2016). The activation of Th1, Th2, and

Th17 T cell lineages is elicited by different pathogens (Murphy and Weaver 2016). In contrast to these three lineages of effector $CD4^+$ T cells, follicular helper T cells, rather than being activated by a particular pathogen, contribute to the eradication of most pathogens because they provide help to B cells. While antigen-stimulated Th1, Th2, Th17, and follicular helper T cells mediate the activation of other immune cells to actively remove pathogens from the body, regulatory T cells have an inhibitory effect on the immune response (Peter Parham 2021). These T cells mediate the extent of the immune response to prevent excessive T-cell activation and to prevent activation of immune cells in response to self-antigens, thus preventing autoimmunity. In addition, other effector $CD4^+$ T-cell lineages have been described but these are not universally accepted as they often lack proper transcription and cytokine profiles.

When the antigen that stimulates naïve $CD4^+$ T cells is removed, a subset of antigen-experienced T cells differentiate into memory T cells (Peter Parham 2021). These antigen-specific T cells remain in the body long after elimination of the original antigen and are central to adaptive immunity. The memory T-cell subset constitutes a dynamic repository of antigen-specific T cells that accumulate throughout their lifetime. Upon antigen re-exposure, this T cell subset rapidly proliferates and provides a highly specific response. Memory $CD4^+$ T cells can be differentiated from naïve $CD4^+$ T cells by the expression of CD45, a transmembrane protein that confers the ability to respond to recall antigens (Rossjohn et al. 2015). While naïve T cells express a full-length form of CD45, called CD45RA, on their surface antigen-experienced memory T cells express only a short version of CD45, called CD45RO. Memory T cells can be further subdivided into two subsets:

effector memory T cells and central memory T cells (A. C.R. Beitnes et al. 2011). These two subsets differ in their effector function and homing capacity, and hence in the expression of specific surface receptors. Effector memory T cells have immediate effector functions, but limited proliferative potential, whereas central memory T cells have limited effector functions, but high proliferative potential. Central memory T cells express the lymph node homing receptor CCR7 co-expressed with ligand CD62L, allowing them to migrate between secondary lymphoid organs and peripheral blood, whereas effector memory T cells express tissue-homing receptors and migrate to peripheral tissues with primed APCs.

Recently, attention has been given to a memory $CD4^+$ T-cell subset, referred to as tissue-resident memory T cells. This T-cell subset resides in non-lymphoid tissues, such as the lungs, intestines, and other mucosal surfaces, where it persists even in the absence of the driving antigen to provide a rapid local response (Rossjohn et al. 2015)(Dai et al. 2008)(S. W. Qiao et al. 2014). The hallmark marker of tissue-resident memory $CD4^+$ T cells is the surface expression of CD69 (Chatzileontiadou et al. 2021). This membrane-bound receptor has been implicated in the differentiation of T cells and retention of T cells in lymphoid organs (Dendrou et al. 2018). Normally, CD69 is expressed on $CD4^+$ effector T cells shortly after its activation in lymphoid tissues. CD69 prevents activated effector memory $CD4^+$ T cells from migrating out of lymphoid organs (Chatzileontiadou et al. 2021) (Lindeman et al. 2021). Similarly, it has been demonstrated that it is required for retention of $CD4^+$ memory T cells in non-lymphoid tissues (Høydahl et al. 2019).

Altogether, it can be concluded that innate and adaptive immune responses work together to sustain homeostasis in the gut and in a synchronized manner to maintain normal immune function and tolerance to luminal antigens that are innocuous while defending against pathogens. When this synchronization fails, it leads to immune-mediated diseases such as CeD.

1.3 Intestinal epithelial cells (IECs)

The intestinal epithelium consists of a single layer of columnar IECs of different lineages, which act as a physical barrier together with a layer of mucus that overlays it (König et al. 2016). IECs populate all mucosal surfaces and are the first line of defence against environmental antigens (Rescigno 2011). IECs are composed of at least six different mature cell types, including Paneth cells, goblet cells, enteroendocrine cells, M cells, tuft cells, and enterocytes (Liao, Zhao, and Gregersen 2009)(Wang et al. 2020). Each cell type displays unique microscopic characteristics and functions. For example, Paneth cells are located at the base of the crypts in the small intestine (Haber et al. 2017). They protect intestinal stem cells (ISCs) by releasing antimicrobial peptides (AMPs) and supporting the stem cell niche by secreting growth factors essential for ISCs self-renewal and proliferation (Sato, Van Es, et al. 2011). Goblet cells are responsible for generating mucins that contribute to the formation of a protective layer on the surface of the intestinal epithelium (Johansson and Hansson 2016)(Neutra and Leblond 1966). These mucins act as barriers, protecting the underlying tissues from luminal factors and, as such, perform innate immune functions (Mcdermott and Huffnagle 2014). Enteroendocrine cells, comprising

approximately 1% of the IEC population, produce hormones that regulate various digestive functions (Worthington, Reimann, and Gribble 2018). M cells contribute to gut immunity by preferentially allowing the uptake of luminal antigens via phagocytosis and transcytosis (Miller et al. 2007). They are strategically located above PPs, and thus traffic antigens to the underlying professional APCs in the PPs (Mabbott et al. 2013). Tuft cells represent a rare population of IECs that express chemosensory receptors and are involved in defense against helminth infections (Sato, Van Es, et al. 2011). Lastly, enterocytes account for approximately 80% of mature IECs that populate the intestinal epithelium (Ross and Pawlina 2011).

Additionally, IECs as a selective barrier play functional roles, such as mediating paracellular and transcellular permeability pathways. Paracellular permeability pathways are governed by apical junction complexes, including adherens junctions, tight junctions, and desmosomes, which connect IECs (König et al. 2016). Tight junctions, lactated at the apical region of the lateral membranes, selectively allow the passage of small molecules, solutes, and ions across the epithelium, while preventing the transport of larger antigens such as microbes, and toxins (Suzuki 2013). Tight junctions are comprised of transmembrane proteins, such as claudins, occludins, junctional adhesion molecules (JAM) along with scaffolding proteins like zonula occludens (ZO) that are anchored to the actin cytoskeleton and are regulated by intrinsic and extrinsic factors such as cellular stress, cytokines, and pathogens (Groschwitz & Hogan, 2009)(Suzuki 2013). An increase in paracellular permeability or epithelial damage, potentially induced by these factors, can allow excessive antigen entry, contributing to the pathogenesis of gut diseases (Groschwitz

& Hogan, 2009). In contrast, the transcellular pathway enables the absorption and transport of nutrients, such as small peptides, amino acids, fatty acids, short-chain fatty acids, sugars, and vitamins across epithelial cells (Suzuki 2013). This pathway is mediated by specific transporters and channels located on the basolateral and apical surfaces of IECs (Suzuki 2013)(Schoultz and Keita 2020). Larger molecules, including dietary peptides, traverse this barrier through endocytosis by enterocytes and are released at the basolatheral surface, where they can interact with immune cells (Pabst and Mowat 2012)(Menard, Cerf-Bensussan, and Heyman 2010). Additionally, specific types of IECs, known as microfold (M) cells, which reside on the epithelium covering the PP or ILFs, are involved in the transport of dietary and microbial antigens to the basolateral membrane (Pabst and Mowat 2012). Proper regulation of the physical intestinal epithelial barrier is critical for maintaining homeostasis, forming of protective immune responses to foreign antigens, and preventing inflammatory conditions (Suzuki 2013). Reduced barrier function has been associated with an increased susceptibility to mucosal pathology (Arrieta, Bistritz, and Meddings 2006). Additionally, the intestinal barrier function is also regulated by the release of inflammatory mediators into the lumen and by cell populations such as IELs, which reside between IECs and have evolved to have an innate-like function and conduct surveillance roles, as mentioned in section 1.2.1.1 (König et al. 2016)(Cheroutre, Lambolez, and Mucida 2011).

Moreover, apart from their role as physical barriers, IEC actively participate in immune defense, primarily by secreting mucins and AMPs. Mucin, which is comprised of mucus, is produced by the goblet cells which express *MUC2.* In the small intestine, mucus

forms a single layer that adheres loosely to the epithelium and is permeable to bacteria. AMPs are mainly secreted by Paneth cells at the base of crypts, but they are also produced by other IECs and innate immune cells (Bevins and Salzman 2011). AMPs play a role in reducing the interaction between luminal antigens and both the epithelial and underlying immune cells (Bevins and Salzman 2011). In addition, dimeric immunoglobulin A (IgA), synthesized by plasma cells in the lamina propria, is transcytoses through IECs before being secreted into the intestinal lumen as secretory IgA (sIgA), where it plays a role in separating luminal antigens from the internal mucosa, thereby enhancing mucosal immunity (Brandtzaeg and Prydzt 1984).

Although small intestinal epithelial cells are not professional APCs, such as B cells or DCs, they have been shown to constitutively express MHC class II molecules (R. M. Hershberg & Mayer, 2000)(L. Mayer 2000)(Lloyd Mayer et al. 1991)(Lloyd Mayer and Shlien 1987)(Biton et al. 2018)(Wosen et al. 2019), particularly under pro-inflammatory conditions (Wosen et al. 2018)(Thelemann et al. 2014)(Malik et al. 2023), implying that IECs have the essential prerequisite to function as non-conventional APCs (Dotan et al. 2007). This characteristic of IECs is discussed in detail in section 1.5.1. The diverse population of IECs collectively contribute to maintaining intestinal homeostasis and performing complex functions within the intestine tract. Thus, a thorough understanding of the specific function of each IEC subtype and their interactions with the immune system is crucial for dissecting their immunomodulatory roles in the pathogenesis of diseases. My thesis focusses on CeD, which primarily affects the first portions of the SI and whether IEC are the target of immune mediated damage. Throughout this thesis, the use of IECs refers

to all mature IECs lineages of the small intestine unless otherwise specified. Although the pivotal role of IECs in maintaining barrier integrity and innate immune pathways is widely recognized, their involvement in antigen-specific immune responses has been a subject of debate. Some studies have attributed MHC class II expression to inflammation, while others have associated it with immunosuppression, epithelial differentiation, or renewal (Biton et al., 2018; Jamwal et al., 2020; Koyama et al., 2019; Thelemann et al., 2014)(Malik et al. 2023). These conflicting results are likely contextual, indicating that thorough understanding will require their investigation in specific diseases and under certain conditions. CeD is a model condition in gastroenterology and medicine, as unlike IBD or type 1 diabetes, with which it associates (Maria Ines Pinto-Sanchez et al. 2020)(Zingone et al. 2024) its main environmental driver, gluten, and the specific molecular pathway with risk genes, MHC class II association (Ludvig M. Sollid and Thorsby 1993), and activation of T cells is well described (Jabri and Sollid 2017)(Ludvig M. Sollid 2022). Although the expression of MHC class II in IECs in patients with CeD has been previously reported (Arnaud-Battandier et al. 1986)(Marley, Macartney, and Ciclitira 1987)(Fais et al. 1992)(Kelly et al., 1988a), confirmation of their role in gluten antigen presentation has been overlooked, in part due to the technical complexity required to answer this question. Thus, in this thesis, I focused on developing an *in vitro* model to investigate the unsolved question regarding the expression of MHC class II expression associated with CeD by IECs, namely HLA-DQ2.5, and its potential to modulate $CD4^+$ T cell activation under different conditions.

1.4 Human MHC molecules

In humans, MHC is located on chromosome 6 and is referred to as human leukocyte antigen (HLA) (Sharon et al. 2016). Human MHC class I and II molecules are called HLA class I and HLA class II molecules, respectively (Dendrou et al. 2018). HLA class I is composed of three isotopes: HLA-A, HLA-B, and HLA-C, comprising a non-covalently linked heavy chain, α, and a light chain (β2-microglobulin), constitutively expressed by all nucleated cells (Peter Parham 2021)(Murphy and Weaver 2016). Conversely, HLA class II has three isotypes, HLA-DR, HLA-DQ, and HLA-DP, consisting of non-covalently linked α and β chains that are constitutively expressed by professional APCs, including DCs, macrophages, and B cells (Dendrou et al. 2018)(Petersen et al. 2016). These two classes of HLA molecules have similar three-dimensional structures with four domains. The peptide-binding site of HLA class I is formed by the α_1 and α_2 domains of the heavy α chain, while the peptide-binding side of HLA class II is formed by α_1 domain of the heavy α-chain and β_1 domain of the βchain (Jabri and Sollid 2017)(Peter Parham 2021). The other side of the HLA class I molecule is called immunoglobulin-like domains, which provide support to the peptide-binding side and project it away from the APC surface, as well as offer binding sites for T cell receptors so the HLA molecules can simultaneously engage with both T cell receptors and co-receptors (C.-Y. Kim et al. 2004)(Hovhannisyan et al. 2008). The peptide-binding site of HLA class I has closed ends and thus can only bind peptides that are eight or nine amino acid residues long (Murphy and Weaver 2016)(Sewell 2012)(Y. C. Liu et al., 2012). In contrast, the HLA class II peptide-binding site has a more open conformation, which allows longer peptides that are 10-25 amino acid residues long

to fit into the groove (Rossjohn et al. 2015)(Janeway et al. 2001)(La Gruta et al. 2018). Since HLA molecules play a pivotal role in immune regulation and function, the diversity in HLA types affects the immune response to diseases. Different types of HLA haplotypes have been identified, which are associated with the protection or susceptibility to autoimmune diseases (Janeway et al. 2001). In this study we use humanized, transgenic mice models that lack all murine MHC class II and solely express human MHC class II in the form of HLADQ8 or DR3-DQ2.5; for simplicity I refer to these HLA class II molecules as MHC class II molecules throughout this thesis.

1.5 MHC class II-mediated antigen processing and presentation

MHC class II proteins are constitutively expressed on the surfaces of professional APCs, including B cells, DCs, and macrophages (A. M. I. Mowat 2003b)(A. M. Mowat and Agace 2014)(Roche and Furuta 2015). Initially, professional APCs take up antigenic peptides into endocytic pathways through either phagocytosis or receptor-mediated endocytosis. The large peptides are then metabolized into sequences that are presented by MHC class II molecules to $CD4^+$ T cells via a tightly regulated process that requires the expression of various accessory molecules (Tortorella et al. 2003). As mentioned in section 1.4, MHC class II molecules are heterodimers of two transmembrane polypeptides, α- and β-chain. MHC class II α and β chains are assembled in the endoplasmic reticulum (ER) into a heterotrimeric complex with a third polypeptide known as the invariant chain (li), which is a chaperone that hinders the binding of peptides into MH the ER (Lamb and Cresswell 1992)(Roche and Cresswell 1991). As such, a portion of li fills the peptide-

binding site of nascent MHC class II molecules. The MHC class II-li complex then traffic through the endosomal pathway and into a low-pH, late-stage endosomal compartment referred to as the MHC II compartment (MIIC)(Jones' et al. 1979)(Neefjes et al. 2011). Within MIIC, proteases selectively degrade li into a shorter fragment called class-II associated invariant-chain peptide (CLIP) (Neefjes et al. 2011)(Roche and Cresswell 1991)(Blum and Cresswell 1988), which temporarily fills the peptide-binding site of MHC class II molecules. Subsequently, a non-classical MHC class II protein known as HLA-DM catalyzes the dissociation of CLIP. Simultaneously, peptides produced from endocytosis or phagocytosis of extracellular pathogens are delivered to MIIC, which contains nascent MHC class II molecules. The removal of the CLIP facilitates the binding of high-affinity peptides to the peptide-binding site of MHC class II molecules (Mellins and Stern 2014)(Denzin and Cresswell 1995) (Sloan et al. 1995). In a subset of APCs such as B cells, certain DCs, and thymic epithelial cells, HLA-DM activity is inhibited by HLA-DO, which competitively binds to HLA-DM and inhibits antigen presentation by MHC class II. Once the peptide-MHC class II complex is formed, MHC class II leaves the MIIC in vesicles that transport it to the cell surface of the APCs. These peptide-MHC class II complex can then be recognized by antigen-specific $CD4^+$ T cells (Figure 1.3) (Sloan et al. 1995)(Sherman, Weber, and Jensen 1995)(Denzin and Cresswell 1995)(Lamb and Cresswell 1992). Of note, the balance between HLA-DM and HLA-DO is controlled by IFN-γ, which induces the expression of HLA-DM but not HLA-DO. During infection, the increased release of IFN-γ upregulates the expression of HLA-DM compared with HLA-DO, thus enhancing the presentation of the antigenic peptides-MHC class II complex to $CD4^+$ T cells.

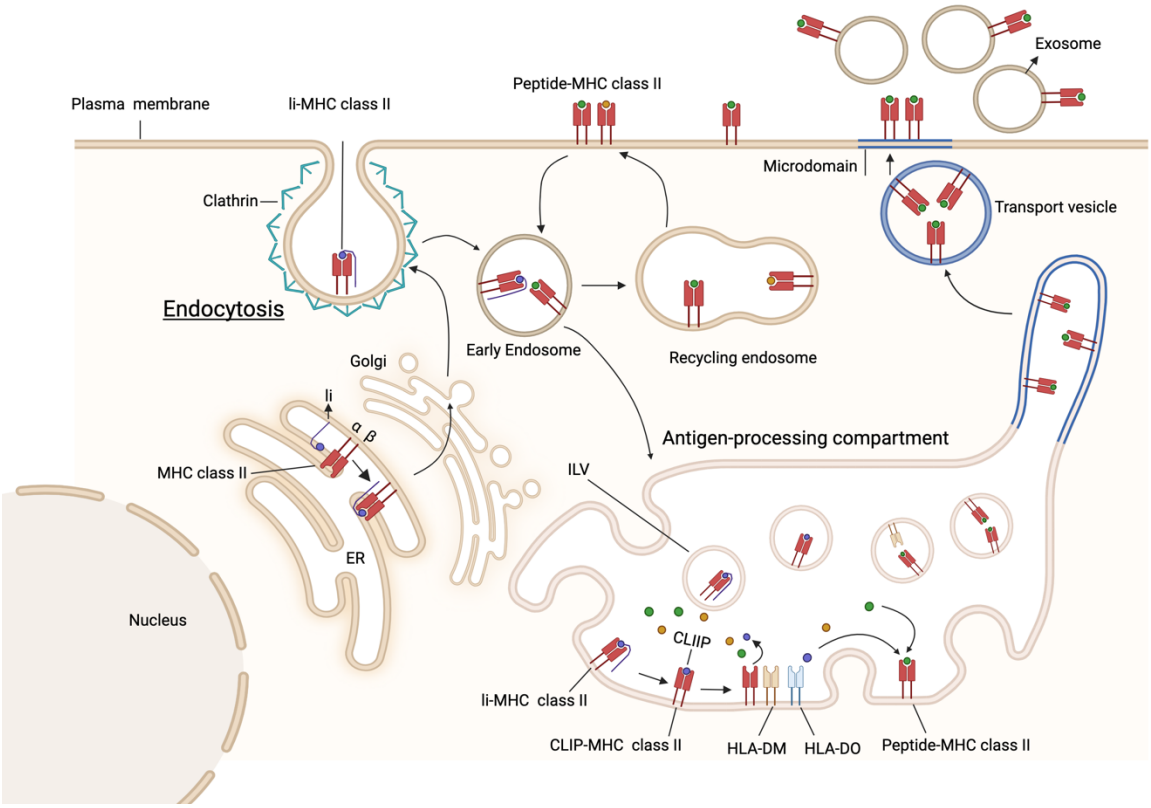


Figure 1.3 Overview of the MHC class II processing and presentation pathways. The MHC class II processing and presentation pathways involve multiple steps. Newly synthesized MHC class II molecules in the endoplasmic reticulum (ER) associate with the invariant chain (li) to prevent premature peptide binding. These li-MHC class II complexes then travel through the Golgi apparatus to the cell surface, where they are internalized via Clathrin-mediated endocytosis. Following internalization, complexes reach multivesicular antigen-processing compartments, with some entering intraluminal vesicles (ILVs) of multivesicular bodies (MVBs). Here, li is cleaved by acid proteases, resulting in the retention of only a short peptide, referred to as the class II-associated invariant chain peptide (CLIP), on the peptide-binding groove. CLIP is eventually displaced by the enzyme, HLA-DM, facilitating the binding of antigenic peptides. HLA-DM function is modulated by HLA-DO. Peptide:MHC class II complexes within the ILVs merge with an MVB membrane and move to the plasma membrane. When an MVB fuses completely with the plasma membrane, it releases LVs as exosomes. Once at the cell surface, these

peptide:MHC class II complexes may be internalized again and recycled, continuing their role in antigen presentation. Image created with. Biorenders.com

1.5.1 Expression of MHC class II in IECs

Epithelial cells express MHC class II molecules, which are preferentially expressed in the small intestine (L. Mayer 2000). MHC class II molecules have been identified throughout the small intestine (Chiba, Iizuka, and Masamune 1988)(Parr and McKenzie 1979)(Wiman et al. 1978)(Graham Mayrhofer, Pugh, and Barclay 1983). IECs constitutively express MHC class II, li, and HLA-DM at homeostasis (Chiba, Iizuka, and Masamune 1988)(MacDonald, Weinel, and Spencer 1988)(Byrne et al. 2002)(R. M. Hershberg et al., 1997)(R. M. Hershberg et al., 1998)(Scott et al., 1980)(Madrigal et al. 1993)(Hundorfean et al., 2007)(Lin, Almqvist, and Telemo 2005)(Cheminay, Möhlenbrink, and Hensel 2005). This highlights that IECs possess the essential prerequisites to function as non-conventional APCs capable of activating $CD4^+$ T cells (Arnaud-battandier et al. 1986)(Gorvel et al. 1985)(Scott et al. 1980)(Wiman et al. 1978)(Parr and McKenzie 1979)(Madrigal et al. 1993). Although the expression of MHC class II has not been detected in the colonic epithelium during homeostasis, this expression has been detected in biopsies from CeD patients (Kelly et al., 1988b)(Fais et al. 1992) (Arnaud-battandier et al. 1986)(Sarles et al. 1987), IBD (Dotan et al. 2007)(Momburg et al. 1988)(Koretz et al. 1987) and graft versus host patients (Koyama et al. 2019b). In response to pro-inflammatory cues, particularly IFN-γ, MHC class II expression is upregulated in the epithelial cells. For example, enhanced levels of tissue IFN-γ in IBD have been associated with an increased expression of MHC class II in IECs (Niessner and

Volk 1995). The most dominant MHC class II isotype expressed in IECs is HLA-DR, followed by HLA-DP, and HLA-DQ is the least expressed isotype in IECs (Madrigal et al. 1993).

Most studies confirming the expression of MHC class II on IECs have been performed in immortalized cell lines. These *in vitro* studies offer a controlled environment to evaluate the effects of the antigen type, exposure route, and dose. Studies using HT29 and T84 cell lines have demonstrated that colonic epithelial cells can induce the expression of MHC class II, HLA-DM, and Ii in the presence of the pro-inflammatory cytokine IFN-γ (R. M. Hershberg et al., 1997). In addition, in the presence of high concentrations of antigens, these cell lines were able to process and present antigens from the apical surface of colonic IECs and stimulate T cell hybridomas without requiring additional stimulation cues (R. M. Hershberg et al., 1997). Of note, T-cell hybridomas do not require co-stimulation, resembling the reduced co-stimulatory requirements of antigen-experienced memory cells in the gut (Croft, Bradley, and Swain 1994)(London, Lodge, and Abbas 2000). Another study showed that transfection of T84 cell lines with class II transactivator (CIITA), which recapitulated proinflammatory stimulation, promoted the uptake and processing of antigens from both the basolateral and apical surfaces. These findings suggest the antigen processing and presentation abilities of IECs can be influenced by various factors, including the presence of inflammation-related stimuli and antigen concentration. Although the expression of MHC class II in IECs has been reported predominantly in enterocytes in the upper villus, a recent study suggested the expression of MHC class II in two subsets of ISCs at the bottom of the small intestinal crypt under homeostatic conditions (Biton et al.

2018). These *in vitro* studies have significant limitations: commonly used cell lines, such as HT29 and T84 are derived from the colon and represent epithelial antigen presentation during inflammation rather than in a non-diseased state because MHC class II molecules are not expressed on colonic IECs under steady-state and healthy conditions and are solely expressed during inflammation and disease states. However, the expression of MHC class II has been induced in both small intestinal and colonic IECs under inflammatory condition such as CeD (Fais et al. 1992)(K.-P. Zimmer et al. 1995)(Arnaud-battandier et al. 1986)(Kelly et al., 1988a), inflammatory bowel disease (Dotan et al. 2007)(Hirata et al. 1986)(Bär et al. 2012)(Smillie et al., 2019a), and graft-versus-host disease (GvHD) (Koyama et al. 2019b), and as well as during infections with *Heligmosomoides polygyrus* or *Salmonella enterica* (Biton et al. 2018). These studies shed light on the dynamic nature of MHC class II expression by IECs and their potential implications for immune responses and inflammatory conditions.

Furthermore, the localization of epithelial MHC class II varies based on different studies. In human colonic cell lines transfected with MHC class II, this molecule was mainly expressed on the basolateral side of (R. M. Hershberg & Mayer, 2000). Studies on human and rat small IECs revealed a more complex distribution pattern in which MHC class II was detected not only at the basolateral site but also at the lateral and apical sites of IECs, as well as within intracellular vesicular structures (Hundorfean et al. 2007)(Hirata et al. 1986)(G Mayrhofer and Spargo 1990). Multiple studies have characterized the localization of MHC class II molecules within IECs, utilizing endoscopic biopsies from both healthy individuals and patients with IBD, as well as human small intestinal organoids

(Hundorfean et al. 2007)(Bär et al. 2012). Additionally, investigations using human small intestinal organoids have provided valuable insights. In the healthy human gut, most MHC class II molecules are observed within the late endosomal structure known as multivesicular bodies (MVB). However, there was also very low expression of MHC class II molecules at the basolateral cell surface, while the expression at the apical surface was limited (Hundorfean et al. 2007)(Bär et al. 2012). In addition, the localization of MHC class II in the late endosomal structure has also been observed in human small intestinal organoids (Bär et al. 2012). In inflamed biopsy samples from patients with CD and UC, it has been demonstrated that the expression of MHC class II increases at the basolateral membrane, accompanied by a decrease in MHC class II expression within MVBs (Bär et al. 2012). These findings support the idea that, during inflammation, MHC class II molecules may be transported from MVBs to the basolateral site, potentially enabling IECs to present antigens to $CD4^+$ T cells in the lamina propria under such conditions. These studies demonstrate the dynamic and context-dependent localization of MHC class II molecules within IECs and provide insights into their involvement in antigen presentation and immune responses within the intestinal mucosa.

Some studies have focused on the *in vivo* evidence of the role of IECs as APCs. In one study, mice infected with bacteria and receiving adoptive transfer of naïve $CD4^+$ T cells were used to compare the ability of IECs and DCs to mediate colitis (Maggio-Price et al. 2013). The results showed that DCs were sufficient to drive colitis, whereas mice with MHC class II exclusively expressed on IECs developed only mild inflammation, possibly because of reduced co-stimulatory signals on IECs (Maggio-Price et al. 2013). Another

mouse colitis model demonstrated that selectively knocking out MHC class II in IECs worsened colitis in mice, resulting in the secretion of higher levels of IFN-γ and decreased levels of Treg cells (Thelemann et al. 2014).

In the context of CeD, stimulation of tissue explants with gliadin (one type of gluten protein) obtained from patients with active CeD induced the upregulation of MHC class II (Fais et al. 1992)(K. P. Zimmer et al. 1995). These alterations were influenced by the active phase of CeD, as there was no difference in the level of epithelial MHC class II expression between CeD patients in remission and non-CeD controls (Arnaud-battandier et al. 1986). Furthermore, a comprehensive analysis of IECs in CeD patients using a genome-wide hybridization bead array analysis revealed the upregulation of 25 defense-related genes during disease, including genes involved in antigen presentation, such as HLA-DOB, HLA-DMB, and CIITA (Pietz et al. 2017). Notably, HLA-DOB levels were also upregulated in treated patients with CeD. These findings suggest that IECs may play an underestimated role in CeD pathogenesis.

In recent years, with the development of organoid culture systems, studies have emerged that evaluate the expression of MHC class II molecules in intestinal organoids and provided valuable insights. However, the functional consequences of MHC class II expression in IECs have been very controversial across different studies. Some studies suggest that MHC class II expression in IECs plays a protective role by enhancing regulatory responses, whereas others indicate a role in promoting effector functions and proinflammatory responses. Despite these conflicting outcomes, which are mostly contextual, there is consensus that epithelial MHC class II molecules can serve as non-

professional APCs. For example, epithelial MHC class II expression exacerbates colitis severity in a *Citrobacter rodentium* infection model of colitis in mice (Jamwal et al. 2020) and contributes to the lethality of GvHD (Koyama et al. 2019b). Conversely, other studies have demonstrated a protective role of IFN-γ-dependent epithelial MHC class II expression in models of colitis induced by DSS and *C. rodentium,* which modulates the development of pathogen-specific $CD4^+$ T cell responses (Malik et al., 2023). Consistent with this, a recent study revealed that the expression of MHC class II in IECs dampened the effector functions of *Heliobacter hepaticus*-specific $CD4^+$ T cells in mice infected with *H. hepaticus* and adoptively transferred with bacteria-specific $CD4^+$ T cells (C. E. Heuberger et al., 2023). In addition, epithelial MHC class II expression mediated by histone deacetylase 3 (HDAC3) promotes the accumulation of commensal-specific Treg cells while reducing the number of commensal-specific $CD4^+$ Th17 cells, thereby protecting against microbiota-specific inflammation (Eshleman et al., 2023).These findings imply that IECs, through the expression of MHC class II molecules, may contribute to $CD4^+$ T cell-mediated adaptive immune responses. Moreover, the expression of MHC class II in IECs follows a diurnal expression pattern and is partly regulated by the circadian clock and dietary intake rhythmicity (Tuganbaev et al. 2020), as well as by diet composition, including a high-fat diet, which has been shown to reduce MHC class II expression in IECs (Tuganbaev et al. 2020)(Beyaz et al., 2021a). Notably, a high-fat diet has been linked to promote intestinal tumor initiation driven by the loss of epithelial MHC class II expression, particularly in malignant ISCs (Beyaz et al., 2021a). Additionally, the expression of MHC class II on two specific ISC subsets has been implicated in determining their fate, which is mediated by

interactions between these cells and lamina propria Treg or Teff $CD4^+$ cell populations or their corresponding signature cytokines (Treg: IL10 or Teff: Th1; FN-γ, Th17: IL17A and Th2; IL13), influencing whether ISCs undergo renewal and maintain an ISC niche or proceed to differentiation (Biton et al. 2018).

However, the exact impact of epithelial MHC class II expression on T-cell responses, as well as the underlying mechanisms governing its expression and function in the context of CeD, remain poorly defined, and further investigation is needed to elucidate these mechanisms.

1.5.2 Co-stimulatory molecule expression in IECs

Co-stimulatory molecules are essential in addition to MHC class II and their ligation to the TCR to fine-tune the function and ability of APCs to effectively activate or inhibit the TCR (L. Chen and Flies 2013). Conventional co-stimulatory molecules on APCs that are required for the activation of naïve T cells include, CD86, CD80, and CD40. The first two interact with CD28 on T cells, while the latter interacts with the CD40 ligand (CD40L). The increased expression of CD80 and CD86 in professional APCs has been associated with exposure to DAMP or PAMP, partly hampering T cell stimulation to the level of tissue damage or infection (Hemmi et al. 2000). Insufficient expression of co-stimulatory molecules upon T cell recognition of the MHC-peptide complex leads T cells to an anergic, hyporesponsive state, and induces tolerance (Alegre, Frauwirth, and Thompson 2001)(Greenwald, Freeman, and Sharpe 2005)(Schwartz 2003). The expression of co-stimulatory molecules by IECs is a subject of debate, as conflicting results have been

reported. Some studies have reported the expression of CD80, CD86, and CD40 in IECs during homeostasis (Nakazawa et al. 1999)(Byrne et al. 2002)(Sanderson et al. 1993). Other studies have associated the expression of these molecules in IECs with tissue inflammation. The selective expression of CD86 and CD40 on IECs was detected during inflammation associated with IBD (Cayabyab, Phillips, and Lanier 1994) as well as in colonic cell lines following stimulation with IFN-γ (Borcherding et al. 2010)(Nakazawa et al. 1999)(Bloom, Simmons, and Jewell 1995). Recently, CD80 expression was shown to be upregulated in IECs during GvHD (Koyama et al. 2019b).

Additionally, APCs also express other co-stimulatory molecules, including inducible co-stimulatory ligand (ICOS-L) and programmed death ligand 1 (PD-L1) (Keir et al. 2008). The expression of the former molecule on APCs leads to the activation of effector $CD4^+$ T cells, whereas the latter promotes the suppression of $CD4^+$ T cell effector function (Sharpe 2009)(Crawford and Wherry 2009)(Keir et al. 2008)(Van Seventer et al. 1990). Thus, the expression of an array of co-stimulatory molecules adjusts the interaction of APCs with $CD4^+$ T cells and gives APCs the capacity to induce the activation or inhibition of $CD4^+$ T cells according to the immunological context. It has been shown that IECs express ICOS-L and PD-L1 molecules (Sharpe 2009)(Ponce de León et al. 2019), the ability of IECs to provide sufficient co-stimulatory signals to activate naïve $CD4^+$ T cells remain inconclusive. Of note, effector T cell reactivation relies less on co-stimulation molecules, and since the intestinal epithelium is predominantly populated with activated effector and regulatory T cell phenotypes, this would rationalize the potential role of IECs as non-conventional APCs, capable of activating effector, but not naïve $CD4^+$ T cells that

reside in the LP (Nakazawa et al. 1999)(Sanderson et al. 1993)(Croft, Bradley, and Swain 1994)(L. Chen and Flies 2013).

1.5.3 Modulation of MHC class II expression in IECs

The expression of MHC class II molecules is closely modulated by a transcriptional coactivator known as the CIITA (Reith, LeibundGut-Landmann, and Waldburger 2005). CIITA is regulated by three independent promoters: pI, pIII, and pIV (J. M. Waldburger et al. 2000). The expression of pI and pIII has been detected in professional APCs, whereas pIV has primarily been identified in non-hematopoietic cells, such as IECs, in response to IFN-γ (J.-M. Waldburger et al. 2001). In the intestine, IFN-γ can be released from various immune cells, including ILC1s, NK, γδT cells, $CD8^+$ αβ T cells, and specific subsets of $CD4^+$ αβ T cells (Vivier et al. 2008)(Fuchs et al. 2013)(Ferrick et al. 1995). It has been shown that upon adoptive transfer of $CD4^+$ T cells into T cell-deficient mice, the expression of MHC class II on IECs significantly increased, whereas the transfer of IFN-γ-knockout T cells did not elicit the same response (Thelemann et al. 2014). IL-27 is another cytokine that may induce the expression of MHC class II molecules in non-hematopoietic cells. The former belongs to the IL-12 superfamily of cytokines and is secreted by activated DCs especially during intestinal inflammation (Pflanz et al. 2002). It has been found that IL-27 increases the expression of CIITA in colorectal cancer cells and induces the expression of HLA-DQ, -DR, -DP, -DM, and of invariant chain in endothelial cells (Diegelmann et al. 2012)(Feng et al. 2007). The corresponding effect of IL-27 and IFN-γ on MHC class II

expression can be attributed to the fact that both cytokines promote Th1 $CD4^+$ cell responses (Hunter 2005).

Moreover, the microbiome modulates the expression of MHC class II molecules. Commensal bacteria contribute to intestinal immune system development, which was demonstrated in germ-free mice that have under-developed lymphoid structures in the small intestine (Lorenz et al. 2003) and no MHC class II expression in IECs. However, microbial colonization of germ-free mice promoted γδ T cells to release of IFN-γ, leading to induction of CIITA and MHC class II expression in IECs (Matsumoto et al. 1999)(Matsumoto et al. 1995)(Komano et al. 1995). Specific classes of commensal bacteria, such as segmented filamentous bacteria, can induce MHC class II expression in IECs (Matsumoto et al. 1995). Although it has been shown that colonic epithelium lacks MHC class II expression during non-inflammatory conditions, a recent study showed expression of MHC class II in the colon of specific pathogen free (SPF) mice suggesting presence of pathobionts that drive low-level inflammation can increase MHC class II expression in the colon (Eshleman et al. 2023). This study further revealed that the loss of MHC class II expression in IECs led to increased production of bacteria-specific $CD4^+$ T cells in the LP of the small intestine or colon of mice following colonization with SPF microbiota (Eshleman et al. 2023). These findings suggest that MHC class II in IECs plays a role in preventing commensal microbe-specific T cells under non-inflammatory conditions. Future investigations should address the role of epithelial MHC class II molecules in shaping the microbiome.

Intestinal inflammation can also modulate the expression of the epithelial MHC class II molecules. As mentioned above, different diseases, including CeD, IBD, and graft-

versus-host disease, have been associated with an increase in epithelial MHC class II levels (Kelly et al., 1988a)(Chiba, Iizuka, and Masamune 1988)(Dotan et al. 2007)(Lloyd Mayer et al. 1991)(Mason, Dallman, and Barclay 1981). During homeostasis, epithelial MHC class II is absent from the colon and crypt compartments, while at disease state, its expression is detected (Dotan et al. 2007), which can be attributed to an increased level of IFN-γ during disease (Niessner and Volk 1995). In addition, the change in MHC class II expression is dependent on disease status; for example, the expression of MHC class II in IECs of CeD patients in remission is comparable to that of non-celiac controls (Arnaud-battandier et al. 1986). MHC class II localization can also be altered by intestinal inflammation. For example, in patients with IBD, epithelial MHC class II expression is relocated from the multivesicular bodies (late endosomes) to the basolateral site (Sim et al. 2013). Therefore, in this thesis, I have focused on highlighting the conditions leading to the expression of MHC class II in IECs in the context of CeD and whether MHC class II-expressing IEC have the capacity to activate $CD4^+$ T cells.

1.6 Oral tolerance: The mechanism by which dysregulated immune responses to dietary and microbiota antigens are prevented

The mucosal surface of the intestine is colonized by a large community of resident microbes, collectively called the microbiota, and is continuously exposed to exogenous and transit antigens from dietary and environmental sources, which may include pathogens. The mucosal immune system has coevolved with these antigens and thus has adopted local and systemic mechanisms to not react to them and is immunologically referred to as “oral

tolerance" (Rezende and Weiner 2017)(Pabst and Mowat 2012). Oral tolerance is a state in which the immune system is programmed to develop systemic ignorance and mucosal tolerance to the oral antigens. Upon disruption of this mechanism, an inflammatory immune response to luminal antigens can occur (MacPherson and Uhr 2004). The mechanisms involved in inducing oral tolerance include clonal anergy, deletion of antigen-specific T cells, and induction of Treg cells. Exposure to high doses of orally administered antigens results in antigen-specific $CD4^+$ T cells becoming anergic and clonally deleted (Friedman and Weiner 1994)(Y. Chen et al. 1995). In contrast, repeated exposure to low doses of antigens appears to induce the generation of antigen-specific $FoxP3^+$ regulatory $CD4^+$ T cells as their dominant mechanism, in which subsequent challenges with these antigens promote Treg cells to release anti-inflammatory cytokines IL-10 and TGFβ, leading to immune suppression (Curotto de Lafaille and Lafaille 2009)(Mucida et al. 2007). Activation of Treg cells is induced by $CD103^+$ DCs when they take up oral antigens in the small intestine and traffic to the MLNs to present the antigen to naïve $CD4^+$ T cells and induce Treg cells. In return, Treg cells increase the expression of gut-homing molecules and relocate to the LP, where they induce tolerance (Hadis et al. 2011). In addition, the differentiation of naïve $CD4^+$ T cells to Treg cells and the increased expression of gut-homing molecules are retinoic acid-dependent, which is abundant in the gut, explaining why this tolerance mechanism is associated with the gut (Iwata et al. 2004)(Sun et al. 2007)(Coombes et al. 2007). Thus, Treg cells are pivotal in suppressing inflammation, and the loss of oral tolerance to dietary antigens occurs upon Treg cell dysfunction (Dubois et al. 2010)(Bouziat et al. 2017)(Hadis et al. 2011)(Depaolo et al. 2011).

The mechanism underlying the loss of oral tolerance to luminal antigens is multifactorial and depends on the antigen dose, type, and genetic background of the host. Loss of oral tolerance leads to development of allergies or other food sensitivities, including CeD, a well-known model immune-mediated disease in which there is a loss of oral tolerance to the dietary protein gluten in genetically predisposed individuals. CeD is associated with the Th1 immune response and Treg cell dysfunction because inflammation continues despite the presence of Treg cells following gluten challenge (Cook et al. 2017)(Cook et al. 2020). It has been shown that environmental factors can lead to the loss of oral tolerance to dietary antigen. For example, it has been shown that viral infections in HLA-DQ8 mice at the time of oral antigen exposure, including infection with murine norovirus strain CW3 (Bouziat et al. 2018) or reovirus strain T1L (Bouziat et al. 2017), can induce the loss of oral tolerance and activate antigen-specific Th1 immune responses. Infection of HLA-DQ8 mice with different strains of the same virus, such as murine strain CR6 or reovirus T3D-RV, did not affect their tolerance to gluten. The loss of oral tolerance in this model has been attributed to the upregulation of interferon regulatory factor 1 (IRF1), which ultimately suppresses Treg function (Bouziat et al. 2018; 2017). In another study on the role of IL-15, which is elevated in the small intestine of most patients with CeD (Jabri and Abadie 2015)(Abadie and Jabri 2014)(Mention et al. 2003), conjugation with RA was explored to induce the loss of oral tolerance in susceptible DQ8-D^d-IL15 transgenic mice. Overexpression of the pro-inflammatory cytokine IL-15 in the MLNs and LP of mice was sufficient to induce loss of oral tolerance to gluten (Abadie and Jabri 2014)(Setty et al. 2015). The expression of IL-15 in the presence of RA alters the DCs to release of IL-12p70 and IL-23 cytokines, which

triggers the activation of IFN-γ-producing Th1 responses, leading to the loss of suppressive Treg cells (Depaolo et al. 2011). However, the mice used in this study overexpressed IL-15, making it difficult to investigate the environmental factors that can promote the loss of oral tolerance to antigens. In the following sections, we discuss CeD in more detail and discuss factors that contribute to the loss of oral tolerance in CeD.

1.7 Celiac Disease (CeD)

Finely tuned immunological mechanisms prevent adverse responses to food antigens under normal physiological conditions (Caminero, Meisel, et al. 2019). However, in celiac disease (CeD), which affects approximately 1% of the population worldwide, exposure to dietary gluten, drives $CD4^+$ T cell-mediated inflammation that, in conjunction with innate immune activation (cytotoxic IELs), leads to villous atrophy in the small intestine (Figure. 1.4) (Singh et al. 2018)(Lionetti et al. 2015)(Lebwohl and Rubio-Tapia 2021)(Caio et al. 2019)(Elena F. Verdu and Green 2024). The inflammatory response in CeD is derived by exposure to gluten, a group of proteins present in wheat, rye, and barley, in genetically susceptible individuals carrying CeD risk genes: HLA-DQ2 and/or HLA-DQ8 (E. F. Verdu & Green, 2024)(Ludvigsson et al. 2013)(Tye-Din 2018).

The major susceptibility genes, HLA-DQ2.5 and -DQ8, are necessary for CeD development, and the probability of developing CeD without these alleles is less than 1% (Figure 1.4) (Zhernakova et al. 2011)(Tack et al. 2010)(Trynka, Wijmenga, and van Heel 2010). Approximately 30-40% of the worldwide population expresses one or more CeD susceptibility genes, but only 3-4% of these individuals develop the disease, suggesting the

role of additional genetic or environmental factors must be important in the pathogenesis of the disease (Ludvigsson and Green 2014)(Kamboj and Oxentenko 2017). For example, using transgenic HLA-DQ2^{+} mice, it has been demonstrated that the CD4^{+} T cell response to gluten is tolerogenic in the absence of additional factors (Du Pré et al. 2011).

It has been suggested that bacterial and viral infections (Brown, Jabri, and Dermody 2018) (Kim et al., 2015) (Bouziat et al., 2017)(Tapia et al. 2021)(Kemppainen et al. 2017)(Kahrs et al. 2019)(Stene et al., 2006a)(H. J. Galipeau et al. 2024), in conjugation with high-dose gluten exposure (Andrén Aronsson et al. 2016)(Andrén Aronsson et al. 2019)(Mårild et al. 2019), could potentially break oral tolerance to gluten and contribute to CeD pathogenesis. However, recent studies have shown the method of delivery (C-section or vaginal)(Koletzko et al. 2018)(Lionetti et al. 2017), breastfeeding, and the timing and method of gluten introduction (Lionetti et al. 2014)(Vriezinga et al. 2014) into the infant's diet do not affect the risk of CeD development. Moreover, non-gluten wheat proteins, such as amylase trypsin inhibitors (ATIs), have been implicated as potential co-factors in CeD. ATIs can trigger innate immune responses by activating the TLR-4 on intestinal myeloid cells, thereby inducing the inflammatory response to gluten or contributing to intestinal barrier dysfunction (Caminero, Mccarville, Zevallos, et al. 2019)(Junker et al. 2012). Lastly, variations in the composition of the gut microbiota have been reported in genetically susceptible children who develop CeD compared to those who remain healthy (Olivares, Walker, et al. 2018)(Leonard et al. 2020)(Olivares, Benítez-Páez, et al. 2018). Consequently, in recent years the bacterial components of the gut microbiota have been the focus of many studies as modulators of CeD pathogenesis (H. J. Galipeau et al. 2024)(Elena

F. Verdu and Schuppan 2021). In section 1.7.6, I will discuss recent advances in unraveling microbial-mediated mechanisms, which are still unclear and could potentially influence CeD development.

Altogether, to develop CeD, an individual must ingest gluten, the primary driver in CeD, and must possess specific genetic markers, specifically the HLA-DQ2.5 and/or HLA-DQ8 alleles as well as get exposed to an environmental trigger that is necessary to initiate the disease. Each of these essential elements of CeD development will be elaborated on in the following sections.

1.7.1 Diagnosis, clinical characteristics, and treatment of CeD

The mucosal pathology of CeD is localized in the proximal small intestine and is characterized by various degrees of gluten-dependent small intestinal villous atrophy, crypt hyperplasia, and intraepithelial lymphocytosis (Rostom, Murray, and Kagnoff 2006)(Fasano and Catassi 2012)(Lindfors et al. 2019). The development of CeD lesions follows a pattern described as Marsh stages (Oberhuber 2000)(Marsh and Crowe 1995). Marsh 0 is the normal state, followed by Marsh 1 which is characterized by an increased in number of IELs, without crypt hyperplasia or villous blunting (Oberhuber 2000). Marsh 2 is characterized by crypt hyperplasia without villous blunting. Marsh 3 is divided into three subcategories namely, Marsh3a, Marsh 3b, and Marsh 3c, which are categorized by the degree of villous shortening that increases from Marsh 3a to Marsh 3c, with total loss of villi in Marsh 3c (Oberhuber 2000). CeD in adults is typically diagnosed based on the presence of immunoglobulin (Ig)A and IgG antibodies to tissue transglutaminase 2 (TG2)

and IgG antibodies to deamidated gluten peptide (DGP) in serum, followed by a confirmatory biopsy of the duodenum showing villus blunting (atrophic enteropathy; >Marsh 3)(Rostom, Murray, and Kagnoff 2006)(W. Dieterich et al. 1998)(Walburga Dieterich et al. 1997)(Ludvigsson et al. 2014). Determining the serum level of IgG DGP antibodies is a more appropriate serological marker for individuals with IgA-deficiency (Husby et al. 2012)(Mozo et al. 2012)(Mesin, Sollid, and Di Niro 2012)(Walburga Dieterich et al. 1997). The diagnosis in children relies only on the detection of elevated serum antibody levels, avoiding the need for biopsy-obtaining endoscopy procedures (Husby et al. 2012).

Patients with CeD develop non-specific gastrointestinal symptoms, including abdominal pain, bloating, nausea, malabsorption, and chronic diarrhea (Murray et al. 2004)(Ludvigsson et al. 2013)(Fasano and Catassi 2012)(Therrien, Kelly, and Silvester 2020)(Mearns et al. 2019). Additionally, CeD patients can also experience extraintestinal symptoms, such as dermatitis herpetiformis, osteoporosis, anemia, sterility, iron deficiency, weight loss and neurological disorders, to name a few (Antonio Di Sabatino et al. 2006)(Stefanelli et al. 2020)(Reunala, Hervonen, and Salmi 2021)(Tack et al. 2010)(Kamboj and Oxentenko 2017). Symptoms and pathology do not correlate with CeD, as some patients with CeD experience full atrophy but do not experience any symptoms (asymptomatic patients) (M. Ines Pinto-Sanchez et al. 2021)(Rutz et al. 2002)(Lindfors et al. 2019). Another subpopulation of CeD patients referred to as “potential” CeD patients have TG2 and DGP antibodies in their serum, but do not develop small intestinal villous atrophy (Setty et al. 2015). These individuals may develop an active state of CeD later in

life; however, the factors that lead to disease progression remain unknown. This makes the diagnosis of CeD difficult and leads to long-term complications owing to poorly controlled disease (Catassi, Bearzi, and Holmes 2005)(Kurppa et al. 2024).

The only currently available treatment for CeD is adherence to a strict, life-long, gluten-free diet (GFD), which can reverse intestinal morphology in most patients, normalize antibodies, and alleviate symptoms, especially when diagnosis occurs in childhood (Figure 1.4) (Rubio-Tapia et al. 2010)(M. Ines Pinto-Sanchez et al. 2024). However, a substantial proportion of adult patients with CeD (~34%) remain symptomatic following GFD (Tack et al. 2010)(Green and Jabri 2003b)(Gatti et al. 2024). The main reason for persistent symptoms is that patients cannot remove gluten from their diets and that a small amount (<50mg) of accidental contamination can induce inflammation (Catassi et al. 2007). Patients who do not comply with a GFD, voluntarily or accidentally, can develop complications, such as micronutrient deficiencies and bone fractures, and are at an increased risk for certain cancers (Maria I. Pinto-Sanchez and Bai 2019)(Bozorg et al. 2024). One of the most severe complications of poorly controlled CeD is refractory CeD (RCD), which affects approximately 5% of patients with CeD who do not respond to a strict GFD and continue to experience intestinal damage despite restoring normal levels of anti-TG2 antibody (Rubio-Tapia et al. 2009)(Kaukinen et al. 2007)(Malamut et al. 2024). RCD classified into two types: RCDI, which typically has a benign course (with polyclonal IELs), and RCDII, which is considered a precancerous condition (with monoclonal IELs that have lost the surface expression of CD3 marker) that can progress to jejunitis and, in some cases, to enteropathy-associated T-cell lymphoma, a disease with a high mortality

rate (Tack et al. 2010)(Green and Cellier 2007)(Raiteri et al. 2022)(Rubio-Tapia and Murray 2010)(Malamut et al. 2024). Therefore, the difficulty of adhering to a lifelong GFD and the prevalence of symptomatic individuals highlights the need for the development of better therapies. More importantly CeD patients want a "pill" to help them prevent accidental contaminations and improve their social life (Catassi et al. 2022)(Discepolo et al. 2024). Many potential therapeutic strategies are currently being tested in clinical trials for the treatment of CeD (Robert P. Anderson, Verma, and Schumann 2024)(Discepolo et al. 2024). Preclinical *in vitro* and animal models relevant for understanding CeD pathogenesis would significantly accelerate the process of moving these candidates into approved therapies (Plugis and Khosla 2015)(Tye-Din et al., 2018).

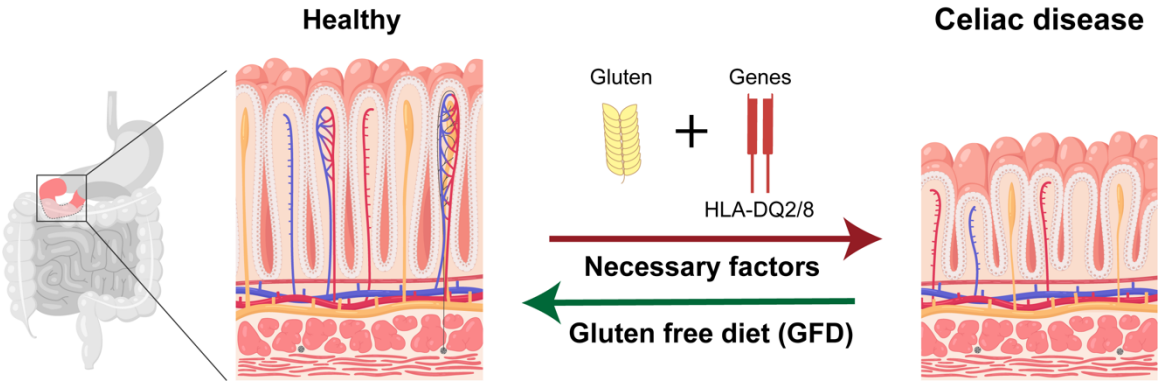


Figure 1.4 Necessary factors for celiac disease development. Gluten proteins and related prolamins present in wheat, barley, and rye are the driving antigens responsible for CeD development. The combined presence of these proteins and the major histocompatibility complex (MHC) class II genes, HLA-DQ2 and HLA-DQ8, are necessary factors for the development of CeD However, additional co-factors including gut microbes and their metabolites, as well as bacterial and viral infections are required.

1.7.2 The main environmental driver: Gluten

Gluten, a storage group of grain proteins, belongs to the prolamin family found in wheat, barley, and rye (Wieser 2007)(Biesiekierski 2017)(Daly et al. 2020)(Frits Koning 2005)(Bromilow 2017). In wheat, gluten accounts for up to 90% of the total protein content (Biesiekierski 2017). Wheat gluten, a well-characterized protein, is composed of two structural proteins, gliadin and glutenin (Balakireva and Zamyatnin 2016)(Wieser 2007). Gliadin is comprised of smaller proteins, including α-, β-, γ-, and ω-gliadins, while glutenin can be categorize into high- and low-molecular weight glutenin (Balakireva and Zamyatnin 2016). Gluten in barley and rye are known as hordeins and secalins, respectively (Dahal-Koirala et al. 2020)(W. A. Wahab et al., 2016)(Van De Wal et al., 1999)(Hardy et al., 2020). α-gliadin is the best characterized protein of gluten and is rich in glutamine and proline residues, making it proteolytically resistant (Shan et al., 2002). The breakdown of α-gliadin yields a peptide sequence known as "33-mer," which is 33 amino acids long. Since the discovery of the 33-mer within α-gliadin, it has become one of the key peptides in CeD studies, as it contains the 6 six copies of the three main immunogenic epitopes in HLA-DQ2^{+} patients with CeD (S.-W. Qiao et al. 2005)(Shan et al. 2002). The term gliadin used throughout this thesis refers to whole gliadin present in wheat.

1.7.3 The necessary genes: HLA association

Human leukocyte antigen (HLA) is a molecule that presents pathogenic- and self-derived peptides within antigen-binding pockets to T-cells (Sharon et al. 2016). HLA is the human counterpart of murine MHC, which are expressed on APCs and present antigens to

T cells (Dendrou et al. 2018)(Peter Parham 2021). In CeD, HLA genes, namely HLA-DQ2.5 and/or HLA-DQ8, are necessary but not sufficient to cause CeD development. CeD is known to have a multifactorial etiology, including both genetic (HLA and non-HLA loci) and environmental factors (pathogenic infection and dysbiosis) that can contribute to disease pathogenesis, similar to many chronic inflammatory diseases (Abadie et al. 2011)(Jabri and Sollid 2009)(Wacklin et al. 2013)(Di Biase et al., 2021)(Chibbar & Dieleman, 2019)(Di Biase, 2021, Chibbar, 2019). CeD has one of the strongest HLA associations compared with other HLA-associated autoimmune disease such as type 1 diabetes, rheumatoid arthritis, multiple sclerosis, and Goodpasture's disease (Dendrou et al., 2018)(Koning et al., 2015)(Seldin, 2015). Several genes within the HLA gene complex on chromosome 6 can be attributed to CeD development, which is primarily associated with alleles HLA-DQA (*DQA1*05:01*) and HLA-DQB (*DQB1*02:01*) alleles. These two alleles are carried on the DR3-DQ2.5 haplotype that encodes the heterodimer HLA-DQ2.5 molecules, which is expressed by the majority (~90%) of CeD patients (Sollid et al., 1989)(Tye-Din et al., 2018). Patients negative for HLA-DQ2.5 usually express HLA-DQ8 (~5%) (encoded by the alleles DQA1*03:01/DQB1*03:02) or HLA-DQ2.2 (~5%) (encoded by the alleles DQA1*02:01/DQB1*02:02) and confers low risk to develop CeD (Lundin et al. 1994)(Kagnoff 2007)(Fallang et al. 2009)(Petersen et al. 2015)(Ting et al. 2020)(Kooy-Winkelaar et al. 2011). Individuals who are homozygote for HLA-DQ2.5 have an increased risk of developing CeD compared with individuals who are heterozygous for HLA-DQ2.5, implying a gene-dosage effect in CeD development, which may impact

disease severity in patients (Karell et al. 2003)(Kuja-Halkola et al. 2016)(Murray et al., 2007)(Ploski et al., 1993).

Apart from the HLA genes, non-HLA genes also play a role in the genetic risk of CeD. Through genome-wide association studies, 42 non-HLA loci implicated in CeD have been identified, mainly involving single nucleotide polymorphisms (Ricaño-Ponce et al., 2020)(Withoff et al., 2016). The collective contribution of these non-HLA genes loci to the overall risk of developing CeD is lower compared with HLA genes alone (Withoff et al., 2016). However, these genetic factors also play a role in regulating different aspects of the immune system and barrier function. Thus, they have the potential to modify the way CeD manifests and influences its phenotype (Cerqueira et al., 2021).

Immune activation in CeD is preferentially initiated following the interaction of gluten peptides with TG2, a key autoantigen in CeD (Abadie et al., 2011b). TG2, an enzyme expressed intracellularly in various cell types, is secreted into the extracellular space, either the lamina propria or the gut lumen (Molberg et al., 1998) by shedding enterocytes (Iversen et al., 2020) during inflammation and mechanical stress to the tissue. Once released, TG2 is activated by elevated extracellular Ca^{2+} levels, facilitating tissue stabilization by cross-linking extracellular matrix proteins. In the context of CeD, native gluten peptide is the substrate for TG2, in which TG2 facilitates the cross-linking of glutamine-rich gluten peptides to extracellular matrix proteins or to itself or to undergo deamidation process by converting gluten peptide glutamine residues to negatively charged glutamic acid (Molberg et al., 1998)(van de Wal et al., 1998) . This deamidation process significantly enhances the affinity of negatively charged gluten peptide to bind to positively charged pocket of HLA-

DQ2 or HLA-DQ8 molecules on the surface of professional APCs and increase peptides' immunogenicity (C.-Y. Kim et al., 2004)(Henderson, Tye-Din, et al., 2007)(Lee, Wucherpfennig, and Wiley 2001). Essentially, HLA-DQ8 molecules preferentially bind to negatively charged glutamate residues at positively charged P1 and P9 pockets, while HLA-DQ2.5 and HLA-DQ2.2 have affinity to bind glutamate at their P4, P6, and P7 pockets (C.-Y. Kim et al. 2004)(Henderson, Tye-Din, et al. 2007)(Lee, Wucherpfennig, and Wiley 2001)(Henderson, Reid, et al. 2007)(Stepniak et al. 2008). Thus, the strong association of HLA-DQ2.5 with CeD development is because deamidated gluten peptides bind to HLA molecules with strong affinity and higher stability, which in turn provides sustained antigen presentation and enhances the frequency of pathogenic T cell recognition (S.-W. Qiao et al. 2005)(Fallang et al., 2009).

1.7.4 Adaptive immune response in CeD

The adaptive branch of the immune response plays an essential role in CeD pathogenesis, which has been well-characterized, in part because of the known nature of the driving antigen, gluten, and the role of TG2 as the main autoantigen. Central to this response are HLA-DQ2- or HLA-DQ8-restricted $CD4^+$ T cells, which are present in the intestinal mucosa and selectively bind to deamidated gluten peptides rather than native ones (Lundin et al., 1993)(Helene Arentz-Hansen et al., 2000)(van der Wal et al., 1998). This process leads to the expansion and differentiation of gluten-reactive $CD4^+$ T cells, which in conjunction with the formation of anti-TG2 and anti-DGP antibodies activates the

adaptive arm of CeD and derives the development of inflammatory celiac lesions (S. W. Qiao et al., 2012)(Lundin et al., 1994)(Nilsen et al., 1998).

Upon ingestion, gluten peptide is partially digested by the host and bacterial proteases in the lumen of the small intestine (Caminero, Meisel, et al., 2019). The partially digested gluten peptides then enter the lamina propria, possibly via CD71, a transferrin receptor that is overexpressed in untreated CeD patients and facilitates the transport of IgA-gluten complexes through the endocytic route with minimal degradation (Matysiak-Budnik et al. 2008)(Heyman and Menard 2009)(Lebreton et al. 2012) or by paracellular passage due to increased epithelial layer permeability caused by altered tight junctions (Lammers et al. 2008). In the lamina propria, the partially digested gluten peptide interacts with the transglutaminase 2 (TG2) enzyme, which mediates deamidation and transamidation of the gluten peptide. Gluten peptides, specifically T-cell epitopes, are among the preferred substrates for TG2 (Molberg et al. 1998)(Helene Arentz-Hansen et al. 2000)(van der Wal et al., 1998). TG2 enzyme is typically present in a catalytically inactive form in intestine (Siegel et al., 2008). However, in CeD, it is proposed that TG2 becomes activated by dietary gluten in conjunction with inflammatory signals, as demonstrated in IL-15 transgenic mouse model (Abadie et al., 2020)(Abadie et al., 2022). Recent *in vitro* studies have demonstrated that there is an increased expression of TG2 in enterocytes and its release into the intestinal lumen through the shedding of enterocytes could facilitate the interaction of TG2 with gluten-peptide (Iversen et al., 2020)(Amundsen et al., 2023).

Furthermore, TG2 enzyme targets specific glutamine residues of the gluten peptide and either converts them to negatively charged glutamate residues via hydrolysis

(deamidation) or crosslinks them to primary amines (transamidation) (Tollefsen et al., 2006)(Abadie et al., 2011a)(Sollid, 2017). The deamidation process generates immunogenic deamidated gluten peptides (DGPs) that have an enhanced binding affinity for MHC class II molecules in the form of HAL-DQ2.5, HLA-DQ2.2, and HLA-DQ8 expressed on the surface of APCs, including DCs, macrophages, plasma cells, and B cells (Tollefsen et al., 2006)(Abadie et al. 2011)(Ludvig M. Sollid 2017). The CeD lesions exhibit elevated levels of activated $CD11c^+$ conventional DCs and macrophages (A. C.R. Beitnes et al. 2011)(Ann Christin Røberg Beitnes et al. 2012)(Ciccocioppo et al. 2007). Within these lesions, $CD11c^+$ DCs expressing the HLA-DQ2 subset have shown to be more effective as APCs compared with HLA-$DQ2^+$ macrophages (Ráki et al. 2007)(Escudero-Hernández et al., 2020). Conversely, plasma cells, which have lower capacity for antigen presentation than DCs and macrophages, have been identified as the most prevalent APCs expressing HLA-DQ2.5-gluten in the active CeD duodenal mucosa (Di Niro et al., 2012)(Lindeman et al., 2021)(Høydahl et al., 2019). These plasma cells, although they express lower levels of MHC class II than macrophages and DCs, are critical for antigen presentation (Høydahl et al., 2019)(Bujko et al., 2018). Recent studies have indicated that B cells also act as APCs in CeD, as they have been observed to present TG2-DGP complexes through the hapten-carrier hypothesis (TG2 acts as a carrier, while gluten acts as a hapten) to gluten-specific $CD4^+$ T cells (Iversen et al., 2019)(Du Pré et al., 2020).

Following the uptake of DGPs by pro-inflammatory activated DCs in the lamina propria, (Depaolo et al., 2011), they migrate to MLN, process and present DGPs fragments to naïve helper T cells, leading to activation of T cells and generation of gluten-specific

$CD4^+$ effector cells antibodies (Figure 1.5) (Kagnoff, 2007)(S. W. Qiao et al. 2021)(Jabri and Sollid 2009). These activated pro-inflammatory $CD4^+$ Th1 cells then proliferate and secrete Th1 type cytokine such as IFN-γ, IL-21, and metalloproteases (MMPs) (Bodd et al. 2010)(Sarra et al. 2013)(Van Leeuwen et al., 2013). These cytokines may indirectly lead to IECs death and villus blunting by activating cytotoxic IELs (Ciszewski et al., 2020). The presence of elevated levels of IFN-γ has been reported in the intestinal mucosa of patients with CeD (Nilsen et al., 1995), and measuring the level of IFN-γ in *in vitro* T cell assays is a standard technique to evaluate gluten-specific responses (Anderson et al., 2000)(Nilsen et al., 1998). At the same time, cytokines release by activated Th1 cells prime B cells to mature into plasma cells that produce anti-DGP- and anit-TG2-specific antibodies (Figure 1.5). The production of anti-DGP antibodies is driven by interactions between gluten-specific B cells and their corresponding gluten-specific $CD4^+$ T cells (Sollid & Iversen, 2023)(Snir et al., 2017). Interestingly, the formation of anti-TG2 antibodies follows a classic hapten-carrier-like mechanism, in which TG2 serves as the hapten forming a complex with DGP as the carrier (E. F. Verdu & Schuppan, 2021)(Abadie et al., 2024). The resulting TG2-DGP complexes then bind to B cells, which present them to $CD4^+$ T cells that specifically recognized TG2, the hapten protein. These interactions lead to the production of anti-TG2 antibodies, contributing to the autoimmune aspect of CeD (Sollid et al., 1997)(Iversen & Sollid, 2020)(Lindstad et al., 2021)(Gefen et al., 2015). As such, the B cells can profoundly amplify the T cell responses to gluten as APCs (Iversen et al., 2019). The production of CeD-associated antibodies is a consequence of T-cells and B-cells interactions (Jabri & Sollid, 2017).

Therefore, the adaptive immune response is an essential part of CeD development; however, it is not sufficient to trigger epithelial cell destruction and requires the synergistic effect of epithelial stress and the innate immune response to license cytotoxic T cells to become killer cells in CeD and cause villus atrophy.

1.7.5 Innate immune response in CeD

Unlike the adaptive immune response, the innate immune response of CeD is less well-characterized, and fewer aspects have been revealed. The main triggers for the activation of innate immune arms remain unknown, and several factors have been speculated. These include gluten sequences that do not engage in MHC class II and are thus not involved in activating $CD4^+$ T cells, known as p31-43 (Araya et al., 2016)(Thomas et al., 2006), immunogenic non-gluten wheat proteins, such as α-amylase trypsin inhibitors (ATIs) that induces the activation of the TLR-4 pathway (Junker et al., 2012)(Jabri & Sollid, 2009)(Green & Jabri, 2006)(Schuppan et al., 2015), and microbial factors. These microbial factors include alterations in the gut microbiota caused by opportunistic pathogens (Wacklin et al., 2014)(Wacklin et al., 2013) and viral or bacterial pathogens antibodies (Figure 1.5) (Caminero, Mccarville, Galipeau, et al., 2019a)(Bouziat et al., 2017)(E. Verdu et al., 2007)(Zevallos et al., 2017)(Fernandez et al., 2017)(Caminero et al., 2016). It is plausible that more than one factor was involved. Therefore, additional studies are required to confirm the innate pathogenic role of gluten in CeD. Thus far, cytotoxic IELs are believed to be activated by IL-15 to mediate the innate immune response antibodies (Figure 1.5) (Schuppan et al., 2009)(Abadie et al., 2024). The phenotype of IELs

and their interactions with IECs in the context of CeD have been studied extensively (Abadie et al., 2012)(Jabri & Sollid, 2006).

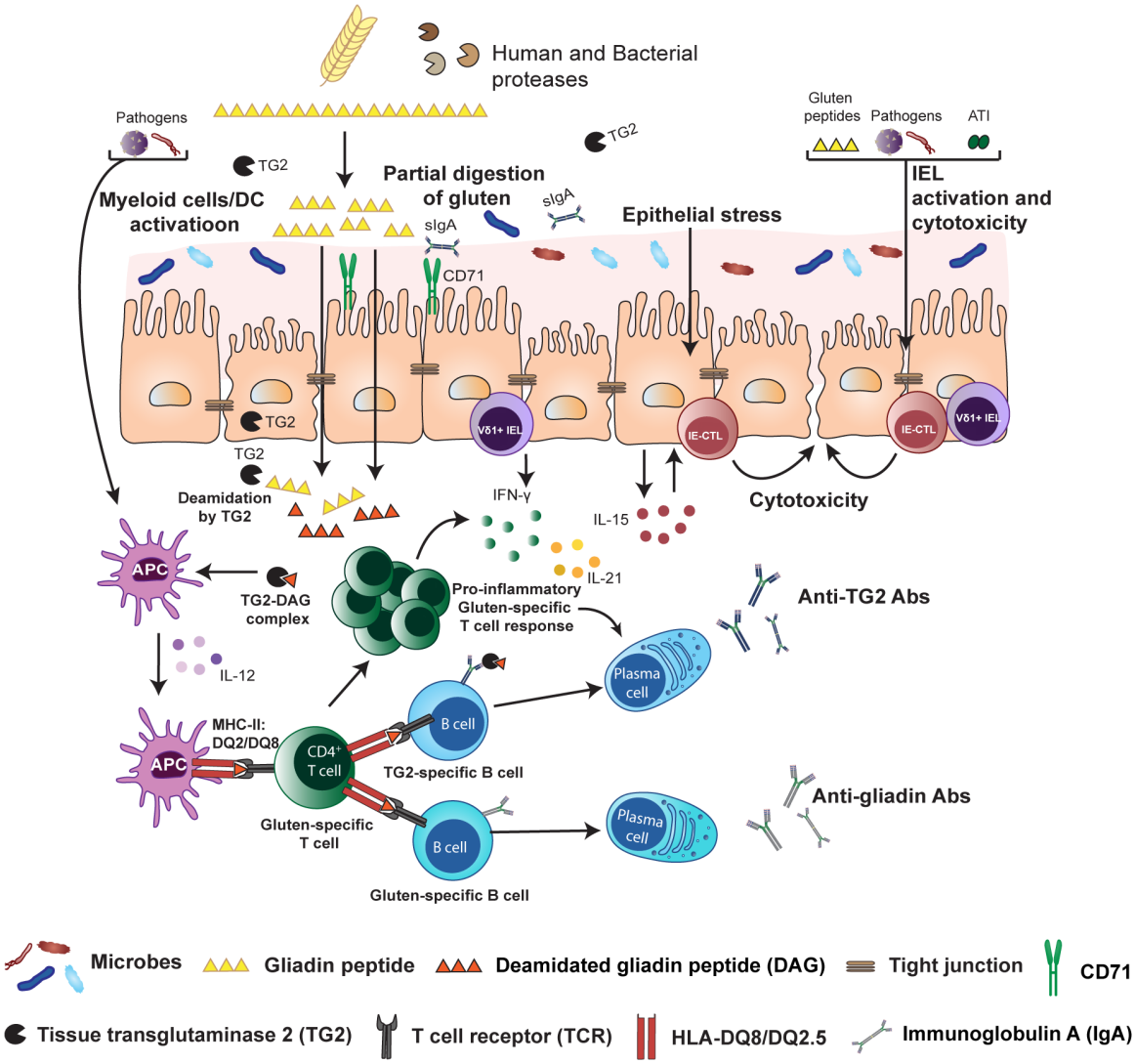


Figure 1.5 Overview of celiac disease Pathogenesis. Upon ingestion, gluten is partially digested in the lumen of the small intestine by both the host and microbial proteases. These proteolysis-resistant peptides then engage with extracellular and enzymatically active transglutaminase-2 (TG2) in the lumen or lamina propria, which is released from activated or damaged cells, such as shaded intestinal epithelial cells, fibroblasts, endothelial cells, and immune cells. The deamidation of gluten peptides by TG2 enhances their binding affinity to HLA-DQ8 or -DQ2.5 molecules on activated antigen-presenting cells (APCs),

such as dendritic cells (DCs), macrophages, and B cells. This interaction triggers the activation and expansion of gluten-specific CD4. T cells, which in turn assist B cells in differentiating into plasma cells, that produce antibodies against both TG2 and deamidated gluten peptides (DAG). Although gluten remains the primary driver of CD4 T cell response in CeD, recent studies have highlighted the role of microbes in modulating this response. Microbes have been proposed to contribute to CeD pathogenesis by participating in gluten metabolism and altering peptide immunogenicity, such as *Pseudomonas aeruginosa*, which possesses elastase-like enzyme (LasB) that metabolizes gluten to an immunogenetic peptide, by inducing cross-reactivity with microbial sequences, such as those found in *Pseudomonas*, or by influencing the breakdown and oral tolerance through viral infections. ATI, amylase trypsin inhibitor; IEL, intraepithelial lymphocytes; Abs, antibodies.

One of the histological hallmarks of CeD is the infiltration of lymphocytes between epithelial cells, with an activated phenotype called intraepithelial cytotoxic T lymphocytes (IE-CTLs) in CeD patients (Jabri & Sollid, 2009)(Green & Jabri, 2003)(Kutlu et al., 1993)(Ettersperger et al., 2016). IE-CTLs are referred to as hyper-activated IELs and express high levels of NK-associated receptors that are transcriptionally licensed to adopt a more NK-like phenotype and, in the absence of inhibitory receptors, induce destruction of the targeted cells independent of TCR ligand interaction (Meresse et al., 2006)(Meresse et al., 2004), corroborating the finding that IE-CTLs from CeD patients are not gluten-specific (Lundin et al., 1993)(Sollid, 2002). At homeostasis, 80-90% of the IELs in the human small intestine are type A IELs (actively induced), expressing TCR$\alpha\beta^+$ CD8$\alpha\beta^+$ and TCR$\alpha\beta^+$ CD4 that are generated in response to local tissue damage (Mayassi & Jabri, 2018). This type of IELs is believed to be primarily responsible for tissue destruction in the CeD (Jabri & Sollid, 2009). Whereas Type B IELs (naturally occurring) are tissue-resident

lymphocytes that populate the tissue early in life, independent of microbial colonization of the intestine; type B IELs are composed of TCR$\gamma\delta^+$ cells (Hayday et al., 2001)(McDonald et al., 2014)(McDonald et al., 2015)(Mayassi & Jabri, 2018). The levels of both TCR$\alpha\beta^+$ CD8$\alpha\beta^+$ and TCR$\gamma\delta^+$ IELs were dramatically elevated in the intestinal mucosa of patients with CeD (Hüe et al., 2004). The level of TCR$\alpha\beta^+$ IELs reduced in patients with CeD following a GFD (treated CeD) and correlated with the recovery of villous structure, whereas the level of TCR$\gamma\delta^+$ IELs in patients on a GFD remained elevated (Kutlu et al., 1993), which could be attributed to the speculation that TCR$\gamma\delta^+$ is responsible for IEC destruction in CeD (Hayday et al., 2001). Recent studies have found that the repertoire of gut tissue-resident TCR$\gamma\delta^+$ IELs is permanently altered in patient with CeD, though the specific antigens these TCRs respond to remain unclear (Mayassi et al., 2019)(Eggesbø et al., 2020). Typically, these innate-like TCR$\gamma\delta^+$ IELs are replaced by cytotoxic IFN-γ producing, gluten-reactive V$\delta1^+$ IELs, which continue to proliferate even without gluten exposure (Mayassi et al., 2019)(Eggesbø et al., 2020).

Studies on IELs derived from CeD biopsies have demonstrated that these cells have acquired cytotoxic phenotypes, characterized by increased expression of two activating NK-associated receptors, namely natural killer group 2D (NKG2D) and NKG2C/CD94, coupled with very low expression of the inhibitory NK receptor, NKG2A/CD94, which can block TCR and NKG2D activity, indicating that these cells play an active role in mediating cytotoxicity (Meresse et al., 2006)(Meresse et al., 2004)(Jabri et al., 2000). In contrast, IELs from healthy individuals predominantly exhibit a high level of the inhibitory NKG2A/CD94 receptor and low expression levels of activating NKG2D and

NKG2C/CD94 receptors (Jabri et al., 2002)(Roberts et al., 2001). Although the level of TCR$\alpha\beta^+$ reverted in CeD patients adhere to GFD (treated CeD), incomplete or no histological recovery in a substantial group of treated CeD (Rubio-Tapia et al., 2010)(P. J. Wahab et al., 2002) has been attributed to the significant presence of TCR$\alpha\beta^+$ CD8$\alpha\beta^+$ cells compared with healthy controls, which exhibit transcriptional similarity to cytotoxic cells found in active CeD (Abadie et al., 2012)(Jabri & Sollid, 2009). These cells have shown a reduction in the inhibitory NKG2A/CD94 receptor while retaining the expression of activating NKG2C/CD94 receptors (Meresse et al., 2006). However, these TCR$\alpha\beta^+$ CD8$\alpha\beta^+$ cells in treated CeD express notably have lower levels of NKG2D receptors compared with their levels in active CeD (Kornberg et al., 2023)(Meresse et al., 2004). Additionally, it has been shown that some TCR$\alpha\beta^+$ CD8$\alpha\beta^+$ cells that persist and expand in treated CeD express a TCR repertoire similar to IE-CTL found in active CeD (Meresse et al., 2006). This indicates that certain TCR$\alpha\beta^+$CD8$\alpha\beta^+$ cells can function as resident memory populations, capable of being reactivated to mediate cytotoxicity upon gluten exposure (Meresse et al., 2006)(Kornberg et al., 2023). Moreover, the ligands for NKG2D receptors, MHC class I-related chain A/B (MICA/B), are stress-inducible molecules (Bauer et al., 1999)(Groh et al., 1996)(Groh et al., 1998), whereas the ligands for NKG2/CD94 receptors are non-classical MHC class I molecules, HLA-E, which are induced in the presence of IFN-γ (Meresse et al., 2006)(Bauer et al., 1999)(Braun,1998)(Jabri et al., 2020). In patients with active CeD, the upregulated expression of HLA-E and MICA/B on the surface of IECs can elucidate the targeting mechanisms of IE-TCL towards IECs, which facilitates the interaction between IECs and

IE-TCL (Jabri & Sollid, 2009)(Sollid & Jabri, 2013). The mechanism underlying the increased expression of stress ligands on IECs is not well understood, while HLA-E expression is modulated by IFN-γ, which further confirms the need for the activation of gluten-specific $CD4^+$ IFN-γ-producing T cells. The activation of IE-CTLs and their adaptation to the NK-like phenotype are an area of active research that has shown that IL-15 can play an important role in this regard.

Patients with CeD display an upregulation of IL-15 in both the small intestinal epithelium and LP and play an important role in CeD development by licensing IE-CTL; however, the driving factors remain unknown (Sarra et al., 2013)(Korneychuk et al., 2014)(Mention et al., 2003)(Malamut et al., 2010). It has been speculated that an increase in IL-15 expression can be a consequence of epithelial stress; however, the driving force for this stress is unknown. In a mouse model overexpressing IL-15 in the MLN and LP, DCs adopt a pro-inflammatory phenotype and release inflammatory cytokines, such as IL12p70 (Depaolo et al., 2011). In addition, naïve $CD4^+$ T cells activated by these inflammatory DCs polarize into Th1 cells and secrete IFN-γ upon exposure to gluten (Depaolo et al., 2011). This finding suggests that increased lamina propria expression of IL-15 may be an innate inducer. Additionally, elevated level of IL-15 in IECs of CeD patients play a fundamental role in activating and proliferating IE-CTL and contribute to gluten-induced tissue damage, as demonstrated in transgenic mice that overexpress IL15 in IECs (Abadie et al., 2020)(Jabri & Abadie, 2015). It is known that IL-15 predominantly functions through cell contact-dependent fashion by binding to IL2/IL15 receptor express on IE-TCL (Jabri & Abadie, 2015)(Tang et al., 2009). This interaction enhances IL-15

signalling, leading to increased expression of NKG2D and licensed IELs to transform into a cytotoxic phenotype, IE-CTL, and destroy distressed IECs based on stress signals, leading to villous atrophy during CeD progression (Jabri & Abadie, 2015)(Tang et al., 2009)(Tang et al., 2015). However, the exact mechanism underlying increased expression of non-classical MHC class I and IL-15 in IECs remains unidentified. Future studies should focus on identifying the crosstalk between IECs, gluten-reactive $CD4^+$ T cells, and IELs, their role in tissue destruction, and uncovering the mechanism of epithelial alterations in CeD.

Altogether, many aspects of the adaptive immune system and some details of the innate immune response involved in CeD are well understood; however, many aspects remain unknown. For example, the role of IECs in the initiation of an immune response at the onset of CeD is not defined, mostly due to the lack of a practical IEC model. Therefore, this thesis focuses on developing such a model and investigating the potential immune role of IEC in inducing $CD4^+$ T cell responses in CeD.

1.7.6 Other environmental factors: Microbial triggers

The GI tract harbors a diverse community of microorganisms known as the microbiota. The gut immune system recognizes the microbiota as harmless and maintains a mutually beneficial relationship, which is pivotal for proper functioning of the GI tract. It has been shown that intestinal microbiota plays a crucial role in maintaining the host's intestinal homeostasis by inducing immune system development, protecting the host against pathogens and contributing to digestion (Lozupone et al., 2012)(Kastl et al., 2020). Studies conducted on germ-free animals have revealed various developmental

abnormalities in intestinal function, metabolism, motility, vasculature, respiratory system, and nutritional requirements (K. Smith et al., 2007). In addition, germ-free animals exhibit immune deficiencies, including reduced numbers of IELs, plasma cells, IgA, and IgA^+ cells in the gut, which leads to impaired immune responses to detrimental stimuli (K. Smith et al., 2007)(Hapfelmeier et al., 2010). However, upon colonization of germ-free mice with non-pathogenic microbes, such as altered Schaedler flora (ASF), which refers to a mixture of naturally occurring gut microbiota in mice, as well as a certain strain of *Escherichia coli*, IgA mechanisms are restored (Hapfelmeier et al., 2010).

Several clinical cohort studies have shown the involvement of gut microbiota in the development and severity of CeD (E. F. Verdu & Schuppan, 2021)(E. F. Verdu & Caminero, 2017)(E. F. Verdu et al., 2015). Several studies have demonstrated altered microbial composition (dysbiosis) in the small intestines and feces of individuals with active CeD, characterized by elevatioin in the relative abundance of pro-inflammatory bacteria such as Proteobacteria and *Bacteroides*, and reduced levels of anti-inflammatory bacteria such as *Bifidobacterium* (Pozo-Rubio et al., 2012)(Sánchez et al., 2013)(Sánchez et al., 2012)(Constante et al., 2022)(Caminero & Verdu, 2019)(Nistal et al., 2016)(Bodkhe et al., 2019)(Nistal et al., 2012). A longitudinal study revealed an increase in the relative abundance of *Bacteroides*, *Salmonella*, *Shigella*, *Klebsiella*, and *Staphylococcus* in the feces of treated CeD patients compared with healthy controls (Di Cagno et al., 2011). These studies demonstrating a variability in gut microbiota compositions between CeD patients and healthy individuals suggested a potential role of bacteria in CeD pathogenesis (Viitasalo et al., 2018). Additionally, in CeD patients, there was variability in the extent of

microbial diversity and dysbiosis, as evidenced by the fact that treated CeD patients that were non-responsive to GFD had a higher abundance of Proteobacteria in their small intestine than treated CeD patients responsive to GFD (Wacklin et al., 2014). The increased abundance of Proteobacteria phyla, particularly *Neisseria flavescens*, an opportunistic pathogen, has been detected in duodenal biopsies of CeD patients compared with controls, with the ability to induce inflammatory responses in mucosal explants and DCs *in vitro* (D'Argenio et al., 2016). *E. coli* is another Proteobacteria that has been identified to be frequently present at high levels in the duodenum of patients with CeD (Nadal et al., 2007)(Collado et al., 2009). *In vitro* studies have shown the inflammatory capacity of *E. coli* obtained from CeD patients by increased production of pro-inflammatory cytokines, IL12 and IFN-γ, upon treatment with peripheral blood mononuclear cells (PMBC), DCs, or intestinal loops (De Palma et al., 2012)(De Palma et al., 2010)(Cinova et al., 2011). These findings imply that the bacteria associated with CeD may have inflammatory characteristics that can contribute to disease severity. These findings support the idea that patients with CeD possess dysbiotic microbiota, which is imbalanced by an increased abundance of bacteria exhibiting pathogenic characteristics. However, it remains unclear whether this dysbiosis is a cause or consequence of the disease. Moreover, a recent study (Constante et al., 2022) identified differences in the microbiota composition between fecal samples and different intestinal segments in CeD patients compared with controls. Specifically, the duodenum of CeD patients exhibited a higher relative abundance of opportunistic pathogens such as *Prevotellla*, *E. coli*, and *Neisseria* compared with controls. (Constante et al., 2022). However, no significant difference was observed in the fecal

samples of the same patients, indicating microbial alterations reported in CeD may be site-specific (H. J. Galipeau et al., 2024).

The role of the microbiota in modulating gluten immunopathology has been investigated in a murine model expressing the CeD risk allele. A study from Verdu lab demonstrated that the colonization status of mice expressing the CeD risk gene, HLA-DQ8, on a non-obese diabetic (NOD) background determined the severity of gluten-induced pathology after mucosal immunization with cholera toxin followed by oral gluten challenges (Galipeau et al., 2011a). In this study, germ-free mice colonized with altered Schaedler flora (ASF) microbiota-commensal bacteria devoid of pathobionts- were protected from gluten-induced immunopathology. In contrast, germ-free mice exhibited severe immunopathology after gluten immunization, and ASF-colonized germ-free NOD-DQ8 mice that were co-colonized with SPF bacteria developed a moderate gluten-induced immune response (Galipeau et al., 2015). These findings suggest that both absence of regulatory protective effects of commensal microbiota (in germ-free mice) and the presence of pathobionts (in SPF-colonized mice) modulates the immune response to dietary antigens. Additionally, when NOD-DQ8 mice colonized with SPF microbiota were co-colonized with pro-inflammatory Proteobacteria, they showed a significant reduction in villous height-to-crypt depth (vh/cd) ratios compared with SPF mice lacking Proteobacteria, which were protected from gluten-induced immunopathology (Galipeau et al., 2015). These results indicate that the microbiota can play a dual role depending on its composition: in the presence of pathobionts, it may augment the immune response towards gluten, while in the presence of benign microbiota, such as ASF, it can alleviate gluten-induced

immunopathology. However, the molecular mechanisms underlying these effects remain unclear. Moreover, the protective role of the ASF microbiota was reversed when NOD-DQ8 ASF-colonized mice were supplemented with an adherent strain of *E. coli* isolated from the small intestine of CeD patients compared with *E. coli* strains obtained from healthy individuals (H. J. Galipeau et al., 2015). Additionally, perinatal vancomycin treatment in NOD-DQ8 SPF-colonized mice enhanced the abundance of Proteobacteria, such as *E. coli*, leading to severe gluten immunopathology. Although these findings did not offer any mechanistic insight into the role of microbes in CeD, they suggest that host immune responses to microbial antigens may contribute to the development of CeD or perpetuating inflammation during the active stage of the disease (Scales et al., 2014).

Among the different bacterial taxa associated with CeD development, the *Pseudomonas* genus has been extensively investigated as a taxa of interest in CeD because of its clinical relevance and its potential microbially mediated mechanism in CeD pathogenesis. Clinically, an increased abundance of *Pseudomonas* has been observed in the small intestine of patients with CeD (Nistal et al., 2012)(Caminero et al., 2015). Additionally, patients with active CeD exhibit high levels of serum antibodies against the I2 sequence of *Pseudomonas* (anti-I2) (Ashorn et al., 2008). These anti-I2 antibodies are identified early at the onset of CeD development (Viitasalo et al., 2014) and remain elevated in individuals with poor responses to the GFD (Viitasalo et al., 2018)(Ashorn et al., 2009). Several molecular mechanisms have been proposed by which *Pseudomonas* genera, such as *P. aeruginosa* may drive, initiate, or perpetuate the immune response in CeD. Gluten-dependent mechanisms include metabolism of gluten by *P. aeruginosa,* which digests

gluten peptides into shorter fragments that retain immunogenicity and can translocate across the epithelial barrier (Caminero et al., 2016). Additionally, molecular mimicry between HLA-DQ2.5-restricted gluten epitopes and peptides from *P. aeruginosa* and *P. fluorescens* has been identified, potentially triggering a cross-reactive immune response. A gluten-independent mechanism involves the virulent LasB elastase, produced by *P. aeruginosa*, which activates the protease-activated receptor-2 (PAR-2) pathway, leading to severe inflammation (Petersen et al., 2020)(Ciacchi et al., 2022). These gluten-specific and bacteria-derived mechanisms are discussed in detail in the subsequent section.

1.7.6.1 *Degradation of gluten by intestinal bacteria*

The small intestinal microbiota is involved in the metabolism of dietary proteins, and under most conditions, the host benefits from the availability of smaller peptide fragments and amino acids, including the digestion of gluten. As mentioned in the previous section, gluten protein, rich in proline residues, resist complete digestion by human gastrointestinal proteases and peptidase, resulting in the formation of large peptides in the in the intestinal lumen (Shan et al., 2002), crossing the epithelial barrier into the lamina propria, and following deamidation with TG2, can potentially activate gluten-specific $CD4^+$ T cells (Frazer et al., 1959)(Dieterich et al., 1997)(Molberg et al., 1998). It is suggested that these undigested food components become substrate for microbial metabolism in the GI tract (Caminero, Meisel, et al., 2019). It is now recognized that the small intestine contains bacteria that can effectively degrade gluten's immunodominant peptides (Herrán et al., 2017)(Fernandez-Feo et al., 2013)(Wei et al., 2020). Caminero *et*

al. (Caminero et al., 2014) identified bacterial stains from GI tract of humans that possess the ability to degrade gluten and found different patterns of gluten digestion in the intestinal content of CeD patients compared with healthy controls (Caminero et al., 2015). Interestingly, the microbiota of individuals with CeD exhibited a reduced ability to degrade gluten (Constante et al., 2022). This was demonstrated using mice colonized with human small intestinal contents, revealing that the microbiota from CeD patients, even those sharing a similar HLA-DQ background with healthy controls, displayed diminished gluten-degrading capabilities (Constante et al., 2022). Additionally, studies over the past decades have shown that some bacterial enzymes are more potent in digesting gluten peptides and further modulate their immunogenicity of the peptide (Rey et al., 2016)(Caminero et al., 2016; Caminero, Mccarville, Galipeau, et al., 2019a; Caminero, Mccarville, Zevallos, et al., 2019). For example, *P. aeruginosa* is a member of the Proteobacteria phylum and an opportunistic pathogen present in the small intestine that possesses a well-identified elastase capable of partially metabolizing gluten peptides into smaller fragments with retained immunogenicity, as confirmed by *in vitro* functional studies (Wei et al., 2015)(Herrán et al., 2017)(Caminero et al., 2016). It has been shown that a mutant strain of *P. aeruginosa* lacking *LasR* (regulator of *LasA* and *LasB*) has decreased proteolytic activity, which makes this strain more virulent and causing enhanced inflammation in human and mice explants as well as *in vitro* (LaFayette et al., 2015). In addition, proteases released by *P. aeruginosa* have the capacity to degrade pro-inflammatory cytokines (IL-8 and IL-6) produced by the host (LaFayette et al., 2015), which contradicts an earlier study

in which *P. aeruginosa* significantly increased inflammation in infectious models (Azghani & Connelly, 1990)(Kon et al., 1999).

Furthermore, *P. aeruginosa*-expressing *LasB* elastase (LasB or elastase B is a major enzyme for the metabolism of gluten) and is capable of metabolizing 33-mer peptide into smaller fragments, with increased immunogenicity, which can easily translocate across the epithelium (Caminero et al., 2016). It has been shown that *P. aeruginosa* is not able to metabolize QLP sequence of 33-mer, which is a region contributing to the immunogenicity of the 33-mer peptide. It was found that *P. aeruginosa*-expressing *LasB* elastase-degraded peptides had a stronger ability compared with the 33-mer to stimulate IFN-γ-producing gliadin-specific T cells from patients with CeD *in vitro* (Caminero et al., 2016). In contrast, *Lactobacillus spp*., a small intestinal commensal, potentially show a protective effect, as it decreases the immunogenicity of gluten peptides digested by *P. aeruginosa* (Caminero, Mccarville, Zevallos, et al., 2019). In addition, the presence of elastase from *P. aeruginosa* has been suggested as another bacteria-mediated mechanism that contributes to inflammation in CeD. Small intestinal biopsies from patients with CeD exhibited enhanced proteolytic activity towards gluten compared with those from healthy individuals, which was attributed to the abundance of *P. aeruginosa* with gluten-metabolizing capability in the duodenum of patients with active CeD (Caminero, Mccarville, Galipeau, et al., 2019a).

Moreover, it has also been demonstrated that *P. aeruginosa*-expressing elastase can induce inflammation in mice in a gluten-independent manner. Colonizing germ-free mice with *P. aeruginosa*-expressing *LasB* elastase induced inflammation and increased the level of IELs in the small intestine of these mice compared with the mice colonized with mutant

strain lacking *LasB* elastase. This finding was attributed to the activation of the PAR-2 pathways, as this effect was absent in PAR-2 mutant mice colonized with *P. aeruginosa*-expressing *LasB* elastase (Caminero, Mccarville, Galipeau, et al., 2019a). Additionally, when HLA-DQ8 mice were colonized with *P. aeruginosa*-expressing *LasB* elastase, the gluten-dependent and -independent pathways were synergized to induce more severe inflammation and moderate enteropathy in these mice, suggesting that bacterial elastase has the potential to mediate both gluten-specific and non-specific pathways in CeD (Caminero, Mccarville, Galipeau, et al., 2019a).

Furthermore, a third mechanism by which *Pseudomonas* genus may contribute to CeD pathogenesis, independent of gluten degradation or bacterial elastase, has been suggested. It has shown that HLA-DQ2.5-restricted gluten epitopes exhibit significant sequence similarity to peptides from *P. aeruginosa* and *P. fluorescens* (Petersen et al., 2020)(Ciacchi et al., 2022). These bacterial peptides were found to cross-react with gliadin-specific T cells, either from cell lines transduced with TCRs specific to DQ2.5-restricted gluten peptides or in gluten-reactive $CD4^+$ T cells isolated from patients with CeD. This finding suggests that molecular mimicry between gluten and microbial peptides could initiate or perpetuate CeD (Petersen et al., 2020)(Ciacchi et al., 2022). However, it remains unclear whether these microbial peptides can cross the small intestinal barrier and be presented to $CD4^+$ T cells via HLA-DQ2.5 *in vivo*. Additionally, potential cross-reactivity with other bacterial or viral peptides and gluten requires further investigation.

These findings underscore the intricate mechanisms involved in gluten digestion and the role of gut microbial communities in sequentially degrading gluten, which can

influence the immunogenicity of gluten peptides and their contribution to CeD pathogenesis. Therefore, in this thesis, I investigated the immunomodulatory capacity of gluten digested by intestinal opportunistic pathogens on the activation of gluten-specific $CD4^+$ T cells, mediated by MHC class II expression in IECs.

1.7.7 Gluten challenge in CeD patients

A gluten challenge is the controlled and intentional re-exposure of patients with a CeD to gluten. In most CeD patients, symptoms improve upon GFD and reappear when gluten is re-introduced (Bruins, 2013). Thus, gluten challenge is clinically used in the diagnosis of CeD and in clinical trials (Sollid & Jabri, 2013). The duration of gluten challenge differs depending on its purpose (diagnostic or clinical research), ranging from a day of a single dose to months of repeated administration (Bruins, 2013). Commonly, in adults, gluten challenge involves the consumption of 3 g of gluten/day for a duration of 2-8 weeks (Christophersen et al., 2021). This gluten challenge promotes serological and histological changes in most adult patients with CeD (Leffler et al., 2013). Studies applying gluten challenge have shown several events that happen as a direct effect of gluten exposure (Goel, Tye-Din, et al., 2019)(Christophersen et al., 2014)(Han et al., 2013)(Risnes et al., 2021). Some of these events occurred rapidly within a few hours, and therefore, as such, CeD patients who had previously been on a GFD without symptoms developed GI symptoms (Goel, Tye-Din, et al., 2019)(Goel, Daveson, et al., 2019). The quickest and most dominant cytokine released was IL-2, and its level increases as early as 4 h following oral gluten challenge, which is directly linked to the rapid occurrence of symptoms such as

nausea or vomiting (Goel, Tye-Din, et al., 2019). Reactivated gluten-specific $CD4^+$ T cells are most likely responsible to produce this cytokine in response to gluten challenge. In addition, after three days of gluten challenge, the levels of gluten-specific $CD4^+$ T cells and gut-homing $CD8^+$ αβ and γδ T cells in the serum were found to be elevated, which peaked on day six as confirmed by IFN- enzyme-linked immunospot assay or MHC-tetramers (Han et al., 2013)(Risnes et al., 2021)(Ráki et al., 2007)(Anderson et al., 2005)(Brottveit et al., 2011). Enhanced expression of activation markers, such as CD38, can be measured in gluten-specific $CD4^+$ T cells after a few days of ongoing gluten challenge, and this serve as a marker of gluten exposure (Zühlke et al., 2019). Additionally, measuring CD38 expression at a later point (between days 8 and 14) allowed to distinguish between slow responders and non-responders to the gluten treatment. Gluten-specific $CD4^+$ T cells present in the serum share a TCR repertoire with gluten-specific $CD4^+$T cells in the intestine (Risnes et al., 2018). The gluten-induced $CD8^+$ αβ and γδ T cells in the serum of challenged CeD patients shares phenotypic similarities with IELs in the intestine of active CeD patients including expression of NK receptors (Risnes et al., 2021)(Han et al., 2013)(Kornberg et al., 2023). This shared phenotype is indicative of a memory response that reactivates upon gluten re-exposure (Kornberg et al., 2023)(Risnes et al., 2021). While the gluten specificity of $CD4^+$ T cells in the serum is clear, the gluten specificity is not clear for effector memory $CD8^+$ αβ and γδ T cells in the serum following gluten re-exposure (Jabri & Sollid, 2009)(Mayassi et al., 2019). The presence of these gluten-dependent memory T cells in the serum could reflect changes in the tissue environment triggered by gluten-specific $CD4^+$ T cell activation (Christophersen et al., 2021)(Kornberg et al., 2023).

This implies a coordinated activation model, in which gluten-dependent $CD4^+$ T cell activation promotes the activation and cytotoxic reprogramming of memory $CD8^+$ αβ and γδ T cells, despite these cells not directly recognizing gluten themselves (Christophersen et al., 2021)(Kornberg et al., 2023).

Moreover, activated gluten-specific $CD4^+$ T cells assist DGP- and TG2-specific B cells; thus, gluten challenge can be used to follow the production of antibodies by these B cells (Du Pré & Sollid, 2015). The release of measurable concentrations of antibodies requires longer exposure to gluten. As such, a few weeks of gluten challenge every day is required to detect either TG2- or DGP-specific antibodies in the blood serum of CeD patients who were on a GFD (Bruins, 2013).

1.7.8 Mouse models of CeD

Several animal models have been developed over the past three decades to investigate the specific characteristics of the CeD. This section discusses humanized mouse models that lack all endogenous mouse MHC class II molecules and carry the CeD risk genes: HLA-DQ8 or DR3-DQ2.5.

The first group of transgenic mice that developed features of CeD was the AB^oDQ8 mice (Cheng et al., 2003). The first model in this group was the double transgenic mice, HCD4-DQ8, expressing human CD4 and HLA-DQ8 genes and deficient in mouse MHC class II and mouse CD4. Upon sensitization to gliadin using intraperitoneal (i.p.) injection, HCD4-DQ8 mice produced more anti-gliadin antibodies than did HCD4-DQ6 mice (Black et al., 2002). In addition, T cells isolated from gliadin-sensitized HCD4- DQ8 mice showed

a robust gliadin-specific T cell response and increased production of IL-6, IL-10, and TGF-β (Black et al., 2002). Later, single transgenic HLA-DQ8 mice sensitized and challenged with gluten showed production of anti-gliadin antibodies and increased $CD3^+$ IELs counts and barrier dysfunction (E. F. Verdu et al., 2008). However, these models failed to show changes in vh/cd and the production of anti-TG2 antibodies. The next model also expressed HLA-DQ8 genes, but unlike previous models, AB^oDQ8 mice were crossed with NOD background mice, which increased the risk of developing autoimmunity in these mice. NOD/DQ8 mice showed moderate enteropathy development characterized by decreased vh/cd ratios and increased $CD3^+$ IELs in villi tips following oral sensitization and gliadin challenge (H. J. Galipeau et al., 2011a). The production of anti-gliadin antibodies and barrier dysfunction have been reported in these gliadin-sensitized mice. Similar to previous mouse models, NOD/DQ8 mice failed to develop anti-TG2 antibodies and showed impaired IL-15 production. As shown in previous sections, IL-15 is an integral cytokine in the pathogenesis of CeD, particularly in the innate immune response. Therefore, this model failed to recapitulate a pivotal aspect of the CeD (H. Galipeau et al., 2011)(Suwanai et al., 2010)(Marietta et al., 2004). In addition, an increase in the number of IELs in these models implies that the activation of Th1 immunity may be involved in the recruitment and presence of IELs and the crosstalk between the epithelium and Th1 cells. However, as mentioned earlier, this is insufficient to promote tissue destruction and further requires the licensing of IE-CTL to adopt the NK cell-like phenotype. This has led to the recent generation of triple transgenic mice, characterized by the overexpression of human IL-15 in the LP and intestinal epithelium (Abadie et al., 2020). These mice were established by

crossing HLA-DQ8 mice with villin-IL-15tg mice and subsequent crossing of the resulting mice with D^d-IL-15tg to generate DQ8-D^d-villin-IL-15tg mice. These triple transgenic mice showed overexpression of human IL-15 in the LP and the intestinal epithelium. When orally sensitized and challenged with gliadin, these mice developed partial villous atrophy confirmed by the reduction in vh/cd ratios, suggesting that the overexpression of IL-15 was required for this phenomenon to occur, as it was lacking in previous mouse models. In addition, an increase in *ifng* expression, $CD3^+$ IEL counts, $CD4^+$ T cell proliferation, and production of anti-gliadin and anti-DGP antibodies was reported in this model. Thus, DQ8-D^d-villin-IL-15tg mic is beneficial for investigating the role of IL-15 in CeD patients expressing HLA-DQ8 alleles; however, not all active CeD patients have overexpression of IL-15, and some CeD patients on GFD, despite showing normal histology, have increased expression of IL-15 (Abadie & Jabri, 2014). Another shortcoming of this model is that these mice fail to produce anti-TG2 antibodies after consuming a gluten-containing diet, which is the main drawback of this model as it is the most sensitive diagnostic test in patients with CeD. An additional model was developed using the adoptive transfer of T cells, in which $CD45^{hi}$Th1 cells from peripherally gluten-sensitized mice were transferred into T cell-deficient $Rag1^{-/-}$ knock-out mice (Freitag et al., 2009)(Freitag et al., 2014). Upon oral gluten exposure, these mice exhibited sensitivity, showing key features of CeD, including intestinal inflammation, villous atrophy, crypt hyperplasia, and production of IgG and IgA antibodies against gluten (Freitag et al., 2014)(Freitag et al., 2014). This model has proven valuable for the preclinical evaluation of potential therapies for CeD; however, these mice lack a genetic predisposition for CeD.

Transgenic mice for HLA-DQ2 alleles are essential, because over 90% of patients with CeD carry this allele (Tye-Din, Galipeau, and Agardh 2018). Double transgenic hCD4-DR3-DQ2.5 mice carrying both the human DR3-DQ2 haplotype and human CD4 molecules were previously developed to study the impact of vaccine-based therapy on gluten epitopes to which HLA-DQ2^{+} patients react. These mice showed an increase in the gluten-specific CD4^{+} T cell response following gluten sensitization using i.p. injection but failed to develop villous atrophy. The use of transgenic mouse models has profoundly advanced our understanding of CeD immunopathology. However, owing to the complexity of the disease, no single model has been established to fully recapitulate all aspects of the CeD pathology (N. Korneychuk, Meresse, and Cerf-Bensussan 2015)(Costes et al. 2015).

In this thesis, transgenic DR3-DQ2.5 mice were characterized following gliadin sensitization and challenge. Therefore, experimental mouse models of CeD serve as valuable tools for studying the intricate immunopathogenesis associated with CeD. Many mouse models replicate various aspects of CeD.

1.8 Intestinal organoids

The challenges of culturing primary or isolated IECs *ex vivo* for prolonged periods of time are attributed to the rapid turnover rate of IECs. Within a span of 3-5 days, ISCs differentiate into mature IECs, reach the villus tip, lose contact with neighboring cells, and ultimately undergo a type of cell death refer to as “anoikis” (Barker 2014), which poses challenge to culture primary IECs for long-term. In 2009, Sato et al. (Sato et al. 2009) established a 3D culture system that allowed long-term proliferation and differentiation of

IECs *in vitro*. As mentioned in section 1.3, the identification and isolation of $Lgr5^+$ ISCs make it feasible to study the different types of signalling pathways that govern the functionality of ISCs. These signals originate from the ISCs niche, which refers to the microenvironment surrounding ISCs, including Paneth cells, endothelial cells, blood cells, sub-epithelial myofibroblasts, non-muscle fibroblasts, neurons, and muscle cells (Shaker and Rubin 2010). The ISC niche is regulated by various signalling pathways that maintain the balance between self-renewal and differentiation, such as the Wingless-type MMTV integration site (Wnt), Notch, and bone morphogenetic protein (BMP) (M. R. Walker, Patel, and Stappenbeck 2009)(Yen and Wright 2006). Wnt signalling is required to initiate the crypt-like domain formation and ISCs stemness and proliferation (van Es et al. 2012)(Ireland et al. 2004)(Korinek et al. 1998). Notch signalling conserves ISCs in their undifferentiated state, whereas Notch lateral inhibition controls the balance between secretory and absorptive cell composition. Notch inhibition induces differentiation of intestinal secretory cells (Clevers 2013). BMP signalling is believed to be an adverse regulatory pathway for ISCs self-renewal and proliferation, and the BMP inhibitor, Noggin, promotes crypt formation and ISC differentiation (Haramis et al., 2004). However, the main source of BMP signalling is still undefined and is suggested to be derived from the underlying mesenchyme, such as myofibroblasts or epithelial (Haramis et al., 2004)(Batts et al., 2006)(Thorne et al., 2018). In contrast to BMP, epidermal growth factor (EGF) signalling supports ISCs division and self-renewal. It is speculated that Paneth cells, in addition to releasing antimicrobial compounds, such as lysozyme and defensin, act as a source of several main niche signalling factors, namely, Wnt3a, Notch, and EGF (Sato, Van

Es, et al., 2011)(Farin et al., 2016). It has been shown that incomplete ablation of Paneth cells in mice leads to a partial loss of ISCs, suggesting that Paneth cells comprise the main niche for ISCs and are an essential source of Wnt signalling (Sato, Stange, et al., 2011a)(Sato, Van Es, et al., 2011). However, another study showed that complete ablation of Paneth cells did not affect ISCs and that $Lgr5^+$ ISC function remained intact (Farin et al., 2012)(T. H. Kim et al., 2012). This finding suggests that there may be other sources of Wnt signalling such as intestinal subepithelial myofibroblasts and subepithelial telocytes (Shoshkes-Carmel et al., 2018)(Lei et al., 2014). In addition, the colon lacks Paneth cells; thus, the exact source of Wnt signalling in the colon crypt remains unclear (Sato, Van Es, et al., 2011)(Sasaki et al., 2016).

In recent years, the development of organoid culture systems has eradicated some of these issues by offering long-term 3D cultures for modelling the intestinal epithelium *in vitro*. Intestinal organoids or "mini-guts" are defined as a 3D organ-like structures that undergo self-renewal and self-organization and closely recapitulate the structural, functional, and cellular composition of the intestine (Sato & Clevers, 2013). The resulting 3D organoids were hollow cysts surrounded by budding-like structures corresponding to the crypt domains of the small intestine and villus-like domains that reside between the buds. The organoid crypt domains are populated by $Lgr5^+$ stem cells, Paneth cells, and transit-amplifying (TA) proliferating cells in the crypt neck. During organoid culture, stem cells located at the base of the crypt differentiate and migrate along the crypt-villus axis towards the villus tip and finally shed into the hollow cyst (Sato & Clevers, 2013). Organoid development and long-term propagation require a growth matrix to support their growth:

as such they are embedded within a laminin-rich extracellular matrix such as Matrigel. In addition, organoid growth, proliferation, and differentiation require supplementation with a refined cocktail of growth factors that recapitulate the *in vivo* stem cell niche to maintain stemness in *in vitro* culture, including Want3A, EGF, BMP signalling inhibitor Noggin, and R-spondin (Sato et al., 2009) (Sato, Van Es, et al., 2011). Once embedded in the extracellular matrix in the presence of proper growth factors, 3D organoids are developed in which the apical surface of IECs faces the hollow lumen of 3D organoids, which is not easily accessible, whereas the basolateral surface of IECs faces outwards. This unique characteristic of organoids, replicating the small intestine microenvironment, makes them well-suited for studying the interaction between IECs and other cell types *in vitro.* Recently, various successful organoid co-culture systems have been developed with T cells, macrophages, IELs, enteric nerves, and fibroblasts (Rogoz et al., 2015) (C. E. Heuberger et al., 2023)(Serena et al., 2019) (Nozaki et al., 2016)(Pastuła et al., 2016). In addition, polarized and extracellular matrix-supported organoid monolayers were generated, where the apical surface of the IECs was exposed upward (Moon et al., 2014)(VanDussen et al., 2015). Organoid monolayer cultures enable the introduction of pathogens and other exogenous stimuli directly to the apical surface of IECs, facilitating targeted investigation of the crosstalk between IECs and the components of interest (Ettayebi et al., 2016)(Foulke-Abel et al., 2014)(In et al., 2019).

Organoids are commonly derived from two sources (Rahmani et al., 2019). In the first pathway, organoids derived from embryonic stem cells (ESCs) or human pluripotent stem cells (hPSCs) lead to the formation of human intestinal organoids (HIOs) (Watson et

al., 2014)(Aurora & Spence, 2016)(Takahashi et al., 2018). This type of organoid possesses fetal-like characteristics and is transplanted into mice for further maturation. The second group of organoids is derived from human or mouse organ-restricted adult stem cells as well as crypt tissues, which are also referred to as enteroids (Sato et al., 2009)(Sato, Stange, et al., 2011a)(Jung et al., 2011)(Miyoshi & Stappenbeck, 2013). This type of organoid can be derived to contain only epithelium (Tsai et al., 2018)(Mahe et al., 2015)(Foulke-Abel et al., 2016), or epithelial and mesenchymal components (Ootani et al., 2009)(Dimarco et al., 2014). Organoids have the ability to expand from a limited source and have a lower probability of genomic alterations following propagation (Fatehullah et al., 2016)(Middendorp et al., 2014).Therefore, organoid cultures are invaluable tools for phenotypic studies, disease modelling, drug screening, and personalized medicine. An in-depth introduction of organoids, different culture techniques, and their applications has been addressed in detail in **Appendix II**.

1.9 Gaps in knowledge on the role of IECs in CeD

IECs are not only targets of tissue damage in CeD (Jabri & Sollid, 2009), but they are also central to the pathogenesis of CeD. IECs actively participate in different steps of CeD development, including facilitating the transport of gluten peptides across the epithelium, expression of stress-induced markers (Setty et al., 2015), and release of catalytically active TG2 (Iversen et al., 2020b) into the intestinal lumen. Although IECs are not professional APCs, such as B cells or DCs, they have been shown to constitutively express MHC class II molecules in the small intestine (Scott et al., 1980)(Kelly et al.,

1988a)(Byrne et al., 2002)(Madrigal et al., 1993)(Biton et al., 2018; Mayer et al., 1991), implying that they possess an essential prerequisite to function as non-professional APCs (Wosen et al., 2018)(C. Heuberger et al., 2021). Inflammatory conditions, particularly in the presence of the proinflammatory cytokine, IFN-γ, have been shown to upregulate the expression of MHC class II in IECs, and this expression has been reported in immune-mediated diseases, such as CeD (Arnaud-Battandier et al., 1986)(Marley et al., 1987), and IBD (C. E. Heuberger et al., 2023)(Malik et al., 2023)(Jamwal et al., 2020)(Smillie et al., 2019b), GvHD (Koyama et al., 2019), as well as during infections, including *Heligmosomoides polygyrus* or *Salmonella enterica* (Biton et al., 2018). However, the functional implications of MHC class II expression in IECs remain controversial. Some studies have attributed the expression of MHC class II to inflammation, while others have associated it with immunosuppression, epithelial differentiation, or renewal (Biton et al., 2018; Jamwal et al., 2020; Koyama et al., 2019; Thelemann et al., 2014). These conflicting results are likely contextual, indicating that a complete understanding will require the investigation of specific diseases. In the context of CeD, the expression of MHC class II in the enterocytes of patients with CeD has been previously reported (Arnaud-Battandier et al., 1986)(Marley et al., 1987)(Fais et al., 1992)(Kelly et al., 1988a). Despite this, the role of epithelial MHC class II in gluten presentation and T-cell activation in CeD has been overlooked, partly due to the lack of a proper celiac epithelium expression CeD risk genes. Therefore, in this thesis I developed an *in vitro* model using organoid monolayers that express CeD risk genes, HLA-DQ2, to investigate the functional consequences of this

expression, specifically focusing on MHC class II-mediated activation of gluten-specific $CD4^+$ T cells.

CHAPTER 2 THESIS OBJECTIVES

THESIS OBJECTIVES

2.1 Thesis scope

Celiac disease (CeD) is a chronic multifactorial, T cell-mediated disease driven by gluten in individuals expressing the CeD risk genes HLA-DQ2 or DQ8. While intestinal epithelial cells (IECs) are primarily recognized as targets of tissue damage in CeD, emerging data suggests they also participate in disease pathogenesis by facilitating the transport of gluten peptides, expressing stress-induced markers, and releasing catalytically active transglutaminase 2 (TG2) into the intestinal lumen. Despite not being professional antigen presenting cells (APCs), such as DC, macrophages, or B cells, it has been demonstrated that IECs constitutively express major histocompatibility class (MHC) II, implying their potential to participate in antigen presentation, particularly in pro-inflammatory conditions. However, this has never been proven in the context of CeD. Given the restriction of gluten peptide presentation to HLA-DQ2 or DQ8, an *in vitro* model expressing human MHC class II, HLA-DQ8, and HLA-DQ2 is required. **Therefore, the overall goal of this thesis is to develop organoid monolayers that express only CeD-associated MHC class II molecules, which are necessary for gluten antigen presentation to T cells.** The identification of a gluten-epithelial MHC class II-T cell interaction would reveal a previously unknown immune role of IECs in CeD which will open new lines of drug development targeting this pathway and its environmental determinants. The model would advance investigation into the drivers of IEC dysfunction and microbial triggers, leading to identification of new targets for disease prevention.

2.2 Thesis aims

Aim 1: To evaluate MHC class II expression in the duodenal epithelium of patients with CeD.

Aim 2: To characterize the extent of immunopathology, inflammation, and MHC class II expression induced by gluten immunization in transgenic mice carrying only the human CeD risk gene, DR3-DQ2.5.

Aim 3: To characterize "humanized" organoid monolayers derived from transgenic mice and use this model to investigate the conditions leading to the expression of MHC class II and its co-stimulatory molecules.

Aim 4: To determine whether MHC class II-expressing organoid monolayers activate gluten-specific $CD4^+$ T cells and whether this response is modulated by opportunist pathogens that metabolize gluten.

The materials and methods used throughout this thesis are described in **Chapter 3**. The results of these aims are presented in **Chapter 4** and discussed in detail in **Chapter 5**. The limitations, future directions, and concluding remarks of this study are described in **Chapters 6, 7, and 8**, respectively.

CHAPTER 3 MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 Human

3.1.1 Human biopsy samples

Biopsy samples from the second part of the duodenum (D2) were obtained during endoscopy from five active (elevated TG2 serology and histology reporting of villous atrophy), and four treated (>2 years on a GFD, Marsh score <1) patients with CeD attending the Hospital Bonorino Udaondo, Argentina (Supplementary Table 1). D2 biopsies from three CeD patients diagnosed by TG2 serology and histology (2F, symptomatic, mean age:34; 1F, asymptomatic, 46 years old) were obtained at the McMaster University Celiac Disease Clinic and used for IEC isolation and flow cytometry. The patients had no concomitant autoimmune or chronic inflammatory diseases, such as type 1 diabetes or inflammatory bowel disease. This study was approved by the Research Ethics Committee of the Hospital Bonorino Udaondo (code #6005) or the Hamilton Integrated Research Ethics Board (HiREB #15311).

3.1.2 Intestinal epithelial cell (IEC) isolation from human D2 biopsies

Tissues were obtained with patients' consent, and all experiments were approved by the Hamilton Integrated Research Ethics Board (HiREB #15311). Freshly obtained D2 biopsies were washed in ice-cold Phosphate buffered saline (PBS) and cut into 1-2mm pieces from confirmed celiac patients undergoing endoscopy for persistent symptoms (n=2) or follow-up (n=1). Two patients had confirmed HLA-DQ2^{+} status, and the genotype was not determined in one patient. IECs isolation was performed as previously described with

modifications (Sato, Stange, et al., 2011b). Briefly, the biopsy fragments were incubated in calcium-and magnesium-free PBS supplemented with 2mM Ethylenediaminetetra-acetic acid (EDTA) (Invitrogen) and 0.5 mM dithiothreitol (DTT; Sigma-Aldrich) at 4°C with gentle shaking for 30min. The pieces were then allowed to settle by gravity and the supernatant, containing the dislodged cells, was collected. The remaining pieces were resuspended in 0.1% bovine serum albumin (BSA) and 2mM EDTA in PBS for an additional 30min at 4°C with gentle rocking. This procedure was repeated five times, and the collected supernatants consisting of isolated IECs were pooled and filtered through a 70μm cell strainer (Falcon). Subsequently, the collected cells were dissociated into single cells by incubating in TrypLE Express (Invitrogen) for 2min at 37°C. The single-cell suspension was then passed through a 40μm cell strainer (Falcon) and stained for flow cytometry to evaluate the expression of MHC class II, CD40, CD86, and CD80.

3.2 Gluten preparation

We generated pepsin-trypsin (PT)-digested gliadin or PT-zein by dissolving crude gliadin or zein (Sigma-Aldrich), respectively, in endotoxin-free 0.2N HCl (Sigma Aldrich) for 2h in a 37°C water bath in the presence of pepsin (Sigma Aldrich). After adjusting the pH to 7.4, trypsin (Sigma Aldrich) was added and the solution was boiled for 30min. The obtained PT-gliadin was stored at -20°C for use in *in vivo* gluten immunization. To stimulate the organoid monolayers, the obtained PT-gliadin or PT-zein was deamidated using transglutaminase 2 (TG2). For this purpose, PT-gliadin or PT-zein was incubated with 50μg/ml guinea pig TG2 (Sigma-Aldrich) in a 1mM $CaCl_2$ (PH 8.5) solution for 2h at

37^{o}C. The final products, deamidated PT-gliadin (DAPT-gliadin) and deamidated PT-zein (DAPT-zein), were sterilized by irradiation at McMaster University's hot-cell facility and stored at -80^{o}C until use in *in vitro* experiments.

3.3 Animal studies

3.3.1 Mice

DR3-DQ2.5 mice (8-12-weeks old) on C57BL/6 background were bred at McMaster University's Central Animal Facility (CAF) and used to culture organoids. These mice lack murine MHC class II genes (MHC class II$^{\Delta/\Delta}$) and express only HLA-DQ2.5 and -DR3, as well as murine CD4 (Z. Chen et al., 2002; Madsen et al., 1999). Double-transgenic DR3-DQ2.5-hCD4(de Kauwe et al., 2009) mice (8-12-weeks old) were bred at McMaster University's CAF and used for the isolation of CD4^{+} T cells and culturing of organoid monolayers in some experiments. In addition to expressing human DR3-DQ2.5, these mice also express human CD4. Original breeding pairs of transgenic DR3-DQ2.5 mice were provided by the University of Melbourne, Australia. In some experiments, HLA-DQ8 mice (8-12-weeks old)(Black et al., 2002), bred at McMaster University's CAF (original breeding pairs provided by Mayo Clinic, USA) were used. All mice were fed a low-fat, gluten-free diet (GFD; Envigo Teklad, TD. 05620) for two generations and throughout the experiments. C57BL/6 mice (8-12 weeks old) were purchased from Taconic Bioscience (Rensselaer, NY, USA) and were used as controls. All mice had unlimited access to water and food and were housed in the same room within the CAF. All treatments were performed by the same researcher at the same time of the day. Female and male mice were used for

all experiments, randomly assigned to experimental groups, and monitored weekly during the experiments until the endpoint. Ethical regulations for animal testing and endpoints were followed in the study. In the co-culture experiment, the mice used for organoid culture were age- and sex-matched with those used for splenic $CD4^+$ T-cell isolation. The experiments were approved by the McMaster University Animal Care Committee and McMaster Animal Research Ethics Board (AREB) and were performed under the Animal Utilization Protocol (AUP) #210930.

3.3.2 In vivo experiments

3.3.2.1 *Mouse immunization and challenge to gluten*

DR3-DQ2.5, DR3-DQ2.5-hCD4, or HLA-DQ8 mice were immunized and challenged with gluten using a previously validated protocol (H. J. Galipeau et al., 2011a). Briefly, mice were gavaged with a combination of pepsin-trypsin-digested gliadin (PT-gliadin; 1mg) (Sigma-Aldrich) and cholera toxin from v*ibrio cholerae* (CT; 25µg) (100B; List Biological Laboratories) once a week for three weeks and then challenged by oral gavage with gliadin (10mg) dissolved in 0.02M acetic acid three times a week for three weeks. Non-immunized (NI) mice were used as controls and received sham gavages during the immunization and challenge phases, consisting of CT (25µg) alone or acetic acid, respectively. Naïve mice that did not receive CT or gluten challenge were used as additional controls in some experiments. All the mice were maintained on a GFD throughout the course of the experiment. Mice were sacrificed 24h after the last gavage. For co-culture

experiments, DR3-DQ2.5-hCD4 mice were sacrificed three weeks after the third immunization dose.

3.3.2.2 Mouse intraperitoneal injections with IFN-γ

Gluten-immunized DR3-DQ2.5-hCD4 mice (8-12 weeks old) were intraperitoneally (i.p.) injected with 1×10^5 U recombinant murine IFN-γ (rmIFN-γ; R&D Systems, 485-MI) in 150µl PBS (gluten-Immunized+IFN-γ-injected). Non-immunized mice were injected with 150µl PBS (NI-PBS). IFN-γ was administered once a week during the gluten immunization phase, followed by twice a week during the challenge phase. Mice were sacrificed 24h after the last dose.

3.3.3 In vitro experiments

3.3.3.1 *Anti-gliadin enzyme-linked immunosorbent assays (ELISA)*

Anti-gliadin IgG antibodies in small intestinal washes were measured by ELISA as previously described (H. J. Galipeau et al., 2011a). In brief, small intestinal washes were collected at endpoint. With 5ml of 0.05M EDTA (Sigma Aldrich) in PBS with phenylmethanesulfonylfluoride fluoride (Sigma Aldrich) and soy trypsin inhibitor (Sigma Aldrich). For ELISA measurements, Gliadin (Sigma Aldrich) was dissolved in 70% ethanol and then diluted in PBS. A 96-well Nunc-Immuno Plates (ThermoFisher) were coated with 50µl/well of a 5µg solution of gliadin or were left uncoated to serve as the control. The plate was then incubated overnight at 4°C. Wells were then blocked using 2% BSA (Sigma-Aldrich) in PBS-0.05% Tween-20 (PBS-T) for 2h at room temperature. Subsequently,

intestinal washes, diluted 1:10, were added at 50µl per well in duplicate and incubated for 2h at room temperature. Each plate contained a positive control sample. After washing with PBS-T, plates were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgA (Abcam) (diluted 1:6000) as secondary antibody for an additional 1h at room temperature. The plates were washed with PBS-T, and 50µl of tetramethylbenzidine (TMB) (Dako) substrate was added to each well. Absorbance was measured at 450 nm on a microplate reader (SpectraMax).

3.3.3.2 *Anti-TG2 ELISA*

Anti-TG2 IgA antibodies in serum were measured by ELISA in the serum and intestinal content as previously described (H. J. Galipeau et al., 2011a). Briefly, Seral samples were collected at end point. TG2 derived from guinea pig liver (Sigma Aldrich). For ELISA analysis, TG2 was diluted in PBS at concertation of 0.1µg per well of a 96-well plate and used to coat a 96-well Nunc-Immuno Plate (Thermo Fisher Scientific), and uncoated to serve as the control. The plate was then incubated overnight at 4^{o}C. Wells were then blocked using 2% BSA (Sigma-Aldrich) in PBS-0.05% Tween-20 (PBS-T) for 2h at room temperature. Subsequently, intestinal washes, diluted 1:10, or serum, diluted 1:100, were added at 50µl per well in duplicate and incubated for 2h or 1h at room temperature, respectively. Each plate contained a positive control sample. After washing with PBS-T, plates were incubated with HRP-conjugated anti-mouse IgA or IgG (Abcam) (diluted 1:6000 or 1:8000, respectively) as secondary antibodies for an additional 1h at room temperature.

3.3.3.3 Intestinal tissue processing

3.3.3.4 *Intestinal hematoxylin and eosin staining*

Cross-sections of two to four pieces of proximal jejunum were fixed in 10% formalin for 48 hours at room temperature. Subsequently, the tissues were processed and embedded in paraffin. The tissue sections were cut at 5 μm thickness on charged glass slides. Hematoxylin and eosin (H&E) staining was performed on 5μm thick tissue. Slides were examined using light microscopy (Olympus) at 20x magnification. Two investigators blinded to treatments conducted all histological analyses and made replicate measurements, as previously described (Caminero, Mccarville, Galipeau, et al., 2019b). Briefly, the vh/cd ratios were evaluated using two to four proximal jejunum sections per mouse and measured by dividing the villus height of ten blinded, well-oriented villi by the corresponding crypt depth. Slides were evaluated under light microscopy (Olympus) at 40x magnification, and image analysis was performed in ImageJ.

3.3.3.5 *Intestinal $CD3^+$ IEL staining*

Cross-sections of two to four pieces of proximal jejunum were fixed in 10% formalin for 48 hours at room temperature. Subsequently, the tissues were processed and embedded in paraffin. Immunostaining for $CD3^+$ cells was conducted on 5 μm thick tissue to assess the presence of $CD3^+$ IELs, as previously described (H. J. Galipeau et al., 2011b). Briefly, sections were stained with rabbit anti-mouse $CD3^+$ primary antibody (1:1000, Dako) and incubated overnight at 4^{o}C, followed by incubation with HRP conjugated anti-rabbit antibody (Dako) for 1h at room temperature. The antibody was visualized using 3,3'–

Diaminobenzidine (DAB) (Dako) as the substrate and counterstained with Mayer's hematoxylin (Sigma-Aldrich). Negative control samples were prepared in the absence of a primary antibody. Slides were evaluated under light microscopy (Olympus) at 40x magnification. Two investigators blinded to the treatment groups conducted the immunostaining analyses and made replicate measurements, as previously described (Biagi et al., 2004). Briefly, $CD3^+$ IELs were quantified by randomly selecting ten villi tips and count the number of positive CD3 cells presents per 20 enterocytes in each villi tip. Data was reported as $CD3^+$ cells/100 IECs.

3.3.3.6 *NanoString gene expression in mouse intestine*

To evaluate gene expression, small intestinal tissues from gluten-immunized and NI DR3-DQ2.5 mice were homogenized, and total RNA (ribonucleic acid) was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA purity and concentration were verified using a NanoDrop 2000 Spectrophotometer Bioanalyzer (Thermo Fisher Scientific). Gene expression was measured using a NanoString nCounter Gene Expression CodeSet (Mouse Inflammation Panel, 254 genes; NanoString Technologies) and analyzed using the nSolver software (version 4.0; NanoString Technologies).

3.3.3.7 *IEC isolation from mouse intestine*

IECs were freshly isolated as previously described (Beyaz et al., 2016). Briefly, the duodenum and proximal jejunum were removed from gluten-immunized and NI DR3-

DQ2.5 mice. The intestinal contents were washed, the intestine was opened longitudinally and washed with ice-cold PBS. The tissue was then sliced into 3-5mm fragments and incubated in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 10mM EDTA (Invitrogen) on ice for 90min while shaking. The pieces were then allowed to settle, and the supernatant containing the dislodged cells from the intestinal epithelium was collected. The pieces were then incubated in fresh DMEM/F12-FBS-EDTA at 4^{o}C for 30 min with shaking. This step was repeated at least five times. The collected supernatants containing IECs were pooled and passed through a 70μm cell strainer (Falcon). Subsequently, the collected cells were dissociated into single cells by incubating with TrypLE Express (Invitrogen) for 5min at 37^{o}C. The single-cell suspension was then filtered through a 40μm cell strainer (Falcon) and stained for flow cytometry to assess the expression of MHC class II molecules and other markers in freshly isolated murine IECs.

3.4 Organoid monolayers

3.4.1 Generation of organoids

3D organoids were established, as previously described (Miyoshi & Stappenbeck, 2013)(Moon et al., 2014). Briefly, DR3-DQ2.5, or in some experiments, gluten-immunized+IFN-γ-injected DR3-DQ2.5-hCD4 mouse proximal small intestine, consisting of the duodenum and proximal jejunum, was opened longitudinally, washed with ice-cold PBS, and incubated in 2mM EDTA for 30min on ice. Villi were then gently scraped, tissue was cut into small pieces (1-2mm), and fragments were incubated in collagenase type I

(Tocris) solution (2mg/ml) for 15min at 37°C. These pieces were then suspended in DMEM/F12 medium containing 10% FBS (Gibco), filtered through a 40µm cell strainer (Falcon), and the isolated crypt fractions were enriched by centrifugation (5min at 300g). Subsequently, the crypts were embedded in 20-25µl of Matrigel (Corning) and seeded in a pre-warmed 24-well plate. Once solidified, 500µl of organoid growth medium was added to each well. The organoid growth medium consisted of advanced DMEM/F12 (Gibco) supplemented with penicillin/streptomycin (1%, Gibco), GlutaMAX (Gibco, 1%), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (10 mM), N2 (1X, Gibco), B27 (1X, Gibco), N-acetyl-1-cysteine (1.25 mM, Sigma), Rho kinase inhibitor Y-276432 (10 µM, Sigma), rm-EGF (50 ng/ml, Peprotech), Noggin (100 ng/ml, Prepotech), Wnt3A-conditioned medium (50% final concentration; prepared in-house), and R-spondin 1-conditioned medium (20% final concentration; prepared in-house). The organoids were incubated at 37°C and 5% CO_2 and the medium was refreshed every 2-3 days. Organoids were passaged every seven days by mechanical disruption and enzymatic digestion.

3.4.2 Generation of organoid monolayers

Organoid monolayers were cultured as previously described with modifications (Moon et al., 2014). Briefly, 24-Transwell cell culture inserts (VWR) with a 0.4µm or 3µm pore size were coated with collagen type I (rat tail, 100µg/ml, Sigma) and Matrigel (5%, Corning) for 1h at 37°C. Organoid single-cell suspensions were derived by releasing 3D organoids from the Matrigel dome using Cell Recovery Solution (Corning) and incubating them in TryplE Express (Gibco) for 5min at 37°C. Single cells were then enriched by

centrifugation and resuspended in growth medium at 10^6 cells/ml, and 100µl of the cell suspension was plated onto each coated insert. Next, 60 µl and 100µl of the growth medium were added to the basolateral and apical compartments, respectively. The medium was changed every 2-3 days. Transepithelial electrical resistance (TEER) was measured according to the manufacturer's protocol using an Epithelial Volt-Ohm Meter (Millicell ERS-2) to monitor the integrity of the organoid monolayers. To promote differentiation of organoid monolayers, growth medium was replaced with a differentiation medium composed of growth medium without Wnt3A-conditioned medium and 10% R-Spondin 1-conditioned medium. Downstream analyses were performed on organoids from passage ≥3 that were cultured for 18-21 days.

Single-cell suspensions of organoid monolayers for flow cytometry experiments were obtained by dissociation of organoid monolayers using TrypLE Express (Gibco) for 10min at 37°C, followed by mechanical disruption and passage through a 40µm cell strainer (Falcon). The obtained single-cell suspension was then used for flow cytometry staining.

3.4.3 Organoid monolayer stimulation

Organoid monolayers were stimulated for 24 hours with 150µg/ml of the sterilized solution of deamidated PT-gliadin (DAPT-gliadin) or deamidated PT-zein (DAPT-zein). In separate experiments, organoid monolayers were treated with 10ng/ml of recombinant mouse IFN-γ (rmIFN-γ)(485-MI, R & D systems) for 24h.

3.5 Immunofluorescence staining

3.5.1 Immunofluorescence staining of human D2 biopsy samples

Human biopsy tissues from the second portion of the duodenum (D2) were obtained during endoscopy from five active (elevated anti-TG2 serology and histology reporting villous atrophy) and four treated (>2 years on a GFD) patients with CeD. All the tissues were fixed in 10% formalin for 24-48h at room temperature and embedded in paraffin. Paraffin-embedded sections (4μm) were cut and mounted on coated slides. Heat-induced antigen retrieval was performed by steaming sections in citrate buffer (pH 6.0) for 20min. Non-specific binding was blocked with 2% BSA for one hour at room temperature. The sections were then incubated with monoclonal rabbit anti-EpCAM antibody (1:300; MA5-29246; Invitrogen) overnight at 4^{o}C and then with Alexa 594 conjugated donkey anti-rabbit IgG antibody (1:500; A21207; Invitrogen) for 1h at room temperature. For the second labeling of MHC class II, sections were incubated in FITC-conjugated monoclonal mouse anti-HLA-DR-DQ antibody (1:400; B5329, LS Bio) or PE-conjugated monoclonal mouse anti-HLA-DQ antibody (NBP3-08720, Novus Biologicals) overnight at 4^{o}C. After washing, the sections were mounted using Fluoroshield mounting medium containing DAPI (ab104139; Abcam) for nuclear counterstaining. Negative control staining was performed with isotype controls for HLA-DR-DQ and EpCAM antibodies, using FITC-conjugated monoclonal mouse IgG2a (C742275, LS Bio) and monoclonal rabbit IgG (086199, Invitrogen), respectively. Slides were prepared in the absence of primary antibodies as an additional negative control. All the steps for isotype and negative control staining were the same as those mentioned above. Images were acquired using a Nikon

A1R inverted confocal microscope (Nikon) at 20X magnification and composed using Nikon processing software (NIS elements, Nikon) and Adobe Photoshop (Photoshop, 2020).

Two investigators blinded to the treatment groups performed replicate measurements and quantification. Quantification of MHC class II^+ IECs was performed using two to four D2 sections per patient, and MHC class II^+ cells were counted per 20 IECs in ten randomly chosen villi or crypts. Data are presented as MHC class II^+ cells per hundred IECs.

3.5.2 Immunofluorescence staining of mouse proximal intestine

The duodenum and proximal jejunum were removed from gluten-immunized or NI DR3-DQ2.5 as well as gluten-immunized+IFN-γ-injected or NI+PBS-injected DR3-CD2.5-hCD4 mice, embedded in OCT and frozen in liquid nitrogen. OCT-sections of 10μm thick were cut using a cryo-microtome, mounted on positively charged slides, and fixed with 10% formalin for 10-15min at room temperature. After non-specific blocking with 2% BSA for 1h at room temperature, the sections were incubated with a polyclonal rabbit anti-EpCAM antibody (1:400, 50591, Sino Biological) overnight at 4ºC, followed by incubation with a secondary antibody, Alexa Flour 594 conjugated donkey anti-rabbit IgG antibody (1:500, A21207, Invitrogen), for 1h at room temperature. For double labelling with a specific anti-MHC class II antibody, sections were incubated with FITC-conjugated monoclonal mouse anti-HLA-DR-DQ antibody (1:400, B5329, LS Bio) overnight at 4ºC. After washing, the sections were mounted with Fluoroshield mounting medium with DAPI

(ab104139, Abcam) for nuclear counterstaining. Negative control samples were prepared in the absence of primary antibodies. Negative control staining was performed with isotype controls for HLA-DR-DQ and EpCAM antibodies using FITC-conjugated monoclonal mouse IgG2a (C742275, LS Bio) and monoclonal rabbit IgG (086199, Invitrogen), respectively. Slides were prepared in the absence of primary antibodies as an additional negative control. All the steps for isotype and negative control staining were the same as those mentioned above. Images were acquired using a Nikon A1R inverted confocal microscope (Nikon) at 20X magnification and composed using Nikon processing software (NIS elements, Nikon) and Adobe Photoshop (Photoshop, 2020). Two investigators blinded to the treatment groups performed replicate measurements and quantification. Quantification of MHC class II^+ IECs was performed in 3-4 sections per mouse, and MHC class II^+ cells were counted per 20 IECs in ten randomly chosen villi or crypts. Data are presented as MHC class II^+ cells per hundred mouse IECs.

3.5.3 Immunofluorescence staining of organoids monolayers

The organoid monolayer cell composition and polarity were assessed by immunofluorescence staining, as previously described (Thorne et al., 2018). Briefly, organoid monolayers were fixed in 4% paraformaldehyde for 20min, permeabilized with 0.1% Triton X-100 in PBS (Sigma-Aldrich) for 30min and blocked with 10% normal donkey serum (Invitrogen) and 0.1% Triton X-100 in PBS for 1h at room temperature. Subsequently, the monolayers were incubated overnight with one of the following primary antibodies specific for: E-cadherin (1:400, AF748, R&D Systems), Villin 1 (1:400,

GTX09940, GeneTex), Mucin 2 (1:400, NBP1-31231, Novus Biologicals), Chromogranin A (1:400, NB120-15160, Novus Biologicals) or Lysozyme (1:400, PA1-29680, Thermo Fisher Scientific). The monolayers were then incubated for 1h with one of the following secondary antibodies: Alexa Fluor 488 donkey anti-goat IgG (H+L; 1:1000, A32814, Thermo Fisher Scientific) or Alexa Fluor 647 donkey anti-rabbit IgG (H+L; 1:1000, A32795, Thermo Fisher Scientific). After extensive rinsing, the Transwell membranes were excised from the Transwell inserts using a scalpel and mounted on a glass slide using ProLong Antifade Mountant with DAPI (Invitrogen). Negative control samples were prepared in the absence of primary antibodies. Images were acquired using a Nikon A1R inverted confocal microscope (Nikon) and were composed using the Nikon processing software (NIS Elements, Nikon) and Adobe Photoshop (2020).

3.6 Co-cultures experiment

3.6.1 Bacterial culture and gluten digestion

We used the wild-type (WT) *P. aeruginosa* PA14 and its isogenic non-functional *lasB* mutant strain (*P. aeruginosa las*B$^{\Delta/\Delta}$)(*las*B*::MAR2xT7*) from the available non-redundant transposon mutant library (Liberati et al., 2006). Bacteria were grown in tryptic soy broth, and gentamicin (50µg/ml) was added to select for the Tn5 transposon in the mutant control strain (Caminero et al., 2016). PT-gliadin (15mg/ml) was added to 8ml of Optim-MEM (Gibco) medium in the presence of 10^4 colony-forming units (CFU) of either bacterial strain, followed by incubation at 37^{o}C for 4h. After incubation, the bacteria-digested PT-gliadin substrates were boiled for 15min, followed by deamidation. The final

products were sterilized by irradiation at McMaster University's hot-cell facility and stored at -80°C. Bacterial-digested gliadin preparations were then used in co-culture experiments, as described below.

3.6.2 Organoid monolayer-hCD4$^+$ T cell co-cultures

Organoid monolayers derived from gluten-immunized DR3-DQ2.5, or in some experiments from gluten-immunized+IFN-γ-injected DR3-DQ2.5-hCD4 mice, were treated with 10ng/ml rmIFN-γ (R&D Systems, 485-MI) for 24h before the co-culture assay to induce the expression of MHC class II and co-stimulatory molecules. The IFN-γ-containing medium was then removed and the organoid monolayers were washed three times with expansion media. Organoid monolayers were then treated with 150µg/ml DAPT-gliadin, DAPT-zein, or WT *P. aeruginosa* PA14 digested DAPT-gliadin for 12h. Monolayers treated with media alone, *P. aeruginosa* PA14 supernatant, or DAPT-gliadin digested with the isogenic *P. aeruginos*a non-functional *lasB* mutant (*las*B$^{\Delta/\Delta}$) were used as controls. On the day of the co-culture experiment, hCD4$^+$ T cells were isolated from the spleens of gluten-immunized DR3-DQ2.5-hCD4 mice using column-based magnetic microbeads (CD4$^+$ T cell negative-selection kit; Miltenyi Biotec), according to the manufacturer's instructions. To assess CD4$^+$ T cell proliferation, the CellTrace Violet (CTV) Proliferation Kit (Thermo Fisher Scientific) was used, according to the manufacturer's instructions. Briefly, isolated hCD4$^+$ T cells were incubated for 20min with CTV dye diluted in PBS and washed three times with Roswell Park Memorial Institute medium (RPMI) medium containing 10% FBS. Subsequently, CTV-labeled CD4$^+$ T cells

were seeded at a density of 2.5×10^5 cells per well into the basolateral side of organoid monolayers in the presence of 10ng/ml rhIL-7 (20007, PeproTech) to preserve the viability of cultured $CD4^+$ T cells. After four days of co-culture, the collected $hCD4^+$ T cells were assessed for CTV dilution, as a marker of $CD4^+$ T cell proliferation, and for the expression of T cell activation markers (CD25, CD69, and CD44) by flow cytometry. As a positive control, isolated $hCD4^+$ T cells were seeded on plate-bound anti-CD3 (5µg/ml, 145-2C11, BD Biosciences) and anti-CD28 (2µg/ml, 37.51, BD Biosciences) antibodies for four days.

3.7 Flow cytometry

3.7.1 Flow cytometry on freshly isolated IECs from human D2 biopsy samples

The single-cell suspensions obtained from isolated IECs from human D2 biopsies, as described above, were resuspended in PBS and stained with the fixable viability dye eFluor 780 (1:5000, eBioscience) for 30min in the dark to exclude dead cells. After washing, the single-cell suspension was stained for Fc-receptor blockade by incubation with TruStain FcX (1:20, BioLegend) for 15min. Subsequently, cell suspensions were stained with the following specific anti-human antibodies: CD45 (1:200, CD45-AF700, (2D1)), EpCAM (1:200, EpCAM-AF647, (G8.8)), (1:100, CD86 (1:100, CD86-BV421 (GL-1)), and CD80 (1:100, CD80-BV605 (16-10A1)) (all from BioLegend); HLA-DQ (1:50, HLA-DQ-PE, (rSPVL-3); R&D Systems), and HLA-DR-DP (1:100, HLA-DR-DP-FITC (MEM-136), Invitrogen). The flow cytometry data were acquired on a BD LSR Fortessa using FACSDiva and analyzed using FlowJo software (v10.8.1; TreeStar, Ashland, Ore, USA). The expression of a specific marker in freshly isolated human IECs

from biopsy samples was determined by gating on the live $CD45^{-}EpCAM^{+}$ population. The HLA-DQ population was identified by gating on the live $CD45^{-}EpCAM^{+}HLA\text{-}DQ^{+}$ cells.

3.7.2 Flow cytometry on organoid monolayers or freshly isolated IECs from mice

Single-cell suspension of organoid monolayers or freshly isolated IECs from mouse proximal intestinal tissue were obtained, as described above. The obtained single-cell suspensions were resuspended in PBS and stained with the fixable viability dye eFluor 780 (1:5000, eBioscience) for 30min in the dark to exclude dead cells. After washing, the single-cell suspension was stained for Fc-receptor blockade by incubation with anti-CD16/32 (1:50, BioLegend, (93)) for 15 min. Subsequently, cell suspensions were stained with the following specific anti-mouse antibodies: CD45 (1:200, CD45-FITC or CD45-AF700 (30-F11)), EpCAM (1:200, EpCAM-AF647, (G8.8)), HLA-DQ (1:100, HLA-DQ-PE, (HLADQ1)), CD86 (1:100, CD86-BV421 (GL-1)), CD80 (1:100, CD80-BV605 (16-10A1)), CD71 (1:100, CD71-BV421 (RI7217)), EpCAM (1:200, EpCAM-PE/Cy7, (G8.8)) (all from BioLegend); HLA-DR-DP-DQ (1:100, HLA-DR-DP-DQ-PE (WR18), eBioscience), CD40 (1:100, CD40-BV711 (3/23), BD Bioscience), Qa-1 (1:100, Qa1-BV711 (6A8.6F10.1A6), BD Bioscience), Rae-1 (1:100, RE-1-BV421 (186107), BD Bioscience), Rae-1 (1:100, RE-1-PE (FAB17582), R&D systems). Stained cells were acquired using BD LSR Fortessa flow cytometer (BD Biosciences), and analysis was performed with FlowJo 10 software (v10.8.1; TreeStar, Ashland, Ore). The expression of a specific marker on organoid monolayers was determined by gating on the live $CD45^{-}EpCAM^{+}$ population. The expression of a specific marker in organoid monolayers was

determined by gating on the live $CD45^{-}EpCAM^{+}$ population. The MHC class II population in freshly isolated mouse IECs or organoid monolayers was determined by gating on the live $CD45^{-}EpCAM^{+}HLA\text{-}DR\text{-}DP\text{-}DQ^{+}$ cell population.

3.7.3 Phenotypic analysis of $hCD4^{+}$ T cell

Flow cytometry on $hCD4^{+}$ T cells was performed by collecting the $CD4^{+}$ T cells after four days of co-culture with organoid monolayers and resuspending them in PBS for staining with fixable viability dye eFluor 780 (1:5000, eBioscience) for 30min in the dark to exclude dead cells. After washing, the cell suspension was stained for Fc-receptor blockade by incubation with anti-CD16/32 (1:50, BioLegend, (93)) for 15 min. Subsequently, cell suspension was stained with antibodies specific for lineage markers: CD45 (1:200, CD45-FITC (30-F11)), CD3 (1:200, CD3-BV785 (17A2)), hCD4 (1:200, hCD4-BV605 (SK3)), mCD4 (1:200, mCD4-BV711 (RM4-5)), and phenotypic markers CD25 (1:00, CD25-APC (PC61)), CD25 (1:00, CD25-BV421 (PC61)), CD69 (1:00, CD69-PE (H1-2F3)), and CD44 (1:100, CD44-PE/Cy7 (IM7)) (all from BioLegend). T cell proliferation was assessed using the CellTrace Violet Proliferation Kit (ThermoFisher) per the manufacturer's instructions. In addition, the purity of the isolated $CD4^{+}$ T cells and contamination with other splenocytes was evaluated using the following antibodies: CD19, CD11b, and CD11c, and F9/80 (all from BioLegend). The flow cytometry data were acquired on a BD LSR Fortessa using FACSDiva and analyzed using FlowJo software (v10.8.1; TreeStar, Ashland, Ore, USA). The expression of a specific marker on $hCD4^{+}$ T cells was determined by gating on the live $CD45^{+}CD3^{+}hCD4^{+}$ population.

A fluorescence minus one (FMO) control was included in all the flow cytometry experiments. All gating strategies are presented in the Supplementary Figures. All flow cytometry data were acquired on a BD LSR Fortessa using FACSDiva and analyzed using FlowJo software (v10.8.1; TreeStar, Ashland, Ore, USA).

3.7.4 Cytokines and chemokines secretion analysis

The cytokine and chemokine concentrations in the supernatants of the co-culture or monolayer-only culture experiments were measured using flow cytometry. The concentrations of mouse cytokines and chemokines, including IL-2, IFN-γ, IL-17A, IL-15, Tumour necrosis factor (TNF)-α, IL-6, IL-1α, IL-1β, CXCL-10, CXCL-1, CXCL-5, and CCL-20, were determined using a multiplex cytometric bead-based LEGENDplex™ immunoassay kit (BioLegend) according to the manufacturer's protocol. The data were acquired on a BD LSR Fortessa using FACSDiva (BD Biosciences) and analyzed using LEGENDplex™ software (BioLegend). Only statistically significant data were presented.

3.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism (ver. 9). The data are shown as dot plots, with each dot representing the biological marker of an individual mouse. Data were presented as mean ± standard error of the mean (SEM). Normality of the data was confirmed with the Shapiro-Wilk statistical test. Statistical comparisons were performed with a one-way analysis of variance test (ANOVA) to conduct multiple group comparisons with Tukey's post-hoc correction test. The two-tailed unpaired Student's *t*-test

was applied to measure the differences between the two groups. Nanostring nCounter gene expression analysis was performed by nSolver 4.0 software (NanoString Technologies) using Student's *t-tests*, and only statistically different genes were shown. Pearson correlation coefficients was used to evaluate the linear association between the two variables. A *p*-value of <0.05 was considered statistically significant.

Experiments were planned with the minimum number of animals per group to observe significant differences, as per historical records. No data points were excluded as outliers in this study. Samples where technical issues were encountered (such as unevaluable histological section) were removed from analysis. Information about the biological replicates or the number of animals, specific *p* values, and how data were presented can be found in the figure legends.

CHAPTER 4 RESULTS

RESULTS

4.1 Patients with active CeD have a higher IEC expression of MHC class II

IECs constitutively express MHC class II molecules, which enables them to potentially function as non-conventional APCs (Biton et al., 2018; Byrne et al., 2002; R. M. Hershberg et al., 1998; Hundorfean et al., 2007; Kelly et al., 1988a; Madrigal et al., 1993; Scott et al., 1980). This expression is further amplified in response to inflammation, particularly through the pro-inflammatory cytokine, IFN-γ (Van Der Kraak et al., 2021)(Thelemann et al., 2014)(Malik et al., 2023). Additionally, increased MHC class II expression in IECs has been demonstrated in immune-mediated diseases, such as GvHD (Koyama et al., 2019), CeD (Marley et al., 1987)(Fais et al., 1992)(Arnaud-Battandier et al., 1986), and IBD (Jamwal et al., 2020)(Thelemann et al., 2014)(Smillie et al., 2019b)(Malik et al., 2023). Thus, the role of IECs in expressing MHC class II places them at a key junction for relaying environmental signals to the adaptive immune system (e.g. T cells) within the small intestine. To investigate this, I first evaluated the expression of MHC class II in the epithelium of patients with CeD, which set the stage for further investigation into how IECs contribute to CeD pathogenesis through their interaction with specific immune cells that drive intestinal damage in CeD.

I determined the effect of CeD activity on IEC MHC class II expression by evaluating its expression in D2 biopsies (Figure 4.1A), the most affected site in CeD, obtained from active and treated (on gluten-free diet) CeD patients recruited at C. Bonorino Udaondo Hospital, Argentina (Cohort 1) (Supplementary Table 1). Immunofluorescence staining revealed the expression of MHC class II in the lamina propria of both groups,

corresponding to professional APCs residing within the lamina propria. MHC class II expression was prominently observed in IECs from active compared with treated CeD patients who achieved a Marsh score≤1 (Figure 4.1B), suggesting that increased inflammation is associated with higher epithelial MHC class II expression. No staining was detected in isotype controls (Supplementary Figure 1). Additionally, using flow cytometry on freshly isolated IECs from symptomatic and asymptomatic CeD patients, I confirmed there is expression of the disease-associated isotype of MHC class II, HLA-DQ (Figure 4.2A; Supplementary Figure 2). The expression of HLA-DQ in the epithelium of CeD patients was further evident by immunofluorescence staining on duodenal biopsies from active and treated patients with CeD using HLA-DQ antibody (Figure 4.2B). I also identified the expression of the main co-stimulatory molecules such as CD40, CD80, and CD86 on isolated IEC (Figure 4. A-C). Collectively, these data revealed that IECs in CeD exhibit characteristics of APCs. However, the functional consequences of MHC class II and co-stimulatory molecule expression remain unexplored.

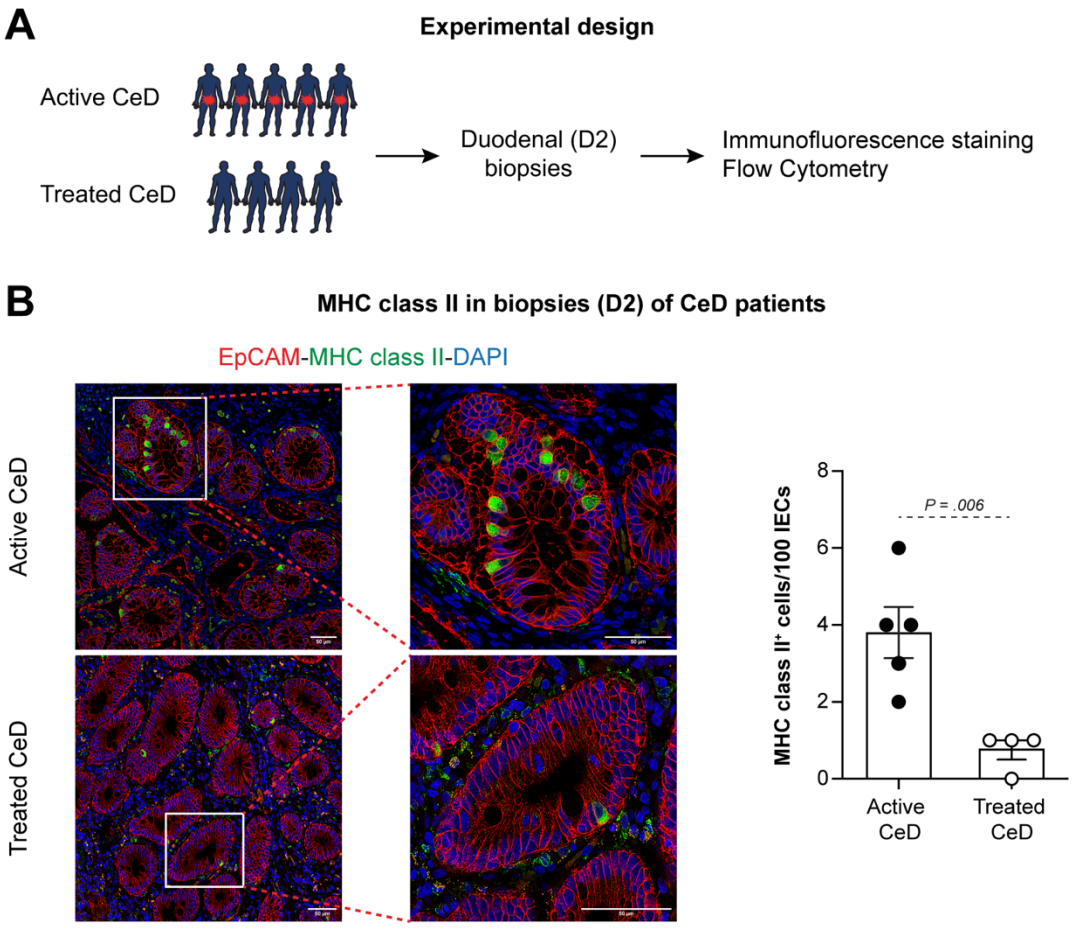


Figure 4.1 CeD activity demonstrates higher expression of MHCII molecules in intestinal epithelial cells (IECs). (A) Duodenal biopsies (D2) were obtained from patients with active (n=5) and treated CeD (n=4), and MHCII or HLA-DQ expression was assessed by immunostaining or flow cytometry. **(B)** Representative immunofluorescence staining of D2 biopsies for $MHCII^+$ using HLA-DR-DQ antibody ($EpCAM^+$: red; $MHCII^+$: green; DAPI: blue; Scale bar, 50µm), and quantification of $MHCII^+$ cells per 100 intestinal epithelial cells (IECs) in D2 biopsies. Data are presented as mean±SEM, with each dot representing an individual patient with CeD. *P*-value was determined using a two-tailed unpaired Student's t-test.

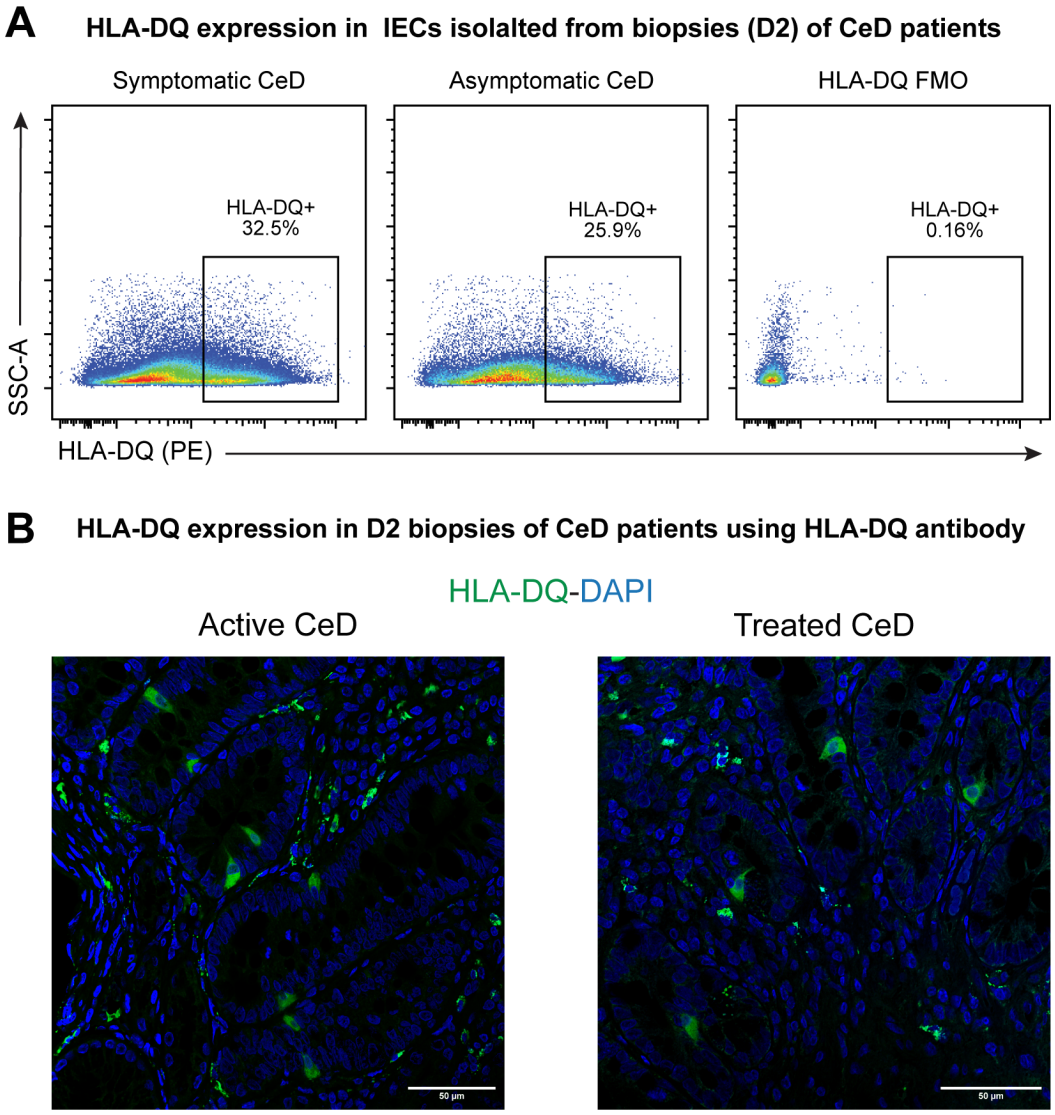


Figure 4.2 Intestinal epithelial cells of patients with CeD express HLA-DQ isotype of MHC class II. (A) Percentage of HLA-DQ+ cells, gated on live $CD45^{-}EpCAM+$ cells using the HLA-DQ antibody from D2 biopsies from symptomatic and asymptomatic CeD patients, assessed by flow cytometry. Representative dot plots are shown. **(B)** Representative immunofluorescence staining of D2 biopsies for $HLA\text{-}DQ^{+}$ using HLA-DQ antibody ($HLA\text{-}DQ^{+}$: green; DAPI: blue; Scale bar, 50µm). See Supplementary Figure 2 for the gating strategy.

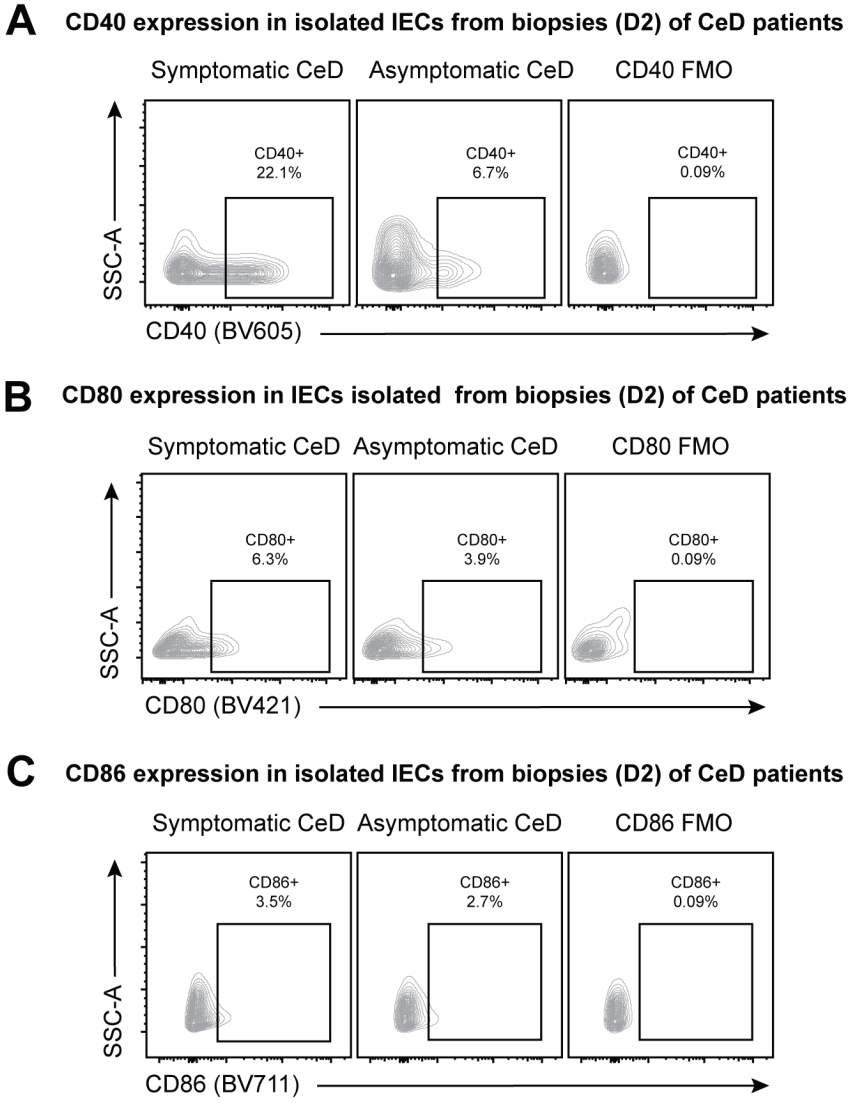


Figure 4.3 Intestinal epithelial cells (IECs) from celiac disease (CeD) duodenal biopsies (D2) express co-stimulatory molecules. Percentage of **(A)** $CD40^+$, **(B)** $CD80^+$, and **(C)** $CD86^+$ cells, gated on live $CD45^-EpCAM^+$ cells from D2 biopsies from symptomatic and asymptomatic CeD patients, assessed by flow cytometry. Representative dot plots and fluorescence minus one (FMO) are shown. See Supplementary Figure 2 for the gating strategy.

4.2 Characterization of DR3-DQ2.5 mice following gluten immunization

4.2.1 Gluten immunization induces adaptive immune response in DR3-DQ2.5 mice

To dissect the functional consequences of class II expression in CeD, I used a humanized mouse model based on transgenic expression of the human CeD risk gene, DR3-DQ2.5. I first determined the level of inflammation induced by gluten immunization in these mice. Gluten-immunized mice were orally gavaged with pepsin-trypsin (PT) digest of gliadin in conjugation with cholera toxin (CT) once a week for three weeks during the immunization phase, followed by a challenge phase in which they received gliadin three times a week for three weeks (Figure 4.4A). NI mice received sham treatment during the immunization and challenge phases and were used as controls (Figure 4.4A). All mice were maintained on a GFD during the experiments. First, the adaptive immune responses to gluten were determined by assessing the production of anti-gliadin (AGA) and anti-translglutaminase (TG2) antibodies. Gluten-immunized DR3-DQ2.5 had high levels of IgA AGA in intestinal content compared with NI mice (Figure 4.4B). While anti-gliadin IgA are common in CeD patients in the gut lumen and represent a local immune response to gliadin (Volta et al., 1985), their specificity is low. However, there was also a statistical trend for higher TG2 IgA in the intestinal content of gluten-immunized DR3-DQ2.5 mice (Figure 4.4C). These results are in accordance with previous reports in other mouse models of gluten sensitivity (E. F. Verdu et al., 2008)(H. J. Galipeau et al., 2011a). Additionally, a significant increase in serum levels of anti-TG2 IgG antibodies in gluten-immunized DR3-DQ2.5 mice was also identified (Figure 4.4D). This increased expression of anti-TG2 IgG finding has not been previously corroborated in transgenic mouse models (E. F. Verdu et

al., 2008)(H. J. Galipeau et al., 2011a)(Abadie et al., 2020). These results indicate elevation of serum TG2 IgG autoantibodies that are specific for diagnosis of human CeD in immunized DR3-DQ2.5 mice.

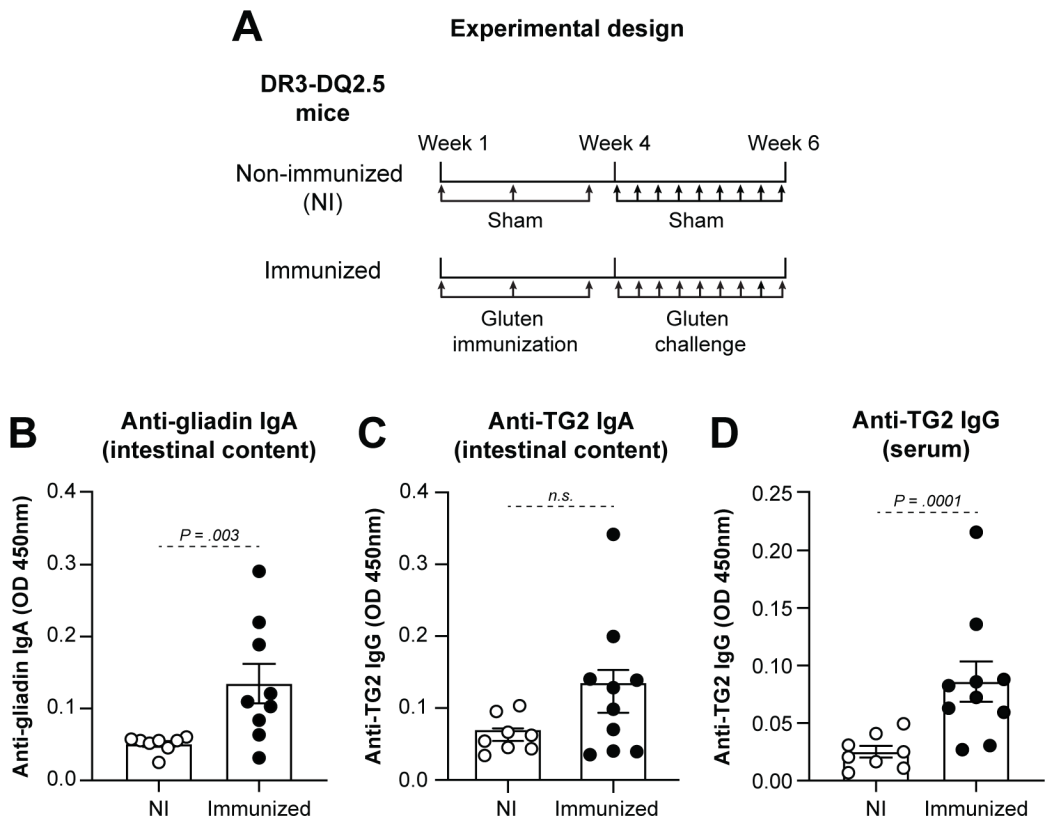


Figure 4.4 Gluten immunization induces production of anti-gliadin and anti-TG2 antibodies in DR3-DQ2.5 mice. (A) Non-immunized (NI) DR3-DQ2.5 mice received sham gavages and were used as controls (open dots). Gluten-immunized mice received PT-gliadin and cholera toxin, followed by gluten challenges (black dots). **(B)** Anti-gliadin IgA levels in the intestinal contents in NS (n=8) and immunized (n=9) mice. **(C)**. Anti-*TG2* Ig*A* levels in the intestinal contents in NS (n=8) and immunized (n=9) mice. **(D)** Serum anti-TG2 IgG levels in the NI (n=8) and immunized (n=10) mice. One sample from the immunized group in (B) was removed from the analysis because of technical issues. Data are presented as mean±SEM, with each dot representing an individual mouse. All *P*-values were determined using a two-tailed unpaired Student's t-test.

4.2.2 Gluten immunization induces moderate immunopathology in DR3-DQ2.5 mice

Immunopathology in these mice was assessed by measuring $CD3^+$ IEL in the tips of the villi and villus height-to-crypt depth ratios. The results indicated that gluten-immunized mice exhibited a significant increase in the number of $CD3^+$ IEL within the tips of villi compared with NI mice (Figure 4.5A). Specifically, gluten-immunized DR3-DQ2.5 mice displayed twice as many $CD3^+$ IEL cells per 100 enterocytes compared with the NI mice, suggesting an elevated presence of IELs within the villi of gluten-immunized mice. Additionally, gluten-immunized DR3-DQ2.5 mice had significantly lower vh/cd ratios (4.7) compared with NI mice (6.2) (Figure 4.5B). These findings suggest that gluten immunization induced moderate immunopathology in DR3-DQ2.5 mice.

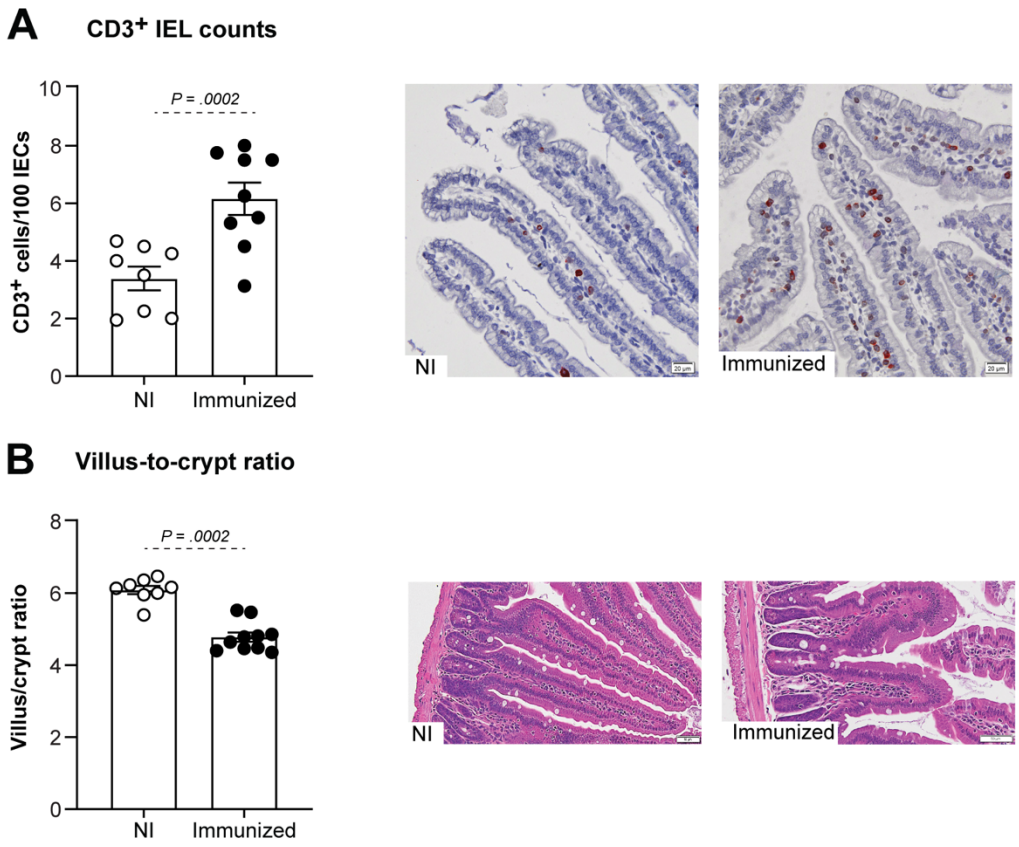


Figure 4.5 Gluten immunization induces moderate immunopathology in DR3-DQ2.5 mice. (A) Quantification of $CD3^+$ intraepithelial lymphocytes (IELs) per 100 intestinal epithelial cells (IECs) from NI (n=8) and immunized (n=9) mice. Representative $CD3^+$ stained sections of the duodenum and proximal jejunum, where $CD3^+$ IELs are stained in red, are shown. Scale bar, 20 µm. **(B)** Quantification of the small intestinal villus height-to-crypt depth (vh/cd) ratios in NI (n=8) and immunized (n=10) mice. Representative hematoxylin and eosin (H&E)-stained small intestinal sections are shown. Scale bar: 50µm. One sample from the immunized group in (B) was removed from the analysis because of technical issues. Data are presented as mean±SEM, with each dot representing an individual mouse. All *P*-values were determined using a two-tailed unpaired Student's *t*-test.

4.2.3 Gluten immunization induces inflammation in DR3-DQ2.5 mice

The level of inflammation induced by gluten immunization in these mice was then determined. For this, I assessed the transcript levels of 254 genes associated with inflammatory pathways in proximal small intestinal tissues obtained from gluten-immunized or NI DR3-DQ2.5 mice. Gluten immunization resulted in the upregulation of several pro-inflammatory genes, such as *Il1a, Tlr3*, and *Cfb*, in gluten-immunized mice (Figure 4.6). Notably, the expression of *Il15*, a crucial cytokine involved in the development of CeD, was increased in gluten-immunized DR3-DQ2.5 mice compared with control group. Genes related to innate immune functions, such as *Rhoa, Tollip*, and MAP kinases (MAPK), including *Mapk3*, *Mapk1*, and *Map3k1*, were also overexpressed in gluten-immunized mice (Figure 4.6). Overall, these findings indicate that gluten immunization in DR3-DQ2.5 mice induced an inflammatory response, as demonstrated by the increased expression of pro-inflammatory genes, some of which contribute to the pathogenesis of CeD.

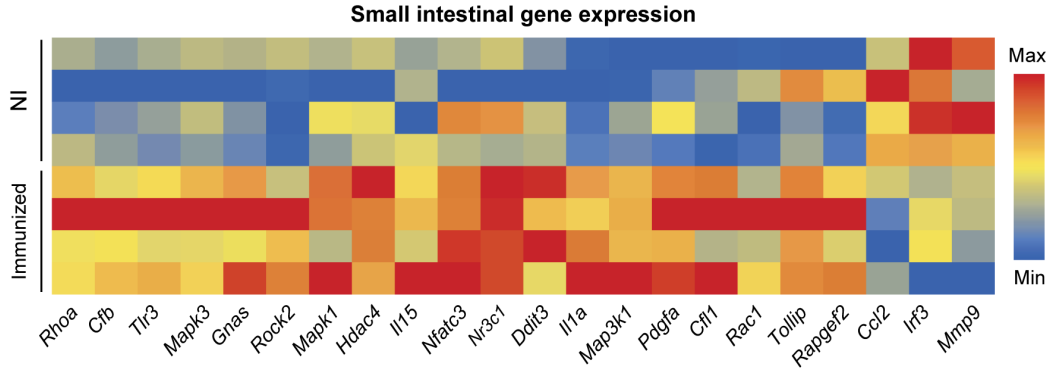


Figure 4.6 Gluten immunization upregulates proinflammatory genes in DR3-DQ2.5 mice. Heat map of significantly altered genes in the small intestinal tissues of NI (n=4) and

immunized (n=4) mice. Only genes with statistically significance expression between the two groups are presented. Each raw represents one mouse.

4.2.4 Gluten immunization induces the expression of epithelial MHC class II in DR3-DQ2.5 mice

Small intestinal epithelial cells are known to constitutively express MHC class II molecules (Scott et al., 1980)(Madrigal et al., 1993)(Kelly et al., 1988a)(Byrne et al., 2002)(Biton et al., 2018)(Hundorfean et al., 2007). This characteristic may potentially enable them to act as non-conventional APCs. The expression of MHC class II in IECs has been found to be upregulated in response to inflammation, particularly in the presence of IFN-γ (Malik et al., 2023)(Thelemann et al., 2014), and in immune-mediated diseases such as GvHD, IBD, and CeD (Koyama et al., 2019b; Smillie et al., 2019b)(Jamwal et al., 2020)(Malik et al., 2023)(Marley et al., 1987)(Arnaud-Battandier et al., 1986). Thus, I evaluated the impact of gluten immunization on MHC class II expression in IECs of DR3-DQ2.5 mice. Phenotypical characterization via immunofluorescence staining of MHC class II on proximal small intestine sections revealed that NI DR3-DQ2.5 mice had lamina propria, but no epithelial MHC class II expression. In contrast, gluten-immunized mice exhibited MHC class II expression in both lamina propria and epithelial cells (highlighted with white arrows in the figure below) (Figure 4.7A). These findings are further supported by the expression of MHC class II in freshly isolated IECs from the duodenum and proximal jejunum of DR3-DQ2.5 mice. Flow cytometric analysis showed enhanced expression of MHC class II in IECs from gluten-immunized mice as compared with that

from NI mice (Figure 4.7B). The representative flow cytometry gating strategy on isolated IECs from murine tissues is shown in Supplementary Figure 4.

I also investigated the expression of Qa-1, a stress-induced non-classical MHC class I marker and a murine ligand for CD94/NKG2 receptors predominantly present on natural killer (NK) cells (Jabri et al., 2000)(Vance et al., 1998), in the epithelium of DR3-DQ2.5 mice following gluten immunization (Figure 4.8A). Using flow cytometry, I found that epithelial expression of Qa-1 was higher in gluten-immunized mice than in NI mice (Figure 4.8B). Therefore, these data highlight that gluten immunization of DR3-DQ2.5 mice induces an inflammatory response in the small intestine that primes the intestinal epithelium to upregulate MHC class II expression and other markers of inflammation.

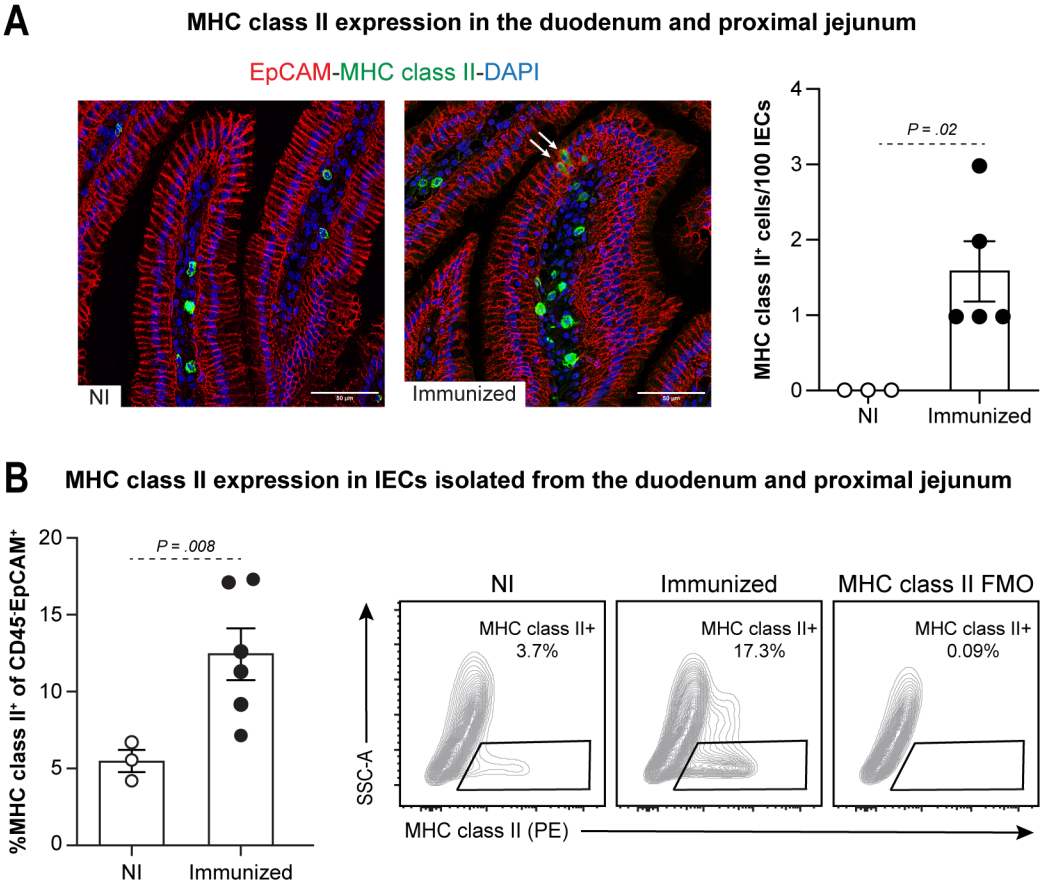


Figure 4.7 Gluten immunization induces MHC class II expression in DR3-DQ2.5. (A) Representative immunofluorescence staining of the duodenum and proximal jejunum for MHC class II$^+$ (white arrows; EpCAM$^+$: red; MHC class II$^+$: green; DAPI: blue; Scale bar, 50µm). Quantification of MHC class II$^+$ cells per 100 intestinal epithelial cells (IECs) from NI (n=3) and immunized (n=5) mice. **(B)** Percentage of MHC class II-expressing cells gated on live CD45$^-$EpCAM$^+$ cells from IECs isolated from the duodenum and proximal jejunum of NI (n=3) and immunized (n=6) DR3-DQ2.5 mice. Representative dot plots showing MHC class II$^+$ cells, gated on live CD45$^-$EpCAM$^+$ cells using an HLA-DR-DP-DQ antibody. Data are presented as mean±SEM, with each dot representing an individual mouse. All *P*-values were determined using a two-tailed unpaired Student's *t*-test. See Supplementary Figure 4 for the gating strategy.

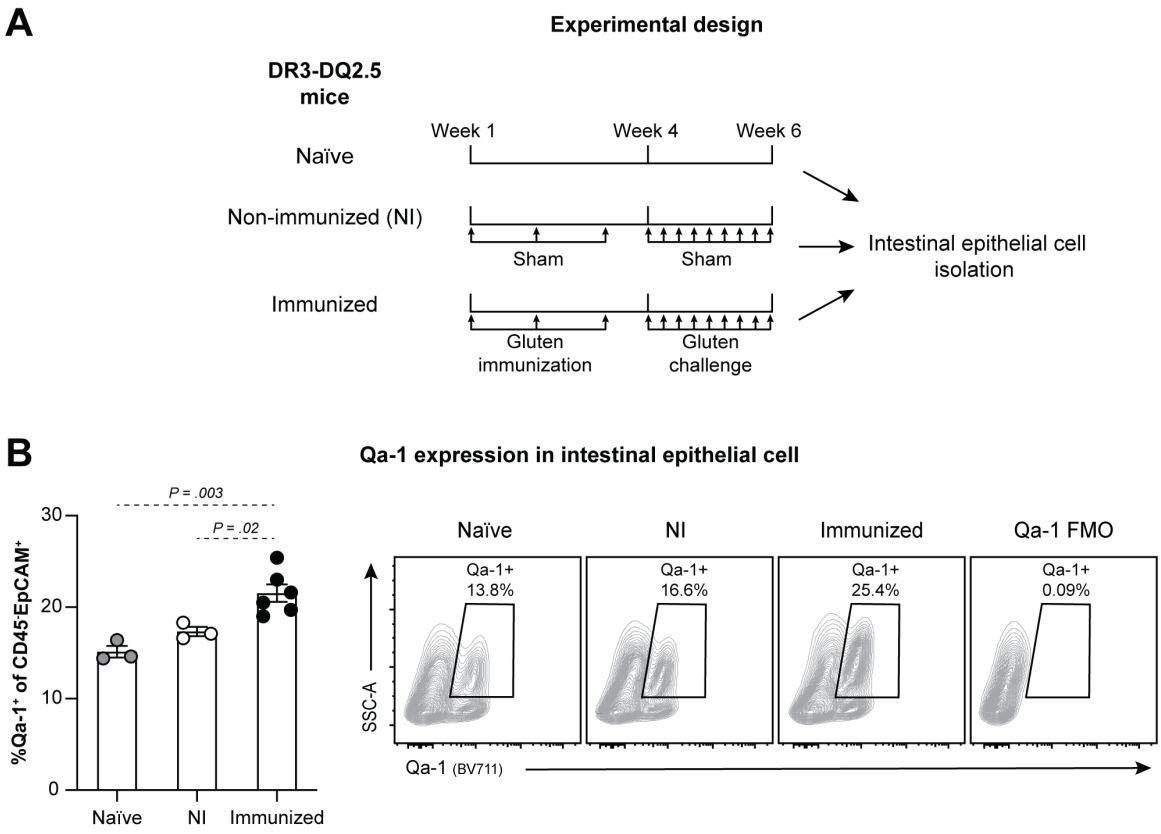


Figure 4.8 Gluten immunization induces the expression of Qa-1 in IECs from DR3-DQ2.5 mice. (A) Non-immunized (NI) DR3-DQ2.5 mice received sham immunization and challenges and were used as controls (n=3; open dots). Gluten-immunized DR3-DQ2.5 mice received PT-gliadin and cholera toxin, followed by gluten challenges (n=6; black dots). **(B)** Percentage of Qa-1-expressing cells gated on live $CD45^{-}EpCAM^{+}$ cells from IECs isolated from NI and immunized DR3-DQ2.5 mice. Data are presented as mean±SEM. Each dot represents an individual mouse. All *P*-values were determined using one-way analysis of variance (ANOVA) with Tukey's post-hoc test for multiple comparisons. Representative dot plots and fluorescence minus one (FMO) control showing $Qa\text{-}1^{+}$ cells, gated on live $CD45^{-}EpCAM^{+}$ cells. See Supplementary Figure 4 for the gating strategy.

4.2.5 Gluten immunization and IFN-γ-injection upregulates the expression of epithelial MHC class II in DR3-DQ2.5 mice

It is known that the pro-inflammatory cytokine IFN-γ, is a potent inducer of MHC class II expression in IECs (Heuberger et al., 2023; Jamwal et al., 2020; Koyama et al., 2019a)(Thelemann et al., 2014) (Malik et al., 2023). To investigate whether stronger *in vivo*-induced inflammation leads to higher small intestinal MHC class II expression, gluten-immunized DR3-DQ2.5-human (h)CD4 mice were intraperitoneally (i.p.) injected with recombinant mouse (rm)IFN-γ (Figure 4.9A). MHC class II immunofluorescence staining was then performed using the HLA-DR-DQ antibody in the duodenum and proximal jejunum tissues collected from immunized+IFN-γ-injected and NI-PBS-injected control mice. MHC class II expression (highlighted with white arrows in the figure below) was higher in immunized+IFN-γ-injected mice than in control mice (Figure 4.9B). Immunofluorescence staining of epithelial MHC class II expression in gluten-immunized (Figure 4.7B) and gluten-immunized-IFN-γ-injected DR3-DQ2.5 mice (Figure 4.9B) revealed that injection of IFN-γ further upregulated MHC class II expression in IECs. No staining was detected in the isotype controls (Supplementary Figure 3). This suggests that *in vivo* inflammation induced by IFN-γ could prime the intestinal epithelium to further elevate MHC class II expression.

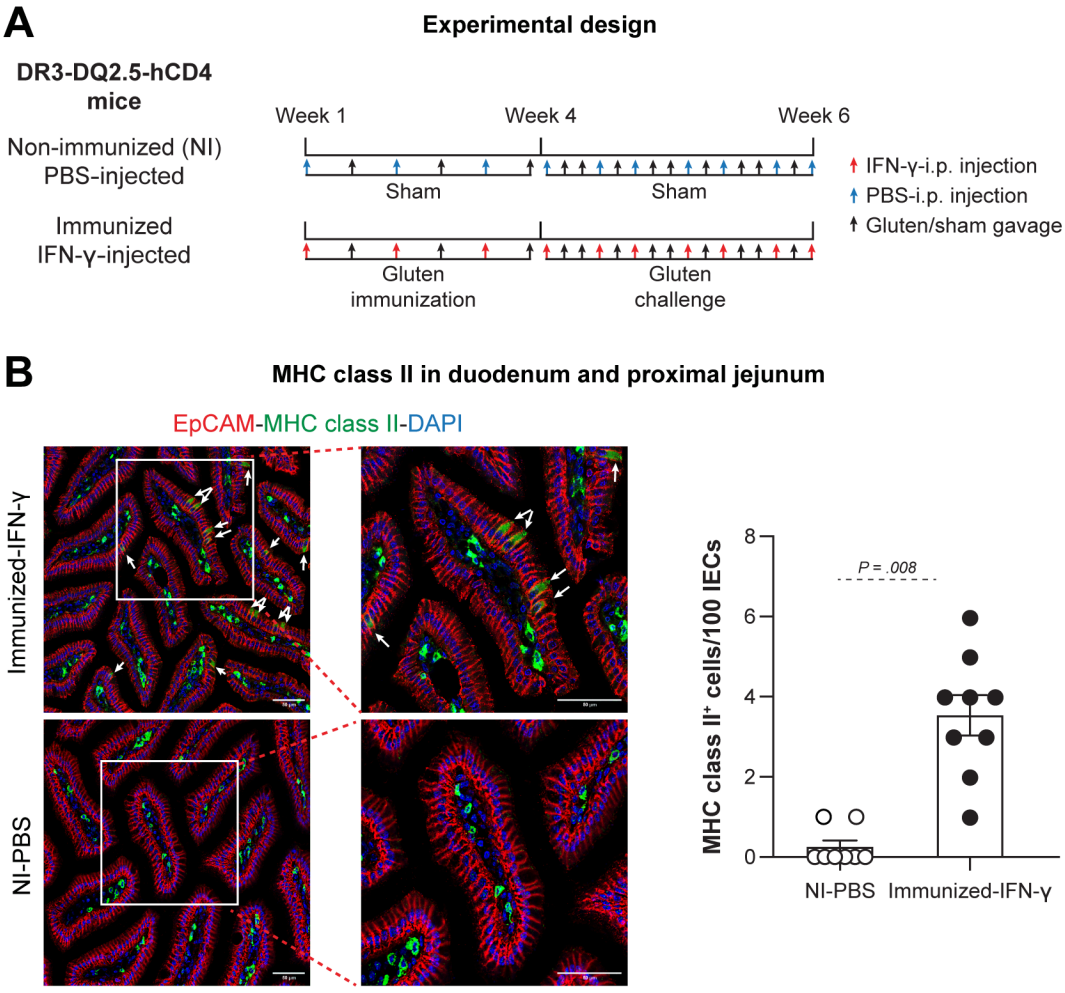


Figure 4.9 IFN-γ injection and gluten immunization enhance epithelial MHCII expression of in DR3-DQ2.5-hCD4 mice. (A) Non-immunized (NI) DR3-DQ2.5-hCD4 mice received sham gavages and were injected with PBS (NI-PBS; open dots). Gluten-immunized mice received PT-gliadin and cholera toxin, followed by gluten challenges. Gluten-immunized mice received i.p. injections of rmIFN-γ throughout the experiment (immunized-IFN-γ; black dots). **(B)** Representative immunofluorescence staining of the duodenum and proximal jejunum for MHCII$^+$ (white arrows; EpCAM$^+$: red; MHCII$^+$: green; DAPI: blue; Scale bar, 50μm). Quantification of MHCII$^+$ cells per 100 intestinal epithelial cells (IECs) from NI-PBS (n=8) and immunized-IFN-γ (n=9) mice. Data are presented as mean±SEM. Each dot represents an individual mouse. All *P*-values were determined using a two-tailed unpaired Student's *t*-test.

4.3 Characterization of organoid monolayers from mice carrying CeD risk genes

4.3.1 Organoid and organoid monolayers derived from mice expressing CeD risk genes express main IEC lineages

The expression of MHC class II in IECs in the context of CeD was investigated by developing organoid monolayers from the duodenum and proximal jejunum of mice carrying CeD risk genes: DR3-DQ2.5 or HLA-DQ8. First, I characterized the 3D organoids and organoid monolayers from the duodenum and proximal jejunum of "naïve" DR3-DQ2.5 and HLA-DQ8 mice on a GFD that had not received adjuvant and gluten. The expression of the main intestinal epithelial lineage markers was verified by immunofluorescence staining of organoid monolayers. Immunofluorescence staining for E-cadherin confirmed the presence of adherens junctions, indicating the establishment of epithelial polarity in both organoids (Figure 4.10A) and organoid monolayers (Figure 4.10B). Staining for Villin1 and Lysozyme showed that enterocytes and Paneth cells populated the organoid and organoid monolayers from DR3-DQ2.5 mice, respectively. The expression of the differentiated cell lineages, including goblet cells and enteroendocrine cells were confirmed with staining for Mucin2 and Chromogranin A. The Ki67 cell expression indicated the presence of proliferative cells at the base of the organoid crypt compartment.

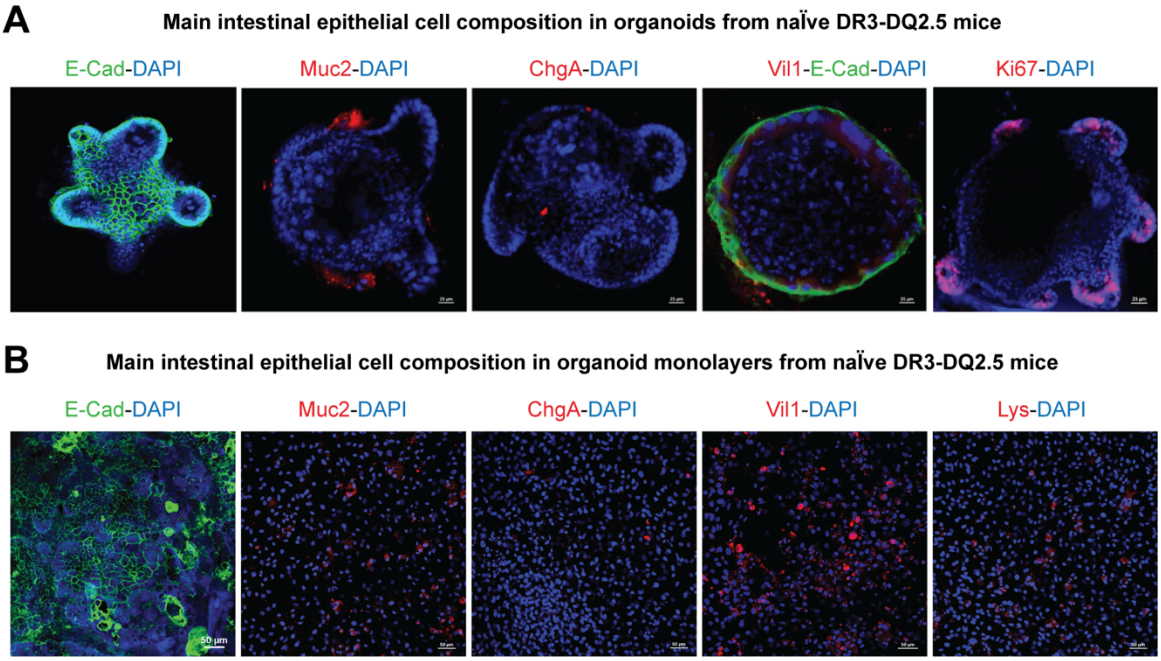


Figure 4.10 Organoid and organoid monolayers derived from naïve DR3-DQ2.5 mice express main IEC lineages. (A) Representative immunofluorescence staining of organoid from naïve DR3-DQ2.5 mice for E-cadherin$^+$ (E-Cad$^+$: green; DAPI: blue); Mucin2$^+$ (Muc2$^+$: red; DAPI: blue); Chromogranin A$^+$ (ChgA$^+$: red; DAPI: blue); Villin1$^+$ (Vil1$^+$: red; E-Cad$^+$: green; DAPI: blue); and Ki67$^+$ (Ki67$^+$: red; DAPI: blue). Scale bar, 25µm. **(B)** Representative immunofluorescence staining of organoid monolayers from naïve DR3-DQ2.5 mice for E-cadherin$^+$ (E-Cad$^+$: green; DAPI: blue); Mucin2$^+$ (Muc2$^+$: red; DAPI: blue); Chromogranin A$^+$ (ChgA$^+$: red; DAPI: blue); Villin1$^+$ (Vil1$^+$: red; DAPI: blue); and Lysozyme$^+$ (Lys$^+$: red; DAPI: blue). Scale bar, 50µm.

Organoid and organoid monolayers were also developed from naïve HLA-DQ8 mice. These mice were previously characterized in our lab as a model of gluten sensitivity (H. J. Galipeau et al., 2011a)(E. F. Verdu et al., 2008). The presence of adherence junctions and the expression of enterocytes, Paneth cells, goblet cells, and enteroendocrine cells were confirmed in organoids (Figure 4.11A) and organoid monolayers (Figure 4.11B) derived

from HLA-DQ8 mice by staining for E-cadherin, Villin1, Lysozyme, Mucin2 and Chromogranin A, respectively. The presence of proliferative cells at the base of organoid crypts were confirmed using Ki67 staining.

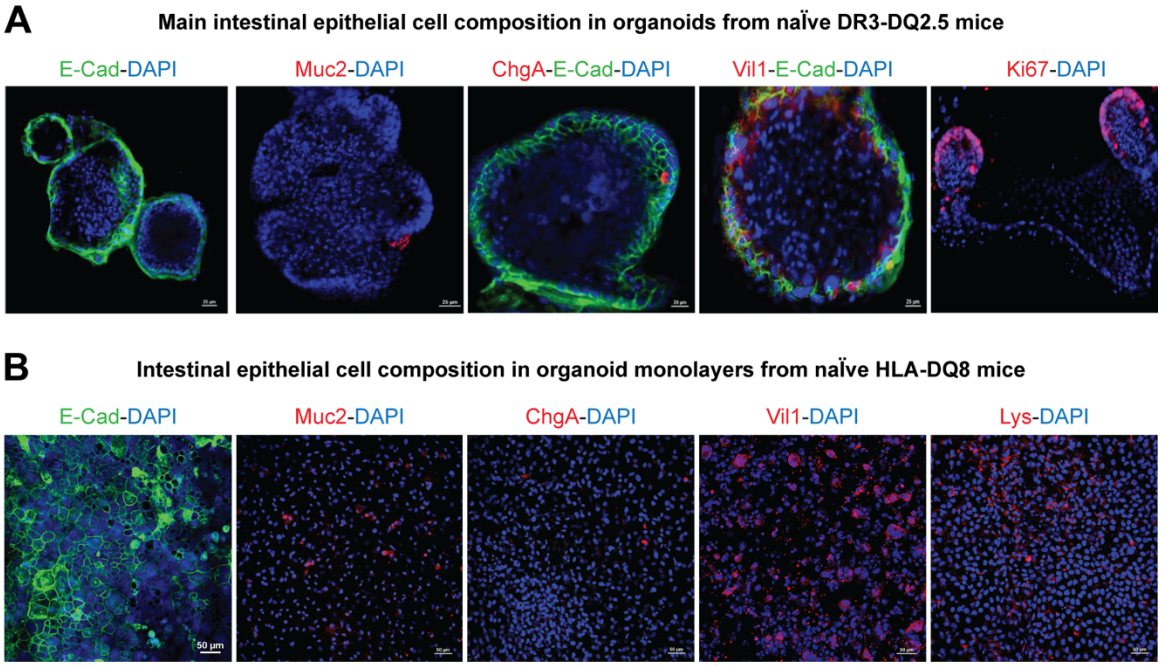


Figure 4.11 Organoid and organoid monolayers derived from naïve HLA-DQ8 mice express main IEC lineages. (A) Representative immunofluorescence staining of organoid from naïve HLA-DQ8 mice for E-cadherin$^+$ (E-Cad$^+$: green; DAPI: blue); Mucin2$^+$ (Muc2$^+$: red; DAPI: blue); Chromogranin A$^+$ (ChgA$^+$: red; DAPI: blue); Villin1$^+$ (Vil1$^+$: red; E-Cad$^+$: green; DAPI: blue); and Ki67$^+$ (Ki67$^+$: red; DAPI: blue). Scale bar, 25µm. **(B)** Representative immunofluorescence staining of organoid monolayers from naïve HLA-DQ8 mice for E-cadherin$^+$ (E-Cad$^+$: green; DAPI: blue); Mucin2$^+$ (Muc2$^+$: red; DAPI: blue); Chromogranin A$^+$ (ChgA$^+$: red; DAPI: blue); Villin1$^+$ (Vil1$^+$: red; DAPI: blue); and Lysozyme$^+$ (Lys$^+$: red; DAPI: blue). Scale bar, 50µm.

4.3.2 Gluten immunization and IFN-γ treatment induce MHC class II and co-stimulatory molecules expression in organoid monolayers

Next, the conditions leading to the expression of epithelial MHC class II in organoid monolayers under different inflammatory conditions were investigated. The first inflammatory condition investigated was induced by gluten immunization of DR3-DQ2.5 or HLA-DQ8 mice. For this, I developed organoid monolayers from gluten-immunized, non-immunized and naïve DR3-DQ2.5 mice and measured the expression level of epithelial MHC class II (Figure 4.12A). Monolayers from gluten-immunized DR3-DQ2.5 mice showed enhanced expression of MHC class II compared with those from non-immunized or naïve mice (Figure 4.12B). Thus, a pro-inflammatory milieu induced by gluten immunization primes IECs to upregulate MHC class II expression, which is then recapitulated in the organoid monolayer. A representative flow cytometry gating strategy for organoid monolayers is shown in (Supplementary Figure 5).

The second induced inflammatory was achieved *in vitro* by stimulating the organoid monolayers with IFN-γ. IFN-γ is one of the main immune mediators in CeD pathogenesis and is secreted by gluten-specific $CD4^+$ T cells in patients with CeD (Nilsen et al., 1995). In addition, IFN-γ is a potent inducer of epithelial MHC class II expression (Heuberger et al., 2023; Jamwal et al., 2020; Koyama et al., 2019a) (Thelemann et al., 2014) (Malik et al., 2023). As such, I determined the effect of IFN-γ on MHC class II expression in IECs. The results showed that *in vitro* IFN-γ stimulation led to higher expression of MHC class II in organoid monolayers derived from NI mice compared with organoid monolayers from the same mice that did not receive IFN-γ treatment or monolayers from naïve mice, as

shown by flow cytometry (Figure 4.12B). Moreover, the expression of MHC class II was further elevated upon IFN-γ treatment when organoid monolayers derived from gluten-immunized mice compared with monolayers from the same mice without IFN-γ treatment or NI or naïve mice treated with IFN-γ, as detected by flow cytometry (Figure 4.12B). These findings demonstrate the influence of IFN-γ on MHC class II expression in IECs.

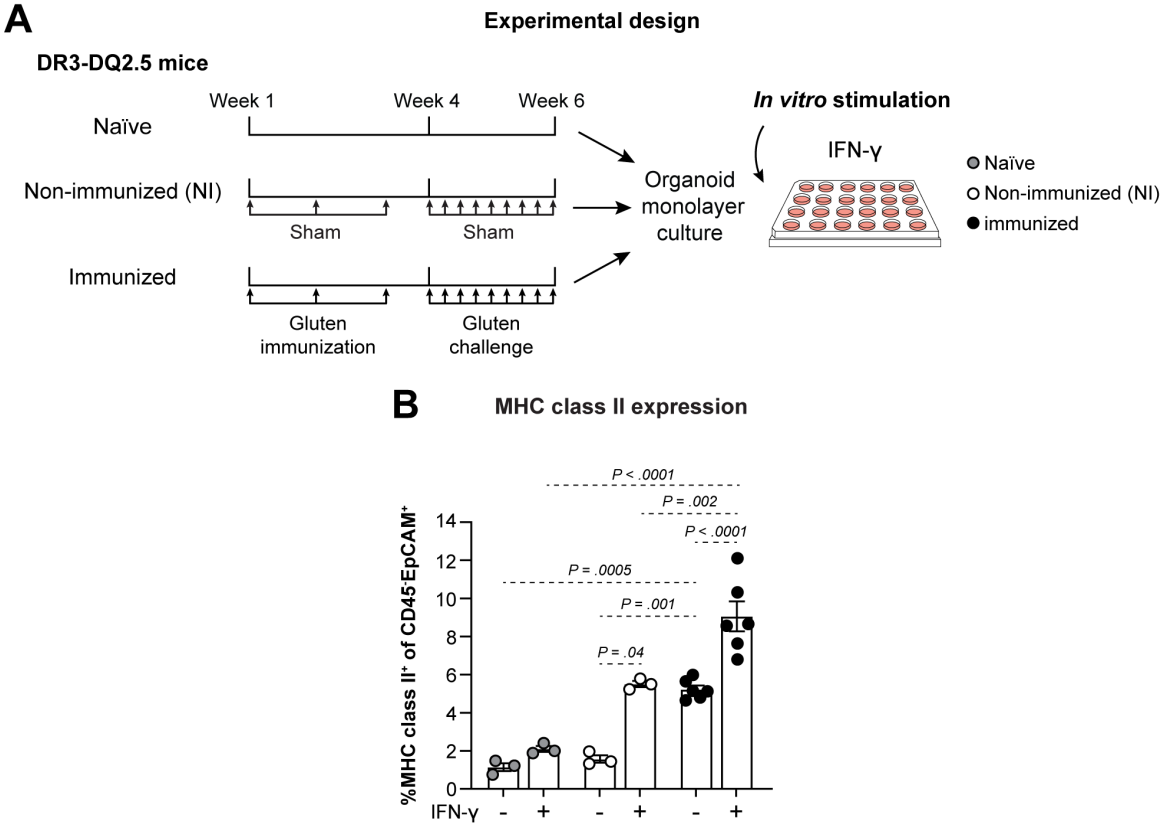


Figure 4.12 Gluten immunization and IFN-γ induce MHC class II expression in organoid monolayers from DR3-DQ2.5 mice. (A) Naïve DR3-DQ2.5 mice received no treatment and were used as controls (n=3; grey dots). Non-immunized (NI) DR3-DQ2.5 mice received sham immunization and challenge and were used as controls (n=3; open dots). Gluten-immunized DR3-DQ2.5 mice received PT-gliadin and cholera toxin followed by gluten challenges (n=6; black dots). Organoid monolayers were then stimulated *in vitro* with or without IFN-γ. **(B)** Percentage of MHC class II-expressing cells using an HLA-DR-

DP-DQ antibody, gated on live $CD45^{-}EpCAM^{+}$ cells from organoid monolayers from naïve, NI, and immunized DR3-DQ2.5 mice stimulated *in vitro* with or without IFN-γ. Data are presented as the mean±SEM. Each dot represents an individual mouse. All *P*-values were determined using ANOVA with Tukey's post-hoc test for multiple comparisons. See Supplementary Figure 5 for the gating strategy.

A similar pattern in the expression of MHC class II was observed in monolayers derived from HLA-DQ8 transgenic mice (Figures 4.13A), which only carry the HLA-DQ isotype of MHC class II. The expression of epithelial MHC class II was found to be higher in monolayers from gluten-immunized HLA-DQ8 mice than in those from NI or naïve mice. Additionally, treatment of organoid monolayers with IFN-γ upregulated the epithelial expression of MHC class II in monolayers from NI mice compared with monolayers from the same mice without IFN-γ treatment or monolayers from naïve mice. This upregulation was more pronounced in organoid monolayers from gluten-immunized mice following IFN-γ stimulation than in untreated monolayers or those from NI or naïve mice (Figure 4.13B). To rule out the possibility that the human MHC class II antibody used here cross-reacts with murine MHC class II expression, I derived monolayers from wild-type C57Bl/6 mice and stimulated them with IFN-γ (Figure 4.14A). Flow cytometry showed no MHC class II expression in IECs even after treatment of the monolayers with IFN-γ (Figure 4.14B).

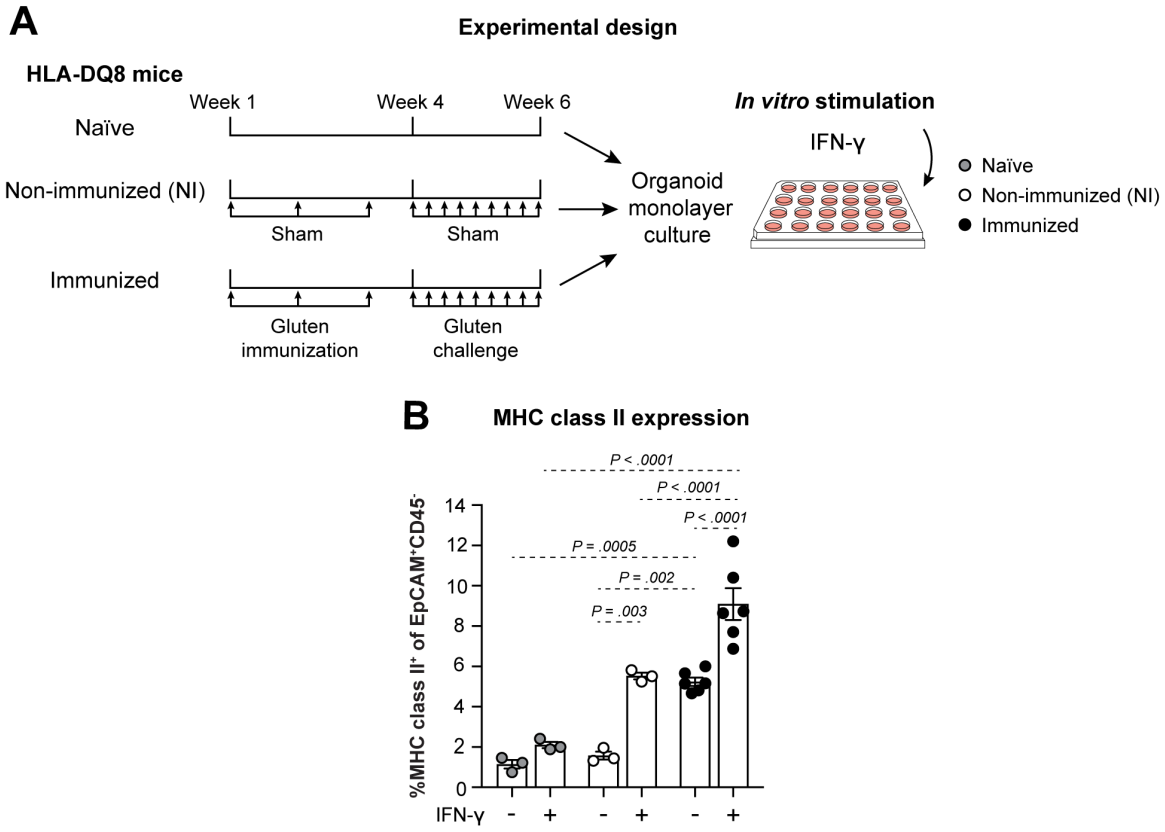


Figure 4.13 Gluten immunization and IFN-γ induce MHC class II expression in organoid monolayers from HLA-DQ8 mice. (A) Naïve HLA-DQ8 mice received no treatment and were used as controls (n=3; grey dots). Non-immunized (NI) HLA-DQ8 mice received sham immunization and challenge and were used as controls (n=3; open dots). Gluten-immunized HLA-DQ8 mice received PT-gliadin and cholera toxin followed by gluten challenges (n=6; black dots). Organoid monolayers were then stimulated *in vitro* with or without IFN-γ. **(B)** Percentage of MHC class II-expressing cells using an HLA-DQ antibody, gated on live $CD45^{-}EpCAM^{+}$ cells from organoid monolayers from naïve, NI, and immunized HLA-DQ8 mice stimulated *in vitro* with or without IFN-γ. Data are presented as the mean±SEM. Each dot represents an individual mouse. All *P*-values were determined using ANOVA with Tukey's post-hoc test for multiple comparisons. See Supplementary Figure 5 for the gating strategy.

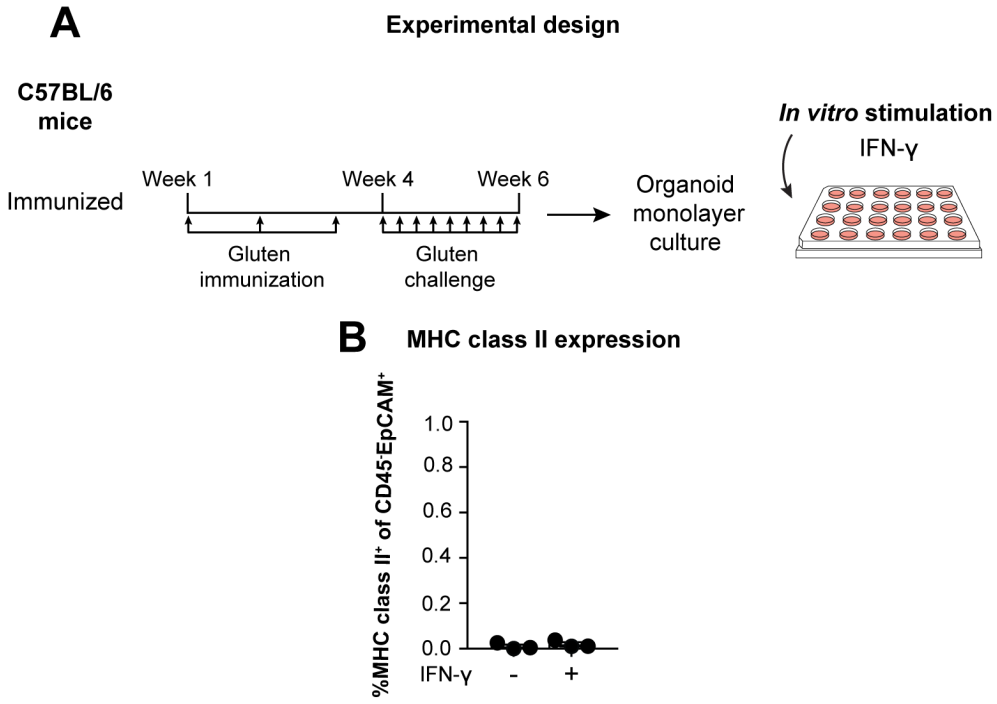


Figure 4.14 Lack of MHC class II expression in organoid monolayers from C57BL/6 mice. (A) Organoid monolayers were derived from gluten-immunized C57BL/6 mice (n=3) and stimulated *in vitro* with or without IFN-γ **(B)** Percentage of MHC class II-expressing cells gated on live $EpCAM^{+}CD45^{-}$ cells from organoid monolayers. Data are presented as mean±SEM. Each dot represents an individual mouse. See Supplementary Figure 5 for the gating strategy.

Subsequently, the level of MHC class II expression in monolayers from gluten-immunized+IFN-γ-injected and control NI-PBS-injected mice was evaluated (Figure 4.15A). The expression of epithelial MHC class II was higher in organoid monolayers from gluten-immunized+IFN-γ-injected mice compared with monolayers from control mice (Figure 4.15B). The expression of MHC class II was also enhanced in monolayers from NI-PBS-injected mice following *in vitro* treatment with IFN- γ compared with untreated monolayers or monolayers from naïve mice. This expression was further increased in

monolayers from gluten-immunized+IFN-γ-injected mice upon *in vitro* stimulation with IFN-γ (Figure 4.14B).

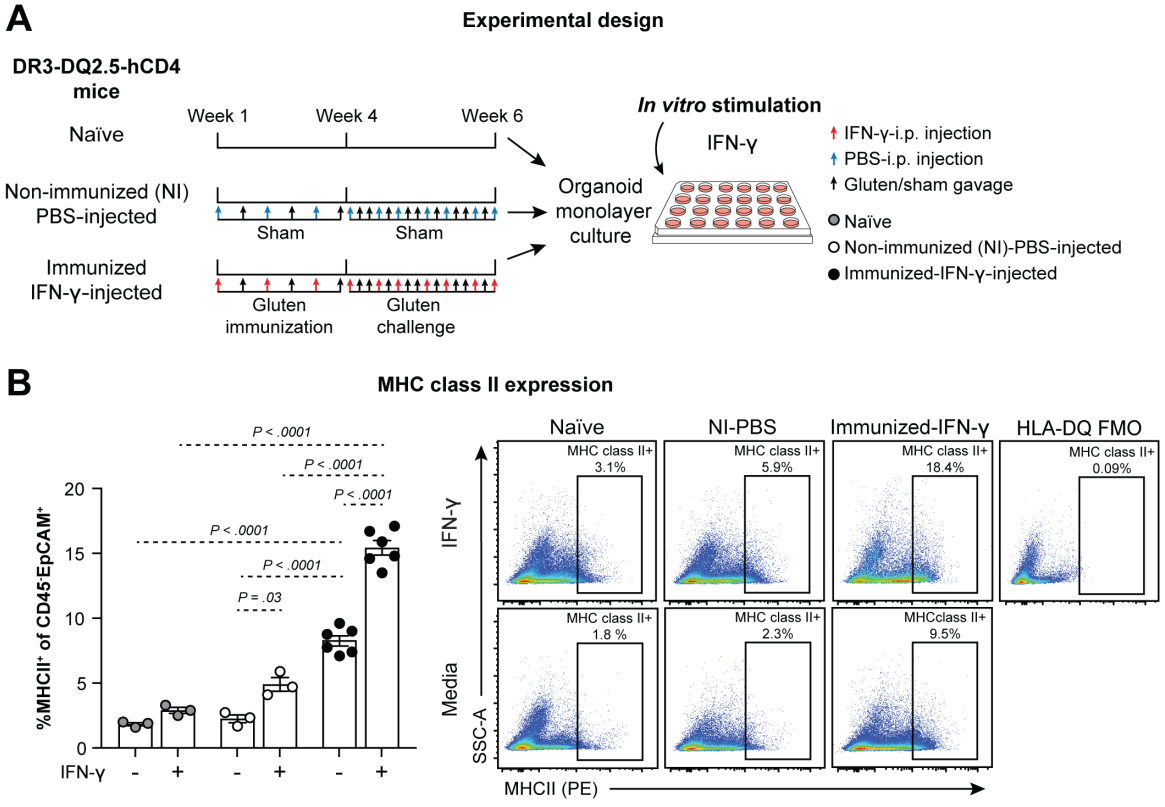


Figure 4.15 Expression of MHC class II in organoid monolayers from gluten-immunized+IFN-γ-injected DR3-DQ2.5-hCD4 mice. (A) Organoid monolayers from naïve (no treatment; n=3; grey dots), non-immunized+PBS-injected (NI-PBS; n=3; open dots), and gluten-immunized+IFN-γ-injected (immunized-IFN-γ; n=6; black dots) DR3-DQ2.5-hCD4 mice. The organoid monolayers were then stimulated with or without IFN-γ. **(B)** Percentage of $MHCII^+$ expressing cells from organoid monolayers, gated on live $CD45^-EpCAM^+$ cells using an HLA-DR-DP-DQ antibody. Representative dot plots showing $MHCII^+$ cells, gated on live $CD45^-EpCAM^+$ cells using an HLA-DR-DP-DQ antibody, gated on live $CD45^-EpCAM^+$ cells from organoid monolayers from naïve, NI-PBS, and immunized-IFN-γ DR3-DQ2.5 mice stimulated *in vitro* with or without IFN-γ. Data are presented as the mean±SEM. Each dot represents an individual mouse. All *P*-values were

determined using ANOVA with Tukey's post-hoc test for multiple comparisons. See Supplementary Figure 5 for the gating strategy.

The expression of epithelial CD71 and Qa-1 in organoid monolayers from DR3-DQ2.5 mice was also assessed. CD71 is a receptor that is implicated in transepithelial gluten peptide transport (Matysiak-Budnik et al., 2008). Organoid monolayers from gluten-immunized mice stimulated with IFN-γ had higher expression of CD71 and Qa-1 compared with untreated monolayers from gluten-immunized mice, monolayers derived from NI, or naïve mice (Figure. 4.16A-C).

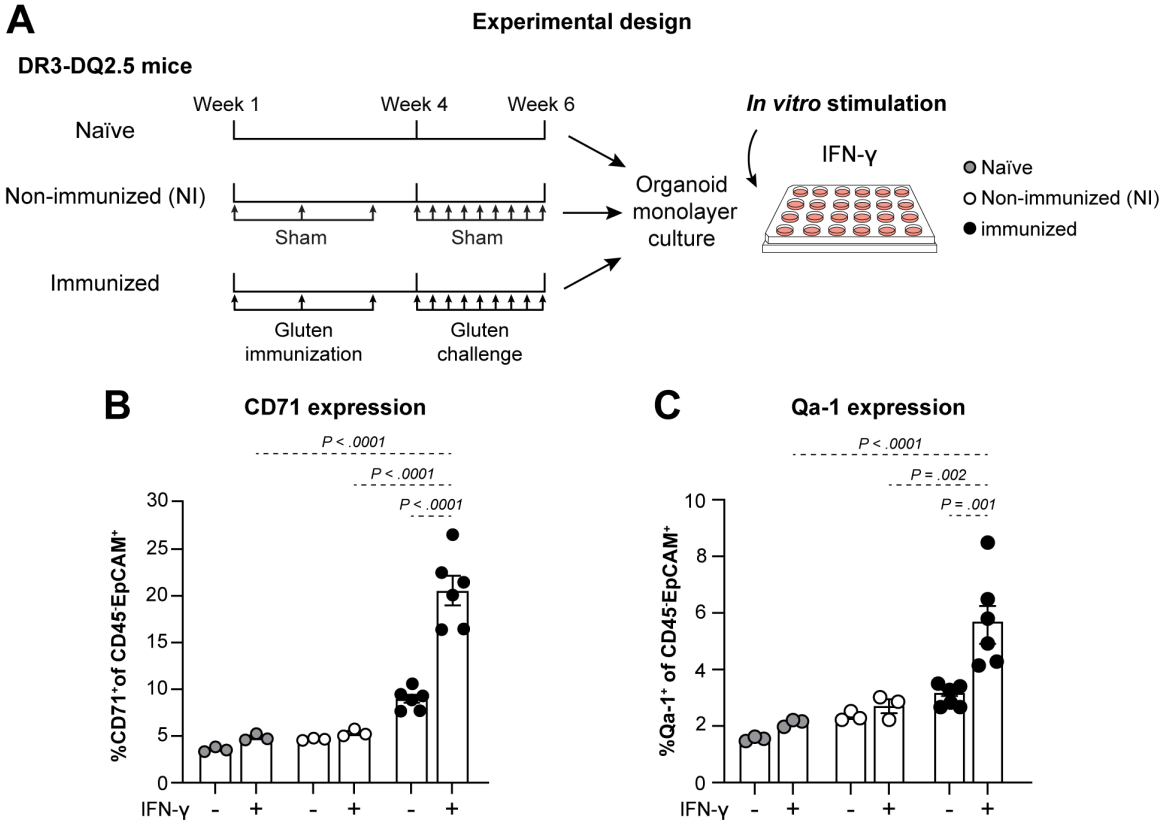


Figure 4.16 Gluten immunization and IFN-γ induce expression of CD71 and Qa-1 in organoid monolayers from DR3-DQ2.5 mice. (A) Naïve DR3-DQ2.5 mice received no

treatment and were used as controls (n=3; grey dots). Non-immunized (NI) DR3-DQ2.5 mice received sham immunization and challenge and were used as controls (n=3; open dots). Gluten-immunized DR3-DQ2.5 mice received PT-gliadin and cholera toxin followed by gluten challenges (n=6; black dots). Organoid monolayers were then stimulated *in vitro* with or without IFN-γ. **(B-C)** Percentage of **(B)** CD71-expressing cells, **(C)** Qa-1-expressing cells, gated on live $CD45^{-}EpCAM^{+}$ cells from organoid monolayers from naïve, NI, and immunized DR3-DQ2.5 mice stimulated *in vitro* with or without IFN-γ. Data are presented as the mean±SEM. Each dot represents an individual mouse. All *P*-values were determined using ANOVA with Tukey's post-hoc test for multiple comparisons. See Supplementary Figure 5 for the gating strategy.

Finally, the expression levels of the classical co-stimulatory molecules, CD80, CD86, and CD40, were determined. The expression of these co-stimulatory molecules is required, in addition to antigens bound to MHC class II molecule, to activate $CD4^{+}$ T cells. The expression of CD40, but not CD80 or CD86, was higher in organoid monolayers from gluten-immunized mice than in monolayers from NI or naïve DR3-DQ2.5 mice (Figure 4.17A). I found that IFN-γ treatment elevated the expression of CD40, CD86, and CD80 in organoid monolayers derived from gluten-immunized mice but not from NI or naïve mice (Figure 4.17B). Similar results were obtained using organoid monolayers from gluten-immunized HLA-DQ8 mice (Figure 4.18A-D). A representative flow cytometry gating strategy on organoid monolayers is shown in Supplementary Figure 5. These results indicate that the expression of co-stimulatory molecules requires several inflammatory stimuli, such as those provided by *in vivo* gluten immunization and a permissive IFN-γ milieu.

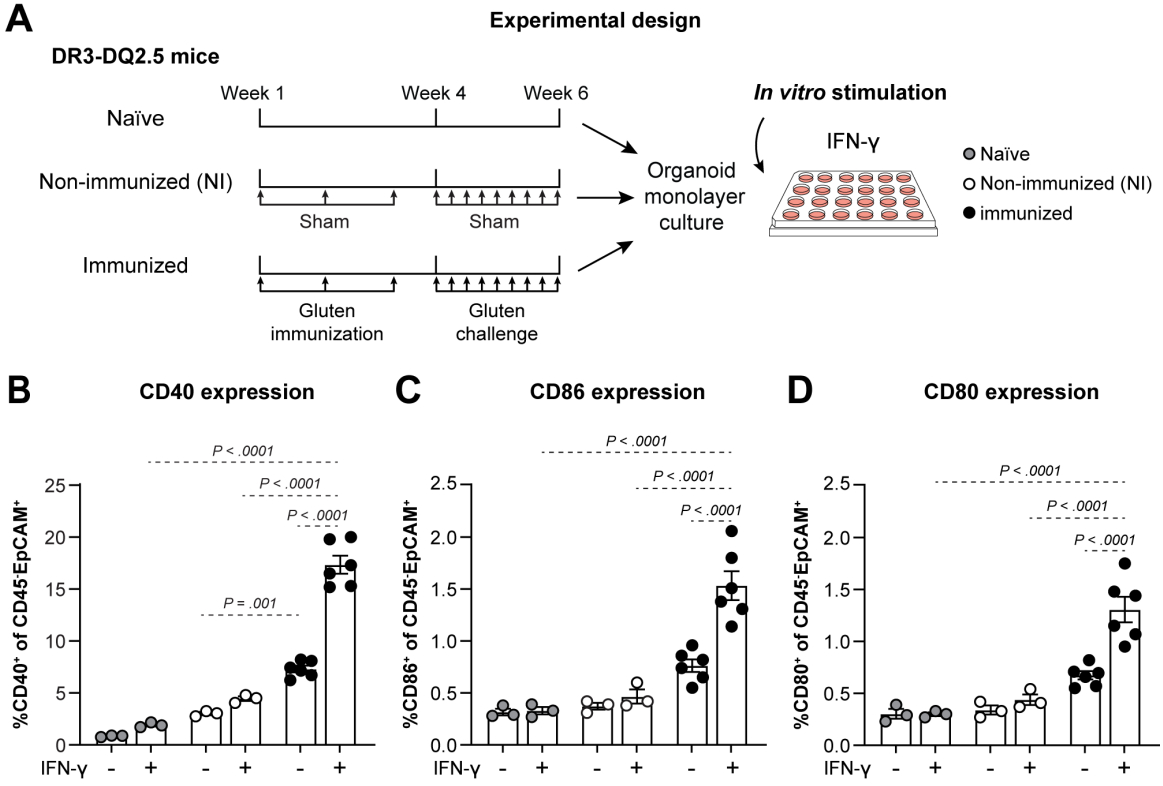


Figure 4.17 Gluten immunization and IFN-γ induce expression of CD71 and Qa-1 in organoid monolayers from DR3-DQ2.5 mice. (A) Naïve DR3-DQ2.5 mice received no treatment and were used as controls (n=3; grey dots). Non-immunized (NI) DR3-DQ2.5 mice received sham immunization and challenge and were used as controls (n=3; open dots). Gluten-immunized DR3-DQ2.5 mice received PT-gliadin and cholera toxin followed by gluten challenges (n=6; black dots). Organoid monolayers were then stimulated *in vitro* with or without IFN-γ. **(B-D)** Percentage of **(B)** CD40-expressing cells, **(C)** CD86-expressing cells, or **(D)** CD80-expressing cells, gated on live $CD45^{-}EpCAM^{+}$ cells from organoid monolayers from naïve, NI, and immunized DR3-DQ2.5 mice stimulated *in vitro* with or without IFN-γ. Data are presented as the mean±SEM. Each dot represents an individual mouse. All *P*-values were determined using ANOVA with Tukey's post-hoc test for multiple comparisons. See Supplementary Figure 5 for the gating strategy.

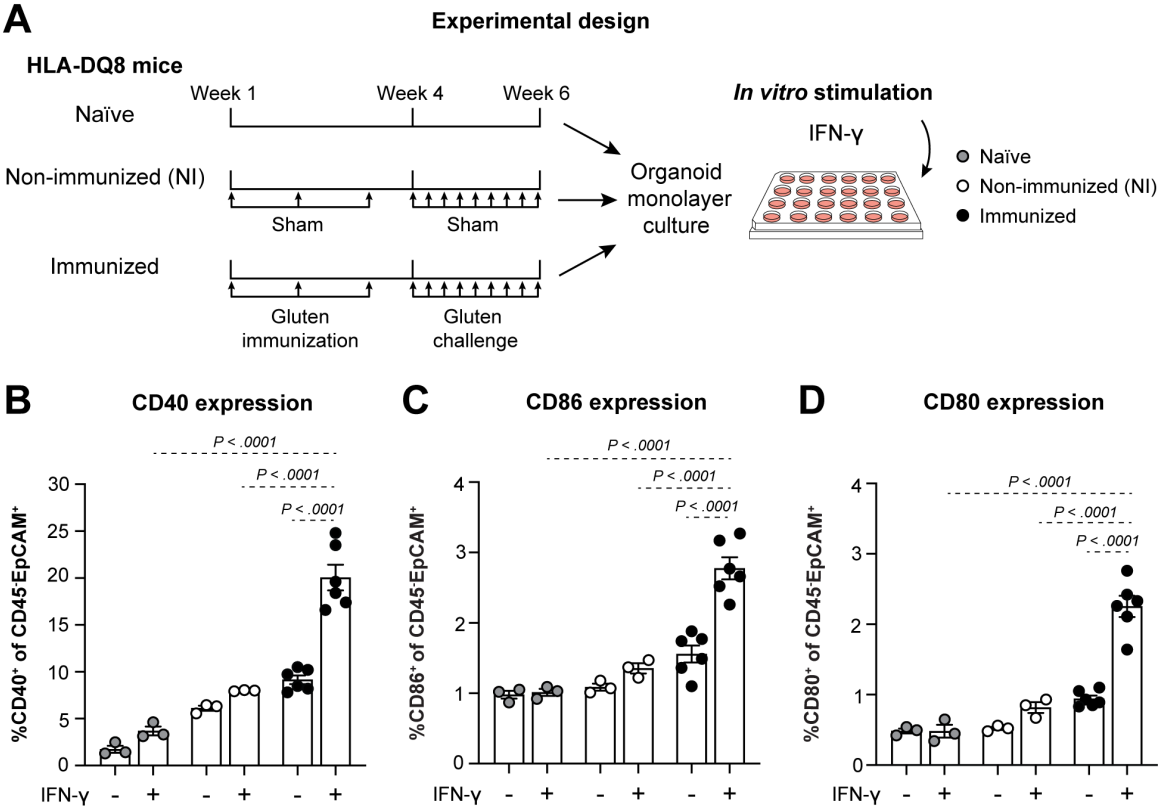


Figure 4.18 Gluten immunization and IFN-γ induce expression of CD71 and Qa-1 in organoid monolayers from HLA-DQ8 mice. (A) Naïve HLA-DQ8 mice received no treatment and were used as controls (n=3; grey dots). Non-immunized (NI) HLA-DQ8 mice received sham immunization and challenge and were used as controls (n=3; open dots). Gluten-immunized HLA-DQ8 mice received PT-gliadin and cholera toxin followed by gluten challenges (n=6; black dots). Organoid monolayers were then stimulated *in vitro* with or without IFN-γ. **(B-D)** Percentage of **(B)** CD40-expressing cells, **(C)** CD86-expressing cells, or **(D)** CD80-expressing cells, gated on live $CD45^{-}EpCAM^{+}$ cells from organoid monolayers from naïve, NI, and immunized HLA-DQ8 mice stimulated *in vitro* with or without IFN-γ. Data are presented as the mean±SEM. Each dot represents an individual mouse. All *P*-values were determined using ANOVA with Tukey's post-hoc test for multiple comparisons. See Supplementary Figure 5 for the gating strategy.

Taken together, these data demonstrate that, under induced *in vivo* or *in vitro* inflammatory conditions, organoid monolayers from DQ2.5- or DQ8-expressing mice exhibit MHC class II expression and its associated co-stimulatory molecules, which are required for T cell activation, as well as other markers that could contribute to the activation of IELs and transepithelial transport molecule. These findings indicate that the epithelium of transgenic mice carrying CeD risk genes possesses the molecular machinery necessary for antigen presentation. However, the functional consequences of upregulated MHC class II expression remain unclear, and their capacity to activate $CD4^+$ T cells is yet to be determined.

4.4 Organoid monolayers and CD4+ T cells co-culture

4.4.1 MHC class II organoid monolayers activate hCD4+ T cells in a gluten-dependent manner

The immune response in CeD is $CD4^+$ T-cell-dependent and HLA-DQ-restricted (Black et al., 2002). To interrogate the MHC class II-$CD4^+$ T-cell interactions, I used double transgenic mice that, in addition to DQ2.5, also carry functional human CD4 receptors on T cells (DR3-DQ2.5-hCD4) (de Kauwe et al., 2009). I established an *in vitro* co-culture system using MHC class II-expressing organoid monolayers from gluten-immunized DR3-DQ2.5 mice and splenic $CD4^+$ T cells from gluten-immunized DR3-DQ2.5-hCD4 mice. IFN-γ-treated MHC class II-expressing organoid monolayers were apically stimulated with DAPT-gliadin, DAPT-zein (unrelated MHC class II antigen in CeD), or media prior to introducing $hCD4^+$ into the basolateral side of the organoid

monolayers (Figure 4.19A). The proliferation of $hCD4^+$ T cells increased by 2.5-fold in response to stimulation of monolayers with DAPT-gliadin compared with DAPT-zein-treated monolayers (Figure 4.19B). Concomitantly, $hCD4^+$ T cells co-cultured with DAPT-gliadin-treated monolayers exhibited an activated phenotype with increased expression of known T-cell activation markers, including CD69, CD25, and CD44, compared with $hCD4^+$ T cells from DAPT-zein-treated monolayers (Figure 4.19C-E). A representative flow cytometry gating strategy for CD4 T cell is shown in Supplementary Figure 6.

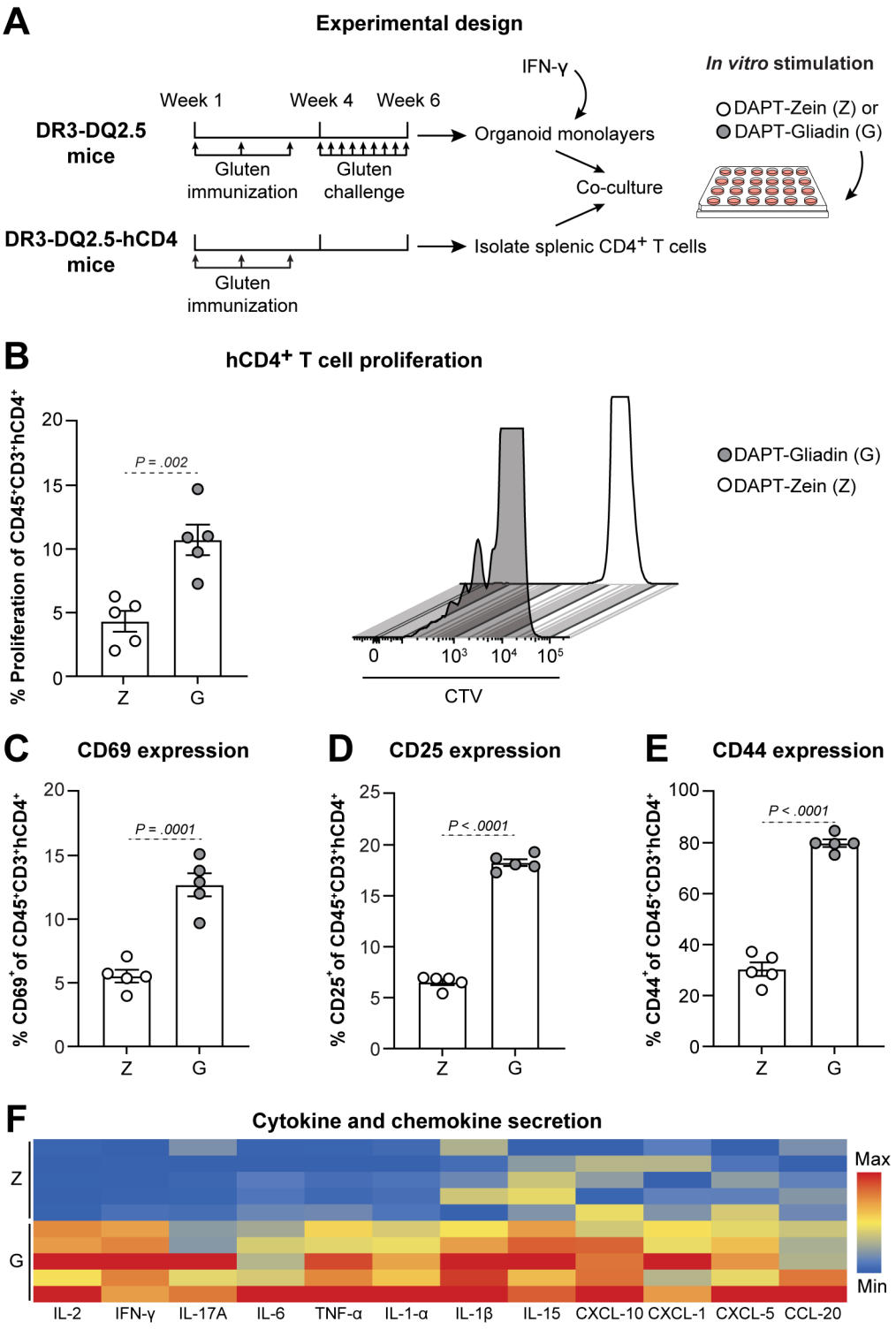


Figure 4.19 MHC class II-expressing organoid monolayers induce a gluten-dependent CD4⁺ T-cell response. (A) Organoid monolayers were co-cultured with splenic hCD4⁺ T

cells in the presence of deamidated pepsin-trypsin-digested (DAPT)-Zein (Z; open dots) or DAPT-Gliadin (G; grey dots). Organoid monolayers from gluten-immunized DR3-DQ2.5 mice (n=5) were further stimulated with IFN-γ for 24h prior to co-culture. Splenic $CD4^+$ T cells were isolated from gluten-immunized DR3-DQ2.5-hCD4 mice (n=5). **(B)** Left panel, percentage of proliferating CellTrace Violet (CTV)-labelled $hCD4^+$ T cells, gated on live $CD45^+CD3^+hCD4^+$ cells that were co-cultured with organoid monolayers in the presence of DAPT-Zein (Z) or DAPT-Gliadin (G). Right panel, representative histograms of CTV-labeled $hCD4^+$ T cells gated on live $CD45^+CD3^+$ cells. **(C-E)** Percentage of **(C)** CD69-expressing cells, **(D)** CD25-expressing cells, or **(E)** CD44-expressing cells gated on live $CD45^+CD3^+hCD4^+$ T cells that were co-cultured with organoid monolayers in the presence of DAPT-Zein (Z) or DAPT-Gliadin (G). **(F)** Heatmap of cytokines and chemokines in co-culture supernatants that were significantly different between (DAPT)-Zein (Z) and DAPT-Gliadin (G). Data are presented as mean±SEM. Each dot represents an individual mouse. All *P*-values were determined using a two-tailed unpaired Student's *t*-test. See Supplementary Figure 6 for the gating strategy.

Stimulation of organoid monolayers with DAPT-gliadin, but not DAPT-zein, induced the secretion of several cytokines and chemokines in the co-culture supernatant (Figure 4.19F). The DAPT-gliadin-stimulated monolayers yielded a 37-fold elevation in IL-2 compared with the DAPT-zein-treated monolayers (Figure 4.20A). IL-2, a cytokine associated with T cell activation and growth (Malek, 2008), rapidly rises in the plasma of CeD patients within two hours of gluten challenges and peaks at four hours (Goel, Tye-Din, et al., 2019). In accordance with clinical studies showing elevated gluten-mediated serum cytokines in CeD patients (Goel, Tye-Din, et al., 2019), I found a 28-fold and 4-fold increase in the secretion of IFN-γ (Figure 4.20B) and IL-17A (Figure 4.20C), respectively, upon treatment of monolayers with DAPT-gliadin compared with DAPT-zein. The

production of IFN-γ and IL-17A in our model corresponded to the increased T helper cell type 1 (Th)1 (Nilsen et al., 1995) and Th17 (Monteleone et al., 2010) T cell phenotype patterns reported in CeD, respectively. IFN-γ, is also known to be the main cytokine released by gluten-specific $CD4^+$ T cells in CeD (Nilsen et al., 1998). In addition, the concentrations of pro-inflammatory cytokines, such as IL-6, TNF-α, IL-1α, and IL-1β, were elevated in response to DAPT-gliadin treatment of monolayers compared with DAPT-zein-treated monolayers (Figure 4.20D-G). In addition, a statistically significant increase in IL-15, a major cytokine in the pathogenesis of CeD (Jabri et al., 2000), was observed upon exposure of monolayers to DAPT-gliadin versus DAPT-zein (Figure 4.20H). The secretion of chemokines known to participate in immune and inflammatory cell recruitment and migration (Oo et al., 2010)(Goel, Daveson, et al., 2019), such as C-X-C motif chemokine ligand (CXCL)-10, CXCL-1, CXCL-5, and C-C motif chemokine ligand (CCL)-20, was higher in the supernatant of DAPT-gliadin-treated monolayers than in the supernatant of DAPT-zein-stimulated monolayers (Figure 4.20I-L). These chemokines are known to participate in immune and inflammatory cell recruitment and migration (Oo et al., 2010).

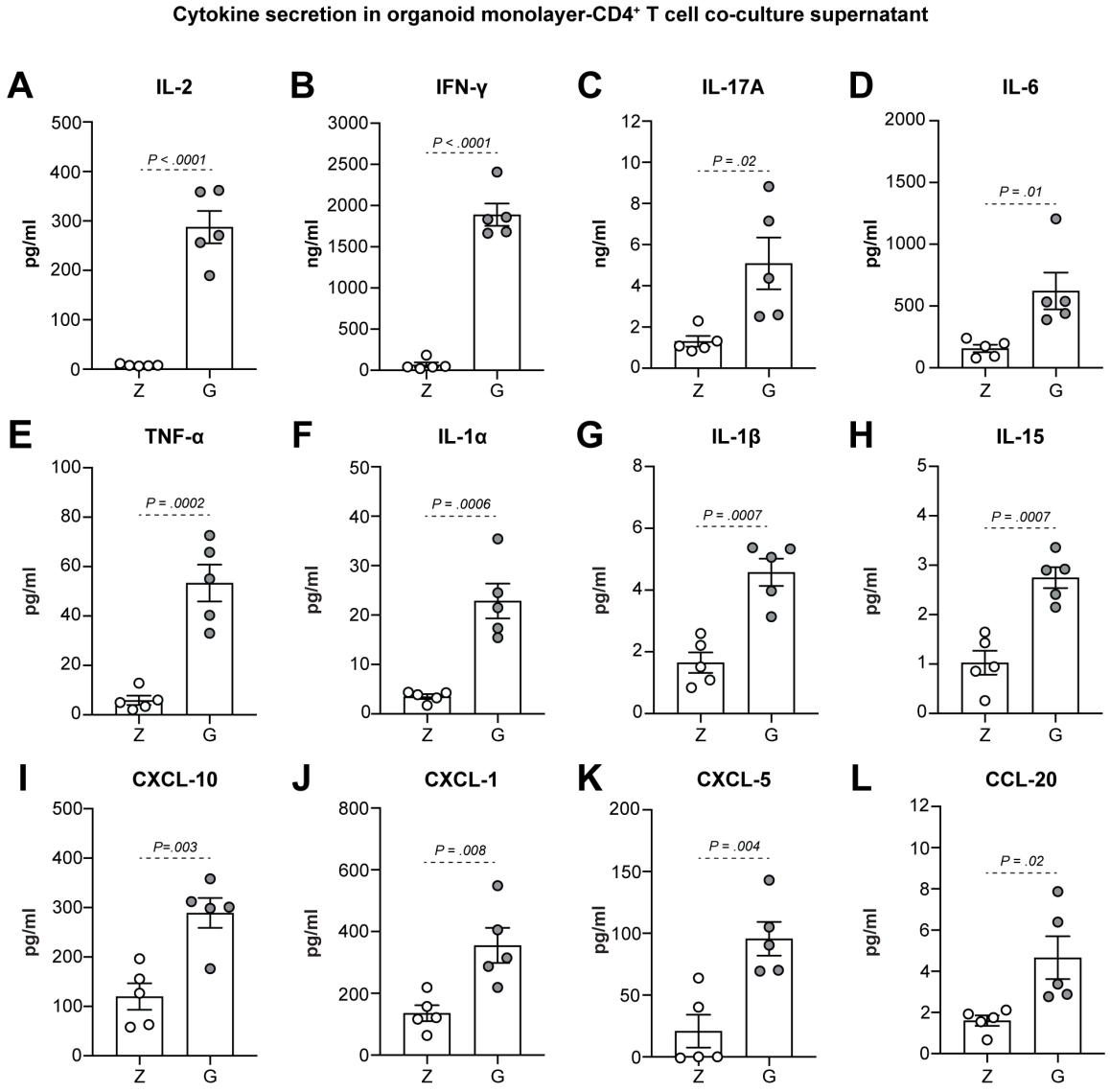


Figure 4.20 Gluten-dependent release of cytokines and chemokines implicated in CeD. (A-L) Quantification of cytokines and chemokines in the supernatant of organoid monolayer-CD4$^+$ T cell co-cultures treated with deamidated pepsin-trypsin-digested (DAPT)-Zein (Z; n=5, open dots) or DAPT-Gliadin (G; n=5, grey dots). Data are presented as mean±SEM. Each dot represents an individual mouse. All *p* values were determined using a two-tailed unpaired Student's *t*-test.

Moreover, the expression of MHC class II and CD71 in organoid monolayers after four days of co-culture with $CD4^+$ T cells was measured (Figure 4.21A). I found enhanced expression of MHC class II and CD71 in organoid monolayers following DAPT-gliadin treatment compared with the media-only group (Figure 4.21B-C). This finding implies that exposure to deamidated gluten (DAPT-gliadin) is associated with activation of $CD4^+$ T cells and release of pro-inflammatory cytokines, in the context of high epithelial MHC class II and CD71 expressions. Indeed, the expression of epithelial MHC class II in monolayers was positively correlated with T cell activation markers, namely, CD69, CD25, and CD44 (Figure 4.22A-C).

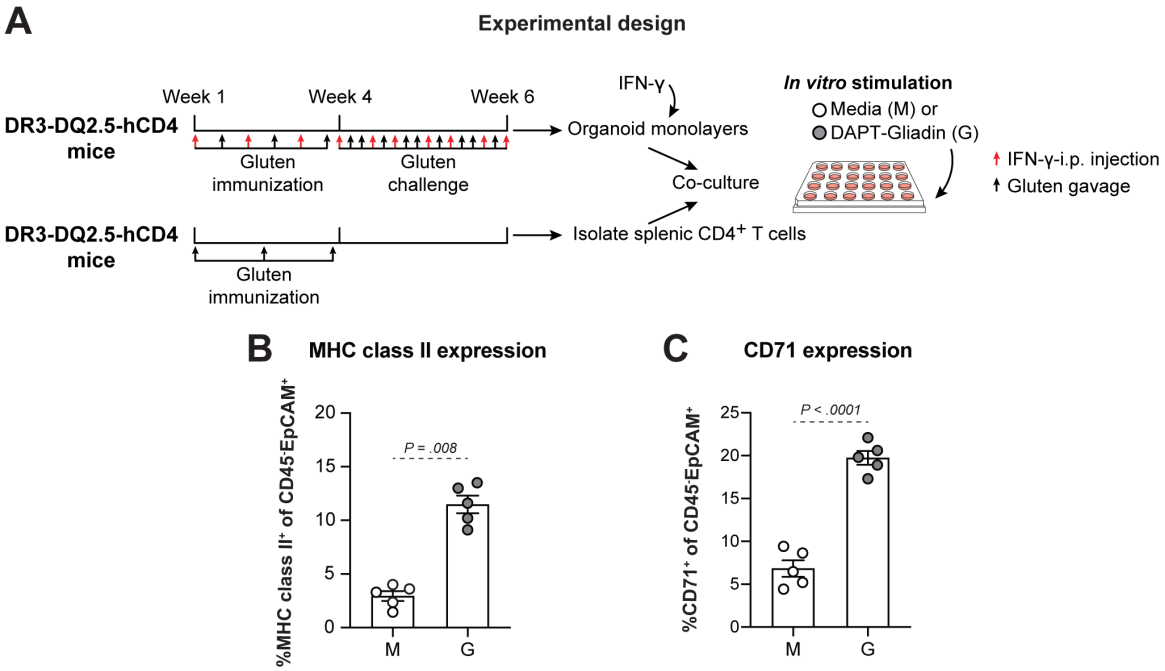


Figure 4.21 Organoid monolayers co-cultured with $CD4^+$ T cells upregulated MHC class II and CD71 expression in the presence of gluten. (A) Organoid monolayers were co-cultured with splenic $hCD4^+$ T cells in the presence of deamidated pepsin-trypsin-digested (DAPT)-Gliadin (G; grey dots) or media (M; open dots). Organoid monolayers from gluten-immunized+IFNγ-injected DR3-DQ2.5-hCD4 mice (n=5) were further

stimulated with IFN-γ for 24h prior to co-culture. Splenic $CD4^+$ T cells were isolated from gluten-immunized DR3-DQ2.5-hCD4 mice (n=5). **(B-C)** Percentage of **(B)** MHC class II-expressing cells using HLA-DR-DP-DQ antibody, **(C)** CD71-expressing cells, gated on live $CD45^-EpCAM^+$ cells from IFN-γ-treated organoid monolayers that were co-cultured with $CD4^+$ T cells in the presence of Media (M) or DAPT-Gliadin (G). Data are presented as mean±SEM. Each dot represents an individual mouse. All *P*-values were determined using a two-tailed unpaired Student's *t*-test. See Supplementary Figure 5 for the gating strategy.

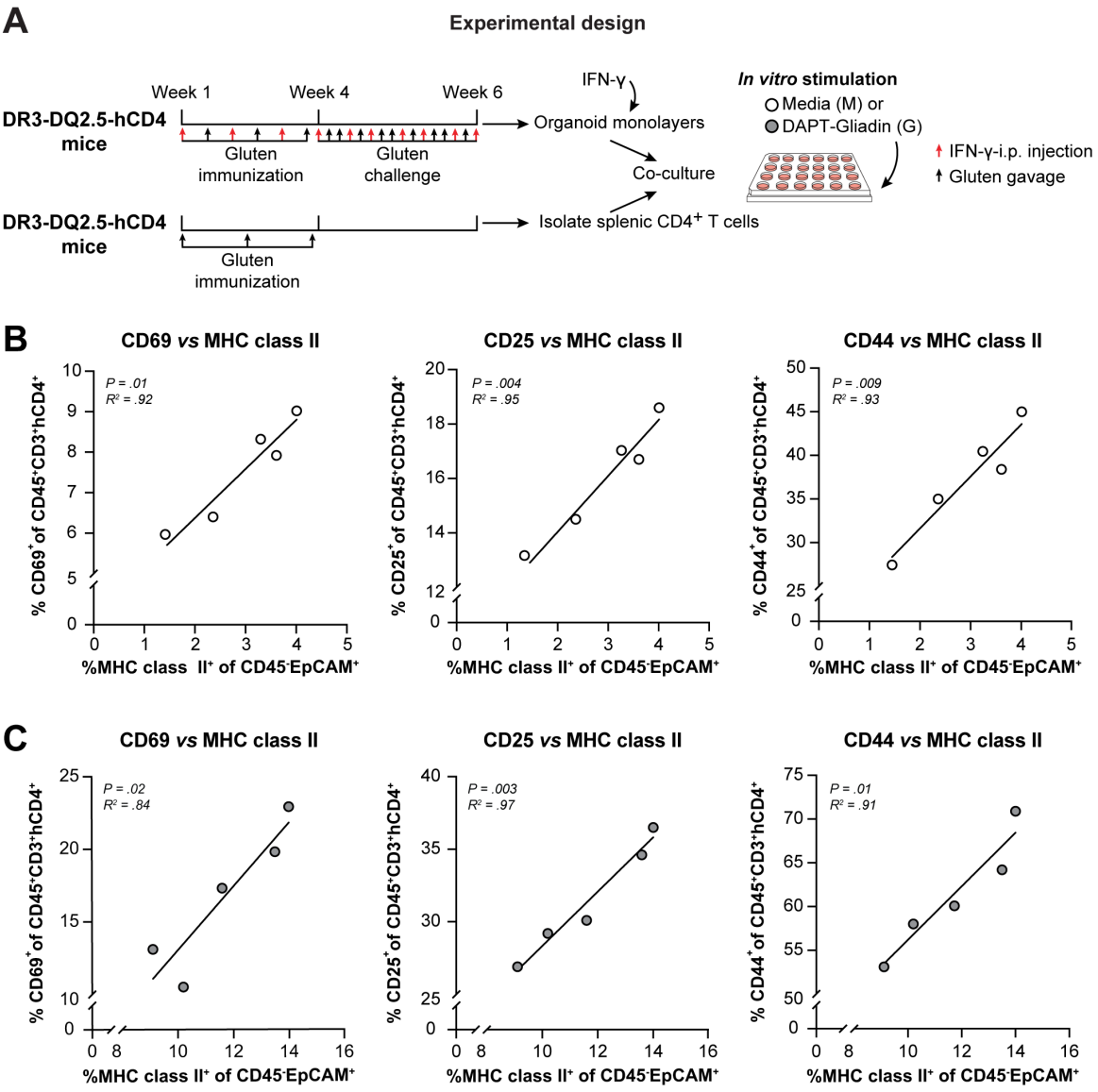


Figure 4.22 T cell activation markers correlate with monolayer MHC class II expression in co-cultures. (A) Organoid monolayers were co-cultured with splenic hCD4$^+$ T cells in the presence of deamidated pepsin-trypsin-digested (DAPT)-Gliadin (G; grey dots) or media (M; open dots). Organoid monolayers from gluten-immunized+IFNγ-injected DR3-DQ2.5-hCD4 mice (n=5) were further stimulated with IFN-γ for 24h prior to co-culture. Splenic CD4$^+$ T cells were isolated from gluten-immunized DR3-DQ2.5-hCD4 mice (n=5). **(B-C)** Correlation between CD69, CD25, and CD44 expression on live

$CD45^+CD3^+hCD4^+$ T cells and MHCII expression on live $CD45^-EpCAM^+$ cells of **(B)** media-stimulated **(C)** DAPT-Gliadin-stimulated co-cultures. Correlations were measured using Pearson correlation coefficients. Each dot represents an individual mouse. All *p* values were determined using a two-tailed unpaired Student's *t*-test. See Supplementary Figure 5 and 6 for gating strategy of organoid and CD4 T cells, respectively.

Subsequently, I evaluated the release of cytokines and chemokines from organoid monolayers derived from gluten-immunized mice upon exposure or not to IFN-γ, with the aim of distinguishing between epithelial-derived cytokines and chemokines and those secreted by T cells, and to ensure the absence of any $CD4^+$ T cells within organoid monolayers (Figure 4.23A). Intriguingly, the T cell-secreted cytokines IL-2 and IL-17A were not detected in monolayer-only cultures, even in the presence of IFN-γ, implying that the presence of these cytokines in the co-culture experiment can be attributed to activation of the underlying $CD4^+$ T cells in the co-culture system. Treating the organoid monolayers with IFN-γ led to higher secretion of epithelial-associated innate cytokines and chemokines, including IL-15, IL1-α, IL-1β, IL-6, CXCL-10, and CCL-20, compared with the control group that received no treatment (Figure 4.23B-K).

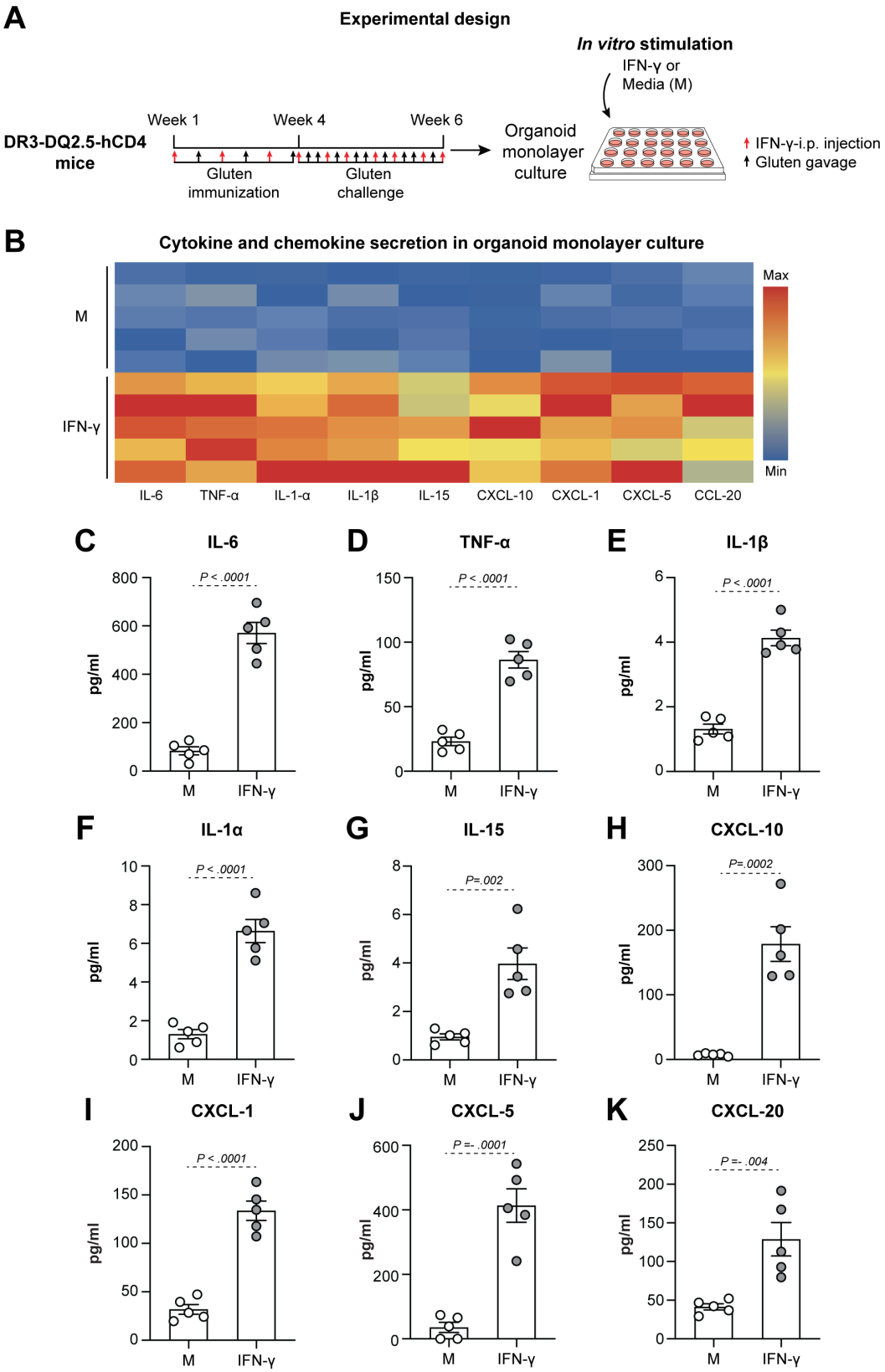

A
Experimental design
In vitro stimulation
IFN-γ or
Media (M)
DR3-DQ2.5-hCD4
mice
Week 1
Week 4
Week 6
Gluten
immunization
Gluten
challenge
Organoid
monolayer
culture
IFN-γ-i.p. injection
Gluten gavage
B
Cytokine and chemokine secretion in organoid monolayer culture
M
IFN-γ
Max
Min
IL-6
TNF-α
IL-1-α
IL-1β
IL-15
CXCL-10
CXCL-1
CXCL-5
CCL-20
C
IL-6
D
TNF-α
E
IL-1β
F
IL-1α
G
IL-15
H
CXCL-10
I
CXCL-1
J
CXCL-5
K
CXCL-20
pg/ml
P < .0001
P=.002
P=.0002
P=-.0001
P=-.004
M
IFN-γ

Figure 4.23 Monolayers stimulated with IFN-γ produce epithelial-associated innate cytokines and chemokines. (A) Organoid monolayers from gluten-immunized+IFNγ-injected DR3-DQ2.5-hCD4 mice (n=5) were stimulated with IFN-γ or media alone (M) for 24h prior to supernatant collection for the measurement of cytokine and chemokine levels. **(B)** Heatmap of cytokines and chemokines in the monolayer supernatant that were significantly different between IFN-γ and media (M) stimulation. Only cytokines with statistically significance secretion between the two groups are presented. **(C-K)** Quantification of cytokines and chemokines in the supernatant of organoid monolayer **only** cultures treated with media alone (M; n=5, open dots) or IFN-γ (n=5, grey dots)**.** Data are presented as mean±SEM. Each dot represents an individual mouse. All *p* values were determined using a two-tailed unpaired Student's *t*-test.

Finally, the expression of the stress-induced markers, Qa-1 and Rae-1, a ligand for NKG2D activating NK cell receptors, was higher in organoid monolayers treated with DAPT-gliadin following a four-day co-culture with $CD4^+$ T cells (Figure 4.24A-C).

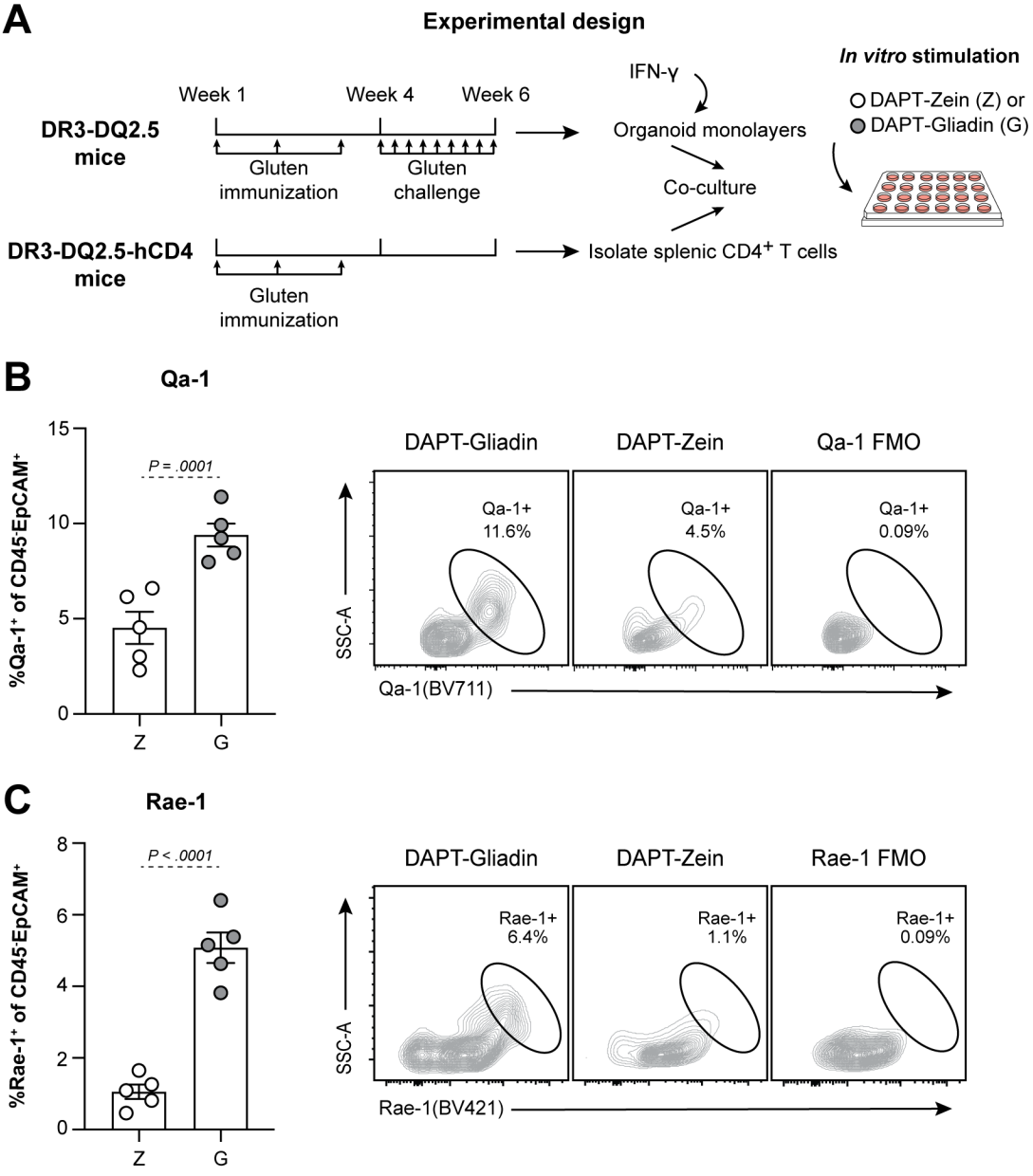


Figure 4.24 Gluten induces the expression of epithelial stress markers in organoid monolayers following co-culture with CD4+ T-cells (A) Organoid monolayers were co-cultured with splenic $CD4^+$ T cells in the presence of deamidated pepsin trypsin-digested (DAPT)-Zein (Z) (open dots) or DAPT-Gliadin (G) (grey dots). Organoid monolayers derived from gluten-immunized DR3-DQ2.5 mice (n=6) were then stimulated with IFN-γ for 24h prior to co-culture. $CD4^+$ T cells were isolated from the spleens of gluten-

immunized DR3-DQ2.5-hCD4 mice (n=6). **(B-C)** Percentage of **(B)** Qa-1-expressing cells, or **(C)** Rae-1-expressing cells, gated on live $CD45^{-}EpCAM^{+}$ cells from organoid monolayers co-cultured with $CD4^{+}$ T cells in the presence of DAPT-Zein (Z) or DAPT-Gliadin (G). Representative dot plots and fluorescence minus one (FMO) controls are shown. Data are presented as mean±SEM. Each dot represents an individual mouse. All *P*-values were determined using a two-tailed unpaired Student's *t*-test. See Supplementary Figure 5 for the gating strategy.

Collectively, these results prove, for the first time, that epithelial monolayers expressing MHC class II induce the proliferation and activation of underlying $hCD4^{+}$ T cells in a gluten-dependent manner, leading to the increased secretion of a panel of CeD-associated cytokines and chemokines.

4.4.2 MHC class II-expressing monolayer is required to promote a T cell response

The purity and phenotype of the isolated splenic $CD4^{+}$ T cells were evaluated using flow cytometry. I assessed the expression of the following markers, CD11b, CD19, and F4/80 to rule out contamination of isolated $CD4^{+}$ T cells with other splenic APCs. The purity of the isolated splenic $CD4^{+}$ T cells was approximately 97.2%, as shown in Supplementary Figure 6, a representative flow cytometry gating strategy for isolated $CD4^{+}$ T cells. Additionally, no traces of CD19 (B cells) or F4/80 (macrophages) were observed in isolated $CD4^{+}$ T cells (Figure 4.25). However, a small population of $CD11b^{+}$ cells (2.16%) was detected, which is a marker expressed by many different cells, including DCs.

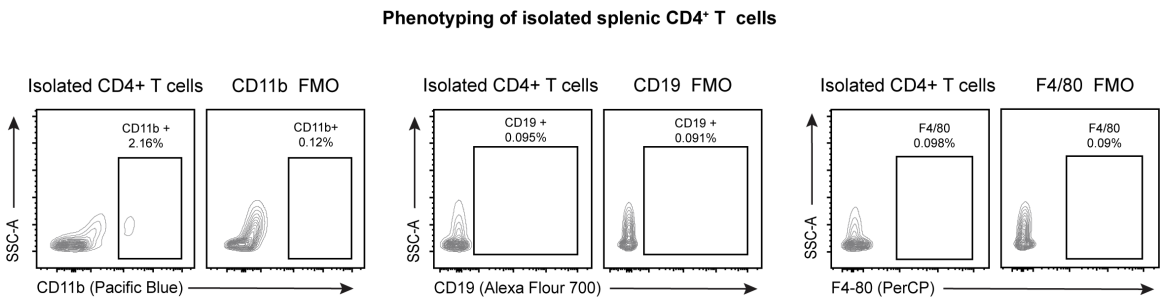


Figure 4.25 No trace of professional APCs in isolated splenic CD4$^+$ T cells from immunized DR3-DQ2.5 mice. The phenotype of the isolated CD4$^+$ T cells from the spleen of gluten-immunized DR3-DQ2.5 mice was determined using flow cytometry to evaluate contamination with other splenic APCs, with the following markers: CD11b, CD19, and F4/80, corresponding to the presence of dendritic cells, B cells, and macrophages. Representative dot plots and fluorescence minus one (FMO) controls are shown.

To confirm that this small fraction of CD11b$^+$ cells did not contribute to the proliferation and activation of CD4$^+$ T cells, isolated CD4$^+$ T cells were cultured in the absence of monolayers and stimulated with or without DAPT-gliadin (Figure 4.26A). After four days of culture, no proliferation of hCD4$^+$ T cells (Figure 4.26B) or expression of activation markers was detected in hCD4$^+$ cells (Figure 4.26C-E). These findings indicate that the presence of MHC class II-bearing organoid monolayers is necessary to present DAPT-gliadin to CD4$^+$ T cells and to promote their proliferation and activation.

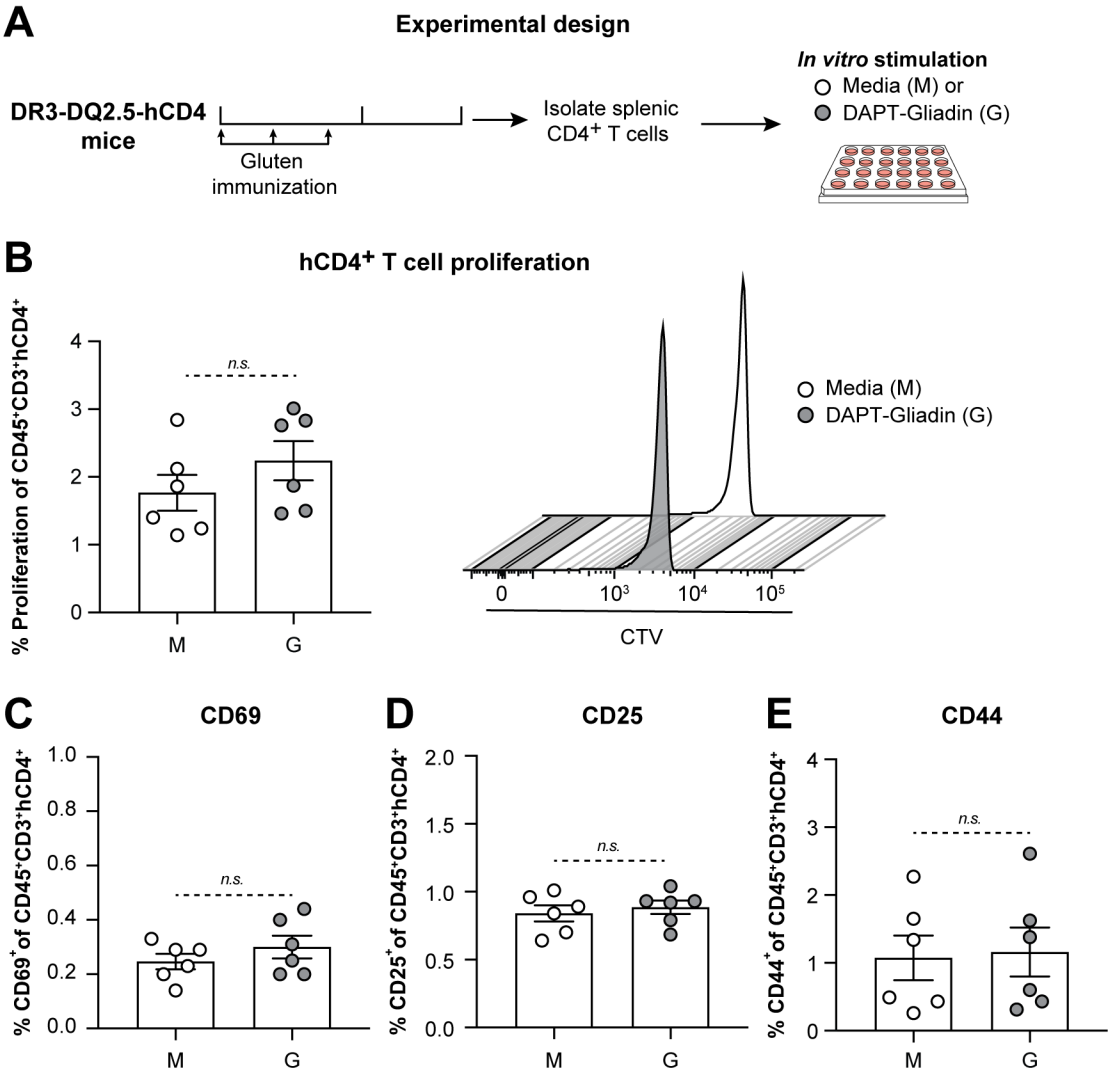


Figure 4.26 CD4$^+$ T cells do not proliferate or activate in the absence of MHCII-expressing organoid monolayers. (A) Splenic hCD4$^+$ T cells from gluten-immunized DR3-DQ2.5-hCD4 mice (n=6) were stimulated with deamidated pepsin-trypsin-digested (DAPT)-Gliadin (G; grey dots) or media alone (M; open dots). **(B)** Left panel, percentage of proliferating CellTrace Violet (CTV)-labelled hCD4$^+$ T cells, gated on live CD45$^+$CD3$^+$hCD4$^+$ cells simulated with DAPT-Gliadin (G) or media (M). Right panel, representative histograms of CTV-labeled hCD4$^+$ T cells gated on live CD45$^+$CD3$^+$hCD4$^+$ cells. **(C-E)** Percentage of **(C)** CD69-expressing cells, **(D)** CD25-expressing cells, or **(E)** CD44-expressing cells gated on live CD45$^+$CD3$^+$hCD4$^+$ T cells that

were stimulated with DAPT-Gliadin (G) or media (M). Data are presented as mean±SEM. Each dot represents an individual mouse. See Supplementary Figure 6 for the gating strategy.

4.4.3 Modulation of hCD4+ T cell activation by opportunistic pathogen-derived elastase

Microbial factors have emerged as modulators of inflammation in CeD (Caminero & Verdu, 2019; E. F. Verdu & Schuppan, 2021). Using peripheral blood mononuclear cells from HLA-DQ2.5^{+} CeD patients, it was shown that gluten peptides metabolized by bacterial elastase have increased immunogenicity (Caminero et al., 2016). I used this knowledge to investigate whether bacterial metabolism influences IEC-T cell interactions in my model. Organoid monolayers were thus stimulated with gliadin that was pre-digested with elastase-producing (wild type; WT) *P. aeruginos*a PA14. As controls, I used DAPT-gliadin, gliadin pre-digested with the *P. aeruginos*a isogenic *lasB* mutant strain lacking elastase activity, referred to as *lasB*$^{\Delta/\Delta}$, and WT *P. aeruginosa* PA14 culture supernatant (Figure 4.27A). Compared with co-cultures treated with DAPT-gliadin pre-incubated with *lasB*$^{\Delta/\Delta}$, DAPT-gliadin, or WT *P. aeruginosa* PA14 supernatant, treatment with DAPT-gliadin pre-digested with WT *P. aeruginosa* PA14 led to 1.7-, 1.4-, and 2.6-fold increases in hCD4^{+} T cell proliferation, respectively (Figure 4.27B). Additionally, increased expression of the activation markers CD69, CD25, and CD44 (Figure 4.27C-E) in hCD4^{+} T cells confirmed a more robust activated phenotype of these cells in co-cultures stimulated with DAPT-gliadin pre-digested with WT *P. aeruginos*a PA14 versus controls.

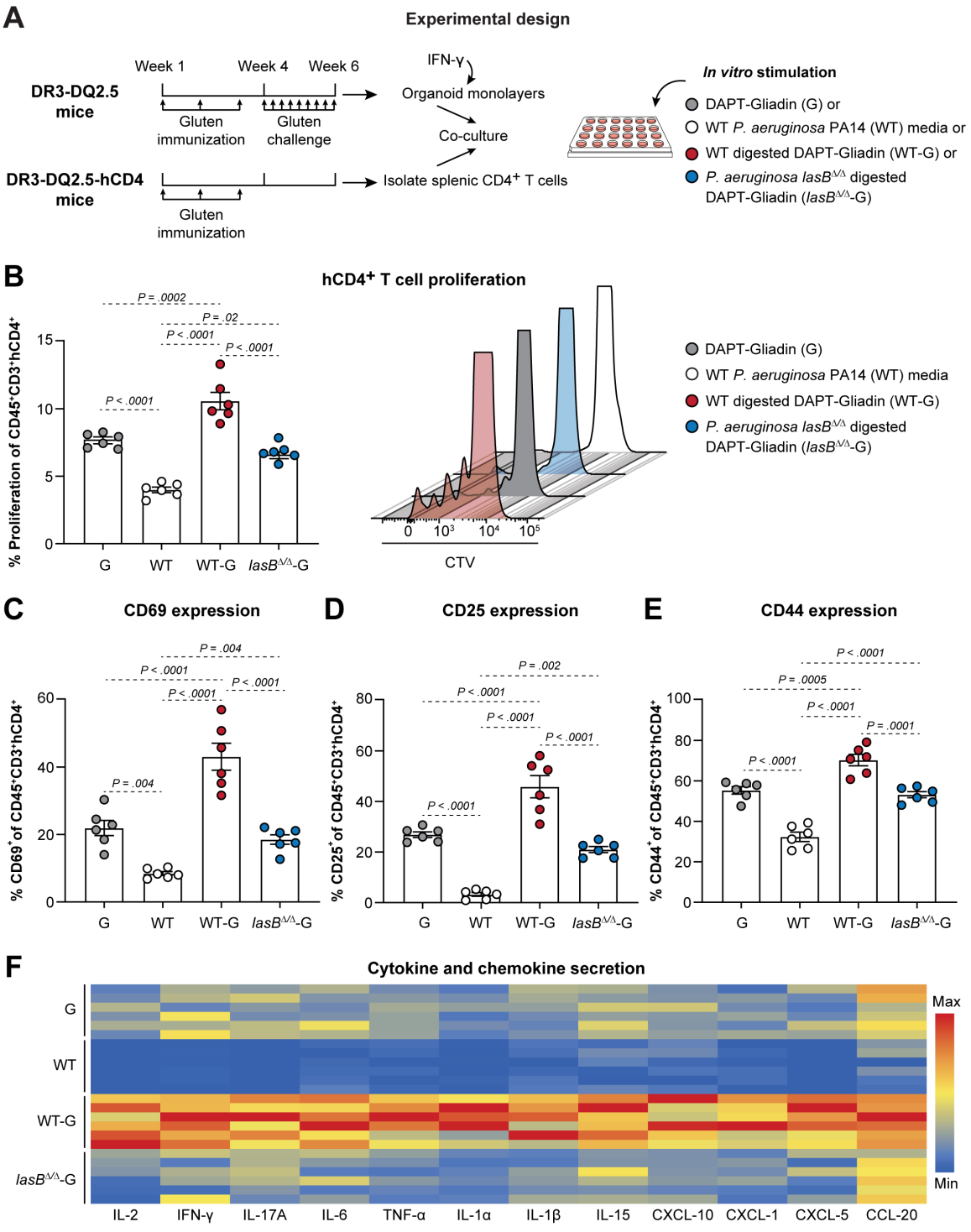


Figure 4.27 Gluten metabolized by opportunistic pathogen-derived elastase induces a robust CD4⁺ T-cell response. (A) Organoid monolayers were co-cultured with splenic

hCD4$^+$ T cells in the presence of deamidated pepsin-trypsin digested (DAPT)-Gliadin (G; grey dots), wild-type *P. aeruginosa* PA14 (WT; open dots) supernatant, WT digested DAPT-Gliadin (WT-G; red dots), or *P. aeruginosa lasB$^{\Delta/\Delta}$* digested DAPT-Gliadin (*lasB$^{\Delta/\Delta}$-G*; blue dots). Organoid monolayers derived from gluten-immunized DR3-DQ2.5 mice (n=6) were stimulated with IFN-γ for 24h prior to co-culture. Splenic CD4$^+$ T cells were isolated from gluten-immunized DR3-DQ2.5-hCD4 mice (n=6). **(B)** Left panel, percentage of proliferating CTV-labelled hCD4$^+$ T cells gated on live CD45$^+$CD3$^+$hCD4$^+$ cells that were co-cultured with organoid monolayers in the presence of DAPT-Gliadin (G), *P. aeruginosa* PA14 (WT) supernatant, WT digested DAPT-Gliadin (WT-G), or *P. aeruginosa lasB$^{\Delta/\Delta}$* digested DAPT-Gliadin (*lasB$^{\Delta/\Delta}$-G*). Right panel, representative histograms of CTV-labeled hCD4$^+$ T cells gated on live CD45$^+$CD3$^+$ cells. **(C-E)** Percentage of **(C)** CD69-expressing cells, **(D)** CD25-expressing cells or **(E)** CD44-expressing cells, gated on live CD45$^+$CD3$^+$hCD4$^+$ T cells that were co-cultured with organoid monolayers in the presence of DAPT-Gliadin (G), *P. aeruginosa* PA14 (WT) supernatant, WT digested DAPT-Gliadin (WT-G), or *P. aeruginosa lasB$^{\Delta/\Delta}$* digested DAPT-Gliadin (*lasB$^{\Delta/\Delta}$-G*). **(F)** Heatmap of cytokines and chemokines that were significantly different between co-cultures stimulated with (DAPT)-Gliadin (G), wild-type *P. aeruginosa* PA14 (WT) supernatant, WT digested DAPT-Gliadin (WT-G), and *P. aeruginosa lasB$^{\Delta/\Delta}$* digested DAPT-Gliadin (*lasB$^{\Delta/\Delta}$-G*). Data are presented as mean±SEM. Each dot represents an individual mouse. All *P*-values were determined using one-way analysis of variance (ANOVA) with Tukey's post-hoc test for multiple comparisons. See Supplementary Figure 6 for the gating strategy.

Furthermore, the activated phenotype of hCD4$^+$ T cells was associated with elevated levels of a panel of cytokines and chemokines (Figure 4.27F), including a 5.4-, 3.7-, and 676-fold increase in IL-2 levels in the supernatant of the co-culture when the organoid monolayers were treated with DAPT-gliadin pre-digested with WT *P. aeruginosa*

PA14 compared with DAPT-gliadin pre-digested with *lasB*$^{\Delta/\Delta}$, DAPT-gliadin, or WT *P. aeruginosa* PA14 culture supernatant, respectively (Figure 4.28A). The secretion of Th1- and Th17-associated cytokines, IFN-γ (Figure 4.28B) and IL-17A (Figure 4.28C), respectively, was highest when organoid monolayers were treated with DAPT-gliadin pre-digested with WT *P. aeruginos*a PA14 versus controls. In addition, organoid monolayers treated with DAPT-gliadin pre-digested with WT *P. aeruginos*a PA14 exhibited elevated release of the pro-inflammatory cytokines IL-6, TNF-α, IL-1α, IL-1β, and IL-15 compared with controls (Figure 4.28D-H). Stimulation of organoid monolayers with DAPT-gliadin pre-digested with WT *P. aeruginos*a PA14 led to higher production of the chemokines CXCL-10, CXCL-1, CXCL-5, and CCL-20 compared with the controls (Figure 4.28I-L).

Taken together, these results demonstrate that MHC class II-expressing organoid monolayers enable more robust activation and proliferation of the underlying hCD4$^+$ T cells when gliadin is partially metabolized by bacterial elastase.

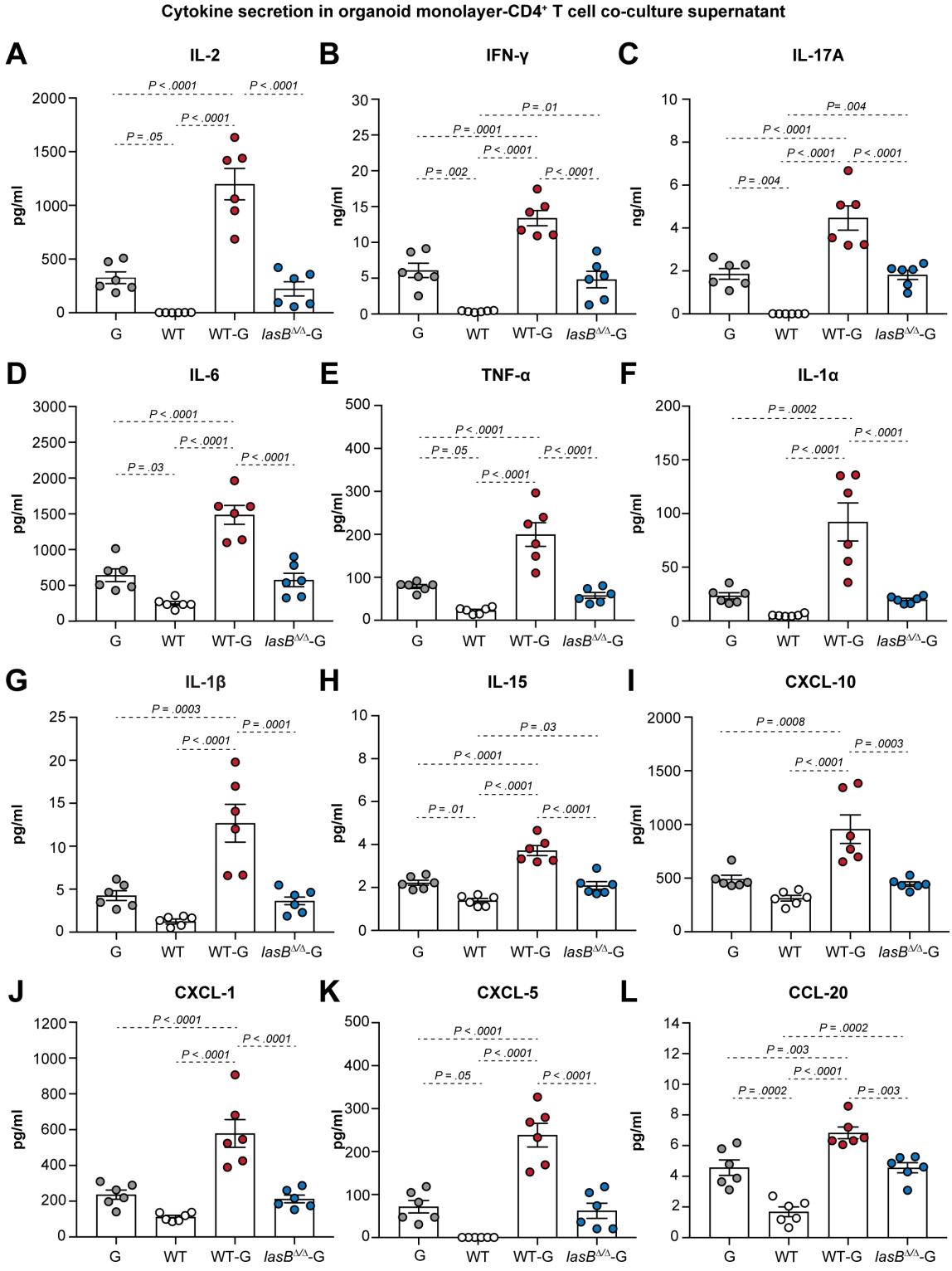

Cytokine secretion in organoid monolayer-CD4⁺ T cell co-culture supernatant
A
IL-2
pg/ml
P < .0001
P < .0001
P < .0001
P = .05
B
IFN-γ
ng/ml
P = .01
P = .0001
P < .0001
P = .002
P < .0001
C
IL-17A
ng/ml
P = .004
P < .0001
P = .004
P < .0001
P < .0001
D
IL-6
pg/ml
P < .0001
P < .0001
P = .03
P < .0001
E
TNF-α
pg/ml
P < .0001
P = .05
P < .0001
P < .0001
F
IL-1α
pg/ml
P = .0002
P < .0001
P < .0001
G
IL-1β
pg/ml
P = .0003
P = .0001
P < .0001
H
IL-15
pg/ml
P = .03
P < .0001
P < .0001
P = .01
P < .0001
I
CXCL-10
pg/ml
P = .0008
P = .0003
P < .0001
J
CXCL-1
pg/ml
P < .0001
P < .0001
P < .0001
K
CXCL-5
pg/ml
P < .0001
P < .0001
P = .05
P < .0001
L
CCL-20
pg/ml
P = .0002
P = .003
P < .0001
P = .0002
P = .003
G
WT
WT-G
lasB^Δ/Δ-G

Figure 4.28 Gluten digestion by opportunistic pathogen-derived elastase promotes secretion of cytokines and chemokines associated with CeD. (A-L) Quantification of cytokines and chemokines in the supernatant of organoid monolayers-hCD4^{+} T cell co-cultures treated with deamidated pepsin-trypsin digested (DAPT)-Gliadin (G; n=6; grey dots), wild-type *Pseudomonas aeruginosa* PA14 supernatant (WT; n=6; open dots), WT digested DAPT-Gliadin (WT-G; n=6; red dots), or *P. aeruginosa lasB*$^{\Delta/\Delta}$ digested DAPT-Gliadin (*lasB*$^{\Delta/\Delta}$*-G*; n=6; blue dots). Data are presented as mean±SEM. Each dot represents an individual mouse. All *P*-values were determined using one-way analysis of variance (ANOVA) with Tukey's post-hoc test for multiple comparisons.

CHAPTER 5 DISCUSSION

DISCUSSION

5.1 Summary of findings

IECs are emerging as potential regulators of $CD4^+$ T cell responses via MHC class II expression. However, until the work performed in this thesis, their role as antigen-presenting cells and in the activation of gluten-reactive $CD4^+$ T cells, had never been proven. I first evaluated the epithelial expression of MHC class II in duodenal biopsies from active and treated (on GFD) CeD patients, and in gluten-immunized and NI (control) DR3-DQ2.5 transgenic mice that lack murine MHC Class II and only carry human HLA-DQ2.5 genes. MHC Class II expression was higher in IEC from active CeD patients *versus* treated and in gluten-immunized DR3-DQ2.5 *versus* NI mice. I then developed humanized organoid monolayers from gluten-immunized, NI, and naïve DR3-DQ2.5 mice, treated with or without IFN-γ *in vitro*, and determined the expression epithelial MHC class II. Organoid monolayers from gluten-immunized mice demonstrated higher expression of MHC class II compared with monolayers derived from NI or naïve mice. This MHC class II expression was upregulated upon treatment of monolayers with IFN- γ, with the highest level detected in monolayers from gluten-immunized mice and accompanied by the expression of co-stimulatory molecules. Next, I evaluated the functional capacity of this expression in terms of T-cell activation by co-culturing MHC class II-expressing organoid monolayers from DR3.DQ2.5 mice with splenic human (h)$CD4^+$ T cells isolated from gluten-immunized double transgenic DR3-DQ2.5-hCD4 mice. Organoid monolayers in the co-culture were treated with zein, gluten, gluten pre-digested with elastase-producing *P. aeruginosa* PA14, or gluten pre-digested with its *lasB* mutant. After four days, T cell

proliferation, activation, and cytokine and chemokine release were assessed. I found that gluten, but not zein, treatment of organoid monolayers increased T cell proliferation, expression of activation markers (CD25, CD44, and CD69), and production of pro-inflammatory cytokines IL-2, IFN-γ, and IL-15. Additionally, organoid monolayers exposed to gluten pre-digested by elastase-producing *P. aeruginosa* PA14 showed increased $CD4^+$ T cell proliferation, activation, and cytokine production compared with monolayers exposed to gluten or gluten pre-digested with the *lasB* mutant. Collectively, IECs upregulate MHC class II under inflammatory conditions, have the capacity to present gluten antigen, and activate gluten-specific $CD4^+$ T cells, which is further potentiated by microbial elastase digestion of gluten.

5.2 General discussion

IECs have been known to constitutively express MHC class II. However, the functional implications of MHC class II expression in IECs remain controversial. Some studies have attributed the expression of MHC class II to inflammation, while others have associated it with immunosuppression, epithelial differentiation, or renewal (Biton et al., 2018; Jamwal et al., 2020; Koyama et al., 2019; Thelemann et al., 2014). These conflicting results are likely contextual, indicating that a complete understanding will require the investigation of MHC class II expression in specific disease and under particular situations. In the context of CeD, the expression of MHC class II in the enterocytes of patients with CeD has been previously reported (Arnaud-Battandier et al., 1986)(Marley et al., 1987)(Fais et al., 1992)(Kelly et al., 1988a). Despite this, the role of epithelial MHC class II in gluten

presentation and T-cell activation in CeD has been overlooked. There are many plausible reasons for this. For gluten presentation to occur, it is essential that the APC expresses the right MHC class II, in the case of CeD, HLA-DQ2 or DQ8. Patients with CeD express different isotypes of MHC class II, in addition to either DQ2 and/or DQ8. Thus, is has been technically very difficult to investigate the immune role of IECs as non-conventional APC in CeD. In the past years however, advances in transgenic animal models and organoid technology as well as in imaging and immunostaining capabilities have prepared the stage for this question to be answered.

I first verified that IECs from patients with CeD express MHC class II (HLA-**DQ**) associated with CeD. I evaluated the expression of MHC class II in duodenal biopsies (D2) of active and treated CeD patients using immunofluorescence staining. The results revealed higher expression of MHC class II in the IECs of active *versus* treated CeD patients compliant with GFD that had achieved histological remission. This finding suggests that CeD activity is associated with upregulation of epithelial MHC class II, which is in line with previous findings that showed expression of MHC class II in the small intestinal epithelium of patients with CeD (Arnaud-Battandier et al., 1986)(Marley et al., 1987)(Fais et al., 1992). I subsequently confirmed the expression of the HLA-**DQ** isotype of MHC class II in duodenal biopsies obtained from active and treated patients with CeD using the HLA-DQ-specific antibody. This finding was further supported by flow cytometry in IECs isolated from biopsies of symptomatic and asymptomatic CeD patients using the HLA-DQ antibody. In addition, the expression of co-stimulatory molecules, including CD40, CD80, and CD86, in IECs isolated from biopsies of symptomatic and asymptomatic patients with

CeD was confirmed using flow cytometry. Together, these findings confirm that the intestinal epithelium of patients with CeD expresses HLA-**DQ**, implying that this epithelium might have the capacity for MHC class II-mediated luminal gluten antigen presentation and T-cell activation during CeD activity; however, this has not been proven.

Next, I investigated specific gluten-epithelial MHC class II-$CD4^+$ T cell interactions by employing humanized organoid monolayers derived from mice carrying the CeD risk allele: HLA-DR-DQ2.5. The use of transgenic mouse models and *ex vivo* organoid monolayer cultures offers a controlled and reproducible platform that complement mechanistically the findings from human biopsies, enabling in-depth investigation of molecular functionalities that cannot be practically explored in humans. These combined approaches allow for the integration of clinical findings with mechanistic understanding, providing novel insight into the immunomodulatory roles of the intestinal epithelium in CeD pathogenesis, which can potentially be targeted therapeutically. To do this, I first determined the impact of gluten immunization and challenge on transgenic mice that lack all murine MHC class II haplotypes and only carry the DR3-DQ2.5 allele, which is prevalent in the majority of CeD patients. Approximately 90% of individuals with CeD carry the HLA-DQ2.5 allele (Sollid et al., 1989), while the remainder express HLA-DQ2.2 and/or -DQ8 (Green & Jabri, 2003b). I then determined the expression of MHC class II in these mice under different induced inflammatory conditions.

DR3-DQ2.5 mice were orally immunized with gluten in conjunction with cholera toxin (CT) to break oral tolerance to gluten, followed by a gluten challenge phase to induce a gut mucosal immune response. Upon gluten immunization, DR3-DQ2.5 developed lower

vh/cd and higher $CD3^+$ IEL cell counts in the villous tip compared with NI mice. In addition, anti-TG2 IgG antibodies were detected in the serum, anti-gliadin IgA antibodies were measured in the intestinal contents, and a trend for increased anti-TG2 IgA antibodies was identified in the intestinal contents of gluten-immunized mice. In patients with CeD, anti-TG2 IgA (and IgG) antibodies are indicative of active CeD, and these antibodies, along with total IgA, are used for the serological diagnosis of patients with CeD (Dieterich et al., 1997)(Dieterich et al., 1998). The development of intestinal antibodies agrees with previous findings in HLA-DQ8, NOD-DQ8 and HLA-DQ8-IL15 transgenic models, indicating that B cells are activated in the mucosa (H. J. Galipeau et al., 2011a)(E. F. Verdu et al., 2008)(Abadie et al., 2020). Results are also consistent with the diverse range of clinical phenotypes observed in CeD, including seroconversion without atrophy as in potential CeD patients, and active CeD characterized by varying degrees of enteropathy (Marsh I-IV)(Catassi et al., 2022)(Malamut et al., 2024).

The increased production of anti-TG2 IgG antibodies in the serum of gluten-immunized DR3-DQ2.5 mice is a unique characteristic of these mouse models, as these antibodies have not been previously detected in other models (H. J. Galipeau et al., 2011a)(E. F. Verdu et al., 2008)(Abadie et al., 2020), suggesting advantages over DQ8 based models. Despite absence of total atrophy, gluten-immunized DR3-DQ2.5 mice developed proximal small intestinal inflammation associated with pro-inflammatory gene expression, including genes that participate in innate immune functions, such as *Tlr3*, *Rhoa*, *Tollip*, and MAP kinases (MAPK), such as *Mapk3*, *Mapk1*, and *Map3k1* (Cognol and Chambard, 2010). Other upregulated genes in gluten-immunized mice were inflammation-

related genes, namely *Il15*, *Il1a*, and *Cfb* (Abadie et al., 2020)(Abadie & Jabri, 2014)(Marafini et al., 2015)(R. Zhou et al., 2007)(Novak et al., 2020). The higher expression of *Il15* in gluten-immunized DR3-DQ2.5 mice is interesting, as this gene encodes IL-15, a key cytokine in CeD that drives intestinal destruction, activates IEL cytotoxicity, and suppresses regulatory T cells (Abadie & Jabri, 2014)(Hu et al., 2018)(Roberts et al., 2001). The observed pattern of pro-inflammatory gene expression in the small intestine of gluten immunized DR3-DQ2.5 mice indicates an inflammatory niche that may promote the expression of MHC class II in IECs. This was confirmed by immunofluorescence staining and flow cytometry of duodenum and proximal jejunum tissues. Specifically, gluten immunization resulted in enhanced MHC class II expression in the inflamed epithelium of gluten-immunized DR3-DQ2.5 mice compared with the non-inflamed epithelium of NI mice, which only showed lamina propria expression of MHC class II molecules. This comparison highlights the role of inflammation in the regulation of MHC class II expression at the epithelial level.

Given that gluten-immunized mice develop moderate inflammation, I hypothesized that a more severe inflammatory stimulus could further enhance MHC class II expression in IECs, thus strengthening the causal link between this expression and active inflammation. To study this, DR3-DQ2.5-hCD4 mice were i.p. injected with rmIFN-γ to induce a stronger inflammatory environment in these mice. Immunofluorescence staining data revealed that gluten-immunized mice injected with IFN-γ (gluten-immunized+IFN-γ-injected) exhibited increased expression of epithelial MHC class II compared with NI mice injected with PBS (NI-PBS+injected). Additionally, the quantification analysis of

immunofluorescence staining results between gluten-immunized+IFN-γ-injected and gluten-immunized mice revealed a notably elevated MHC class II expression in IECs of gluten-immunized mice that received IFN-γ injection (gluten-immunized+IFN-γ-injected) compared with gluten-immunized mice that did not receive the injections. This implies that *in vivo*-induced inflammation caused by IFN-γ injections may prime the intestinal epithelium to further upregulate the expression of MHC class II. This finding is consistent with prior studies indicating that IECs express IFN-γ receptors and that IFN-γ is a potent inducer of MHC class II upregulation in IECs (C. E. Heuberger et al., 2023; Koyama et al., 2019; Wosen et al., 2019)(Malik et al., 2023), further supporting the role of inflammatory signaling in modulating epithelial MHC class II expression. I also measured epithelial expression of Qa-1, a stress-induced non-classical MHC class I marker and a murine homolog of HLA-E that was previously shown to be increased in CeD patients and that signals to innate immune cells (Jabri et al., 2000). Qa-1 was higher in gluten-immunized *versus* NI mice, making this model a relevant platform for studying the interaction between IECs and innate immune cells, such as IELs in CeD, in the future.

The collective results obtained in this section of the thesis suggest that gluten immunization and IFN-γ-injection of DR3-DQ2.5 mice replicate some features of the kinetics of serological and histological characteristics observed in patients with CeD. Altogether, animal models strive to approximate rather than precisely replicate the complexity of multifactorial human diseases. Preclinical models of disease, whether animal, *in vitro*, or *ex vivo*, aim to reproduce key features of the disease under study as closely as possible, providing insights that will always require subsequent validation in

human studies. These models are invaluable tools for uncovering novel mechanisms and conducting preclinical therapeutic screening. In this context, the humanized DR3-DQ2.5 mouse model described in this thesis effectively recapitulates the key aspects of CeD following the breakdown of oral tolerance to gluten. This model will be useful for future studies aimed at exploring the influence of various environmental factors on CeD pathogenesis. However, because MHC class II in these models is expressed in all APC cells, *in vivo* studies will not be useful to discern the isolated role of IECs as non-conventional gluten-presenting cells and the activation of T cells.

Therefore, here, I developed humanized organoid monolayers from the duodenum and proximal jejunum of mice carrying CeD risk alleles: HLA-DQ8 or HLA-DQ2.5. The organoid monolayers derived from both mouse models showed the expression of the main intestinal epithelial cell lineages, including enterocytes, goblet cells, Paneth cells, and enteroendocrine cells. Following the characterization of organoid monolayers, the conditions that lead to the expression of MHC class II and its co-stimulatory molecules, which are crucial for IEC-mediated antigen presentation, were determined. I found that organoid monolayers from gluten-immunized mice strongly expressed MHC class II compared with those derived from control mice, suggesting that the induction of inflammation in these mice prior to organoid development primes the intestinal epithelium to upregulate MHC class II. Stimulation of organoid monolayers with IFN-γ, a Th1-associated cytokine secreted by gluten-reactive $CD4^+$ T cells from patients with active CeD (Nilsen et al., 1998), resulted in the upregulation of MHC class II expression in IECs. Thus, IFN-γ-producing cells or infections that lead to the release of IFN-γ may play a role in

inducing MHC class II in IECs. This finding is consistent with prior studies demonstrating the IFN-γ-dependent upregulation of MHC class II in IECs in response to inflammation (Koyama et al., 2019)(Thelemann et al., 2014; Wosen et al., 2019)(Van Der Kraak et al., 2021)(Malik et al., 2023). The same trend was observed in the expression of MHC class II in organoid monolayers from immunized+IFN-γ-injected mice compared with monolayers from NI+PBS-injected mice following treatment of monolayers with IFN-γ. These results are in accordance with the findings in the epithelial tissue of gluten-immunized versus NI mice. These results suggest that *in vivo* inflammation potentiates the intestinal epithelium of these mice to upregulate MHC class II expression in IECs, which is then recapitulated in organoid monolayers. Moreover, low expression of the co-stimulatory molecules, and Qa-1, were detected in IFN-γ-treated monolayers from immunized mice, implying that the expression of these molecules is a multiple hit and require both inflammatory stimuli of *in vivo* gluten immunization in addition to *in vitro* stimulation with IFN-γ. Taken together, the findings presented here indicate that the intestinal epithelium, under correct inflammatory conditions, possesses the prerequisite machinery to present antigens. However, the next question that arises is whether epithelial MHC class II expression is functional and participates in antigen presentation.

My thesis project confirmed the expression of MHC class II in humans with CeD and mouse models. Other studies have shown epithelial MHC class II expression in infection-induced colitis models (Malik et al., 2023)(Jamwal et al., 2020)(Thelemann et al., 2014), chemically induced colitis (DSS model) (Malik et al., 2023), and T-cell transfer-mediated colitis (Tuganbaev et al., 2020)(Thelemann et al., 2014)(Jamwal et al., 2020).

However, the functional consequences of this expression remained controversial. Some studies suggested that, while MHC class II expression in IECs offers protection against intestinal cancer (Beyaz et al., 2021b), it can exacerbate GvHD by serving as a target for allogeneic T-cells (Koyama et al., 2019). Recent studies have identified that MHC class II expression in IECs is partially regulated by the feeding rhythm and circadian clock, establishing a diurnal pattern of expression (Tuganbaev et al., 2020). In addition, dietary composition, particularly a high-fat diet, significantly reduced MHC class II expression in IECs and disrupted its diurnal pattern (Beyaz et al., 2021b). These studies highlight the complex and context-dependent nature of epithelial MHC class II expression. CeD offers a unique opportunity to investigate functionality of epithelial MHC class II upregulation in a very defined context, as both the MHC class II molecule and its associated environmental driver, gluten proteins, are known. Accordingly, I established an *in vitro* co-culture model that enables the evaluation of the impact of main driver (gluten) on epithelial MHC class II- $CD4^+$ T cell interactions under defined conditions, while concurrently considering the effect of co-factors and triggers, including those derived from bacterial origin.

It is generally accepted that gluten peptides need to gain access to the lamina propria to stimulate inflammation in CeD. Recent studies have challenged the view that deamidation of gluten by TG2 occurs only in the lamina propria, and that this process could be mediated through epithelial TG2 release (Iversen et al., 2020b). However, the mechanism by which gluten fragments translocate across the epithelial barrier remains controversial. Different pathways have been suggested for the translocation of gluten peptides, such as the paracellular pathway via altered tight junctions (Lammers et al., 2008),

and the transcellular pathway via ectopic expression of the transferrin receptor, CD71, in IECs (Matysiak-Budnik et al., 2008)(Lebreton et al., 2012). Intriguingly, the expression of CD71, a transferrin receptor involved in gluten peptide transport across the intestinal epithelium, was identified in organoid monolayers from gluten-immunized mice that were further stimulated *in vitro* with IFN-γ, indicating that the epithelial expression of CD71 requires additional inflammatory stimuli, such as *in vivo* gluten immunization and *in vitro* stimulation with the pro-inflammatory cytokine, IFN-γ. The expression of CD71 molecules in this model recapitulates the finding that CD71 is overexpressed during intestinal inflammation in active but not in potential CeD patients (Matysiak-Budnik et al., 2008).

Traditionally, preclinical models, including animal models or *in vitro* studies, have been designed to serve as an approximation of the disease under investigation and ideally aim to closely replicate the disease characteristics. Their purpose was to provide a new understanding that ultimately requires validation in humans. Evaluating these preclinical models typically involves assessing three key factors: (1) face validity, which examines how well the model represents the characteristics of the disease; (2) construct validity, which investigates the underlying mechanism for the emergence of these characteristics in the model; and (3) predictive validity, which measures the model's ability to predict the human response to treatments or expression of relevant disease pathways. The extent to which these factors overlap varies across different preclinical models. In the context of CeD, organoids and organoid monolayers have been developed from patients with CeD (Dieterich et al., 2020)(Dieterich et al., 2020)(Serena et al., 2019)(Pietz et al., 2017)(Porpora et al., 2022). There is no doubt that patient-derived organoids are invaluable

in advancing the translational research toolbox for the study of CeD; however, these models often develop from individuals with active CeD, where tissues express phenotypic changes, and are already inflamed, and autoimmune processes are activated. This complicates the distinction between changes resulting from disease progression and those resulting from initial mechanisms. In contrast, using humanized organoid monolayers from transgenic mice carrying human CeD risk genes enabled the controlled manipulation of inflammatory conditions, both *in vivo* and *in vitro*, as demonstrated throughout this thesis. This approach offers valuable insights into dissecting the early initiation of inflammation in CeD. However, such manipulations are impractical in human-derived organoids. While organoids from CeD patients are useful for testing new treatments and advancing personalized medicine, humanized organoids such as those developed in this thesis offer significant experimental advantages, enhancing our understanding of specific disease mechanisms under defined conditions. Recently, induced pluripotent stem cell (iPSC)-derived organoids have been developed to investigate the role of mesenchyme in ulcerative colitis (Sarvestani et al., 2021). Although this approach has not yet been explored in the context of CeD, iPSC-derived organoids exhibit fetal-like phenotypes (Takahashi et al., 2018)(C. Liu et al., 2018)(Rowe & Daley, 2019). Currently, studies are exploring the feasibility of transplanting iPSC-derived organoids into mice to promote their maturation and allow them to adopt adult-like characteristics (Fordham et al., 2013)(Watanabe et al., 2022)(Fumagalli et al., 2018). The organoid model presented in this thesis adds unique humanized features and practical advantages to the current toolbox of models relevant to the study of CeD.

Organoids and organoid monolayers are recognized for their ability to closely recapitulate phenotypic characteristics and reflect the genetic conditions of the tissues from which they are derived (J. Kim et al., 2020)(In et al., 2016). As such, they have proven to be powerful *in vitro* models for modeling diseases, screening novel therapeutics, understanding immune-mediated intestinal regeneration and development, dissecting immune responses, and exploring host-microbial interactions (Foulke-Abel et al., 2014)(In et al., 2016)(Schwank et al., 2013)(Günther et al., 2022)(Jowett et al., 2022)(Sasaki et al., 2020)(Li, 2021)(J. Kim et al., 2020). A key limitation of standard organoid monolayer culture systems is the absence of stromal, immune, and microbial components that are known to interact with IECs *in vivo*. Introducing these components in a controlled and defined manner is pivotal to accurately recapitulate the *in vivo* microenvironment in an *in vitro* setting. Additionally, my findings revealed that humanized organoid monolayers, when exposed to appropriate inflammatory conditions, possess the molecular machinery necessary for antigen presentation. This raises critical questions: What are the functional consequences of upregulated MHC class II expression? Does epithelial MHC class II expression lead to $CD4^+$ T cell activation under the right conditions?

To address these questions, I investigated the interactions between organoid monolayers, $CD4^+$ T cells, and dietary antigens, with the aim of elucidating the immunomodulatory role of IECs. To manipulate and control the microenvironment of these interactions, I developed an *in vitro* co-culture system in which MHC class II-expressing organoid monolayers derived from gluten-immunized DR3-DQ2.5 mice were co-cultured with gluten-specific $CD4^+$ T cells. This system provides valuable insights into the impact

of epithelial MHC class II expression on the phenotype and functionality of $CD4^+$ T cells. In addition, the immune response in CeD is $CD4^+$ T cell-dependent and HLA-DQ-restricted (Black et al., 2002); as such I used gluten-immunized double transgenic DR3-DQ2.5-hCD4 mice that carry human CD4 receptors in addition to having DQ2.5 molecules to isolated $CD4^+$ T cells. Although I characterized organoid monolayers derived from both HLA-DQ8 and DR3-DQ2.5 mice, for the co-culture system, I focused exclusively on organoid monolayers derived from DR3-DQ2.5 mice, as this is the genotype expressed in cis or trans by most CeD patients (Sollid et al., 1989) and because the ability of DQ2.5 to bind the gluten peptide repertoire has been extensively characterized (C.-Y. Kim et al., 2004; Tye-Din, Stewart, et al., 2010). Additionally, the well-studied peptide sequence in the adaptive immune response of CeD, the 33-mer in α-gliadin (Shan et al., 2002), has been shown to have a high binding affinity for HLA-DQ2.5 molecule (S.-W. Qiao et al., 2004)(C.-Y. Kim et al., 2004)(Tye-Din, Anderson, et al., 2010)(Hardy et al., 2015).

IECs play a key role in the pathogenesis of CeD by expressing stress-induced markers secondary to inflammation and infections (Allegretti et al., 2013; Hüe et al., 2004; Jabri & Abadie, 2015) and by releasing TG2 into the gut lumen to generate TG2-gluten complexes (Iversen et al., 2020b). A recent study concluded that TG2- and gliadin-specific B and plasma cells are the preferred APCs for gluten in the lamina propria (Høydahl et al., 2019). Although MHC class II expression and function are typically restricted to professional APCs, several studies have shown that non-hematopoietic cells, including IECs, express MHC class II (Wosen et al., 2019)(Wosen et al., 2018)(Biton et al., 2018)(C. Heuberger et al., 2021), and their expression is upregulated by pro-inflammatory stimuli (Marley et al.,

1987)(Wosen et al., 2019). Notably, the expression of MHC class II in the enterocytes of patients with CeD has been previously reported (Arnaud-Battandier et al., 1986)(Marley et al., 1987)(Fais et al., 1992). Despite this, the expression of MHC class II in IECs has been overlooked. As such, the functional implications of epithelial expression of MHC class II molecules in mediating antigen presentation and T cell response in CeD remain unclear. Therefore, a key finding of this thesis is that MHC class II-bearing organoid monolayers are functional and have the capacity to activate $CD4^+$ T cells in a gluten-dependent manner, which did not occur in the absence of MHC class II-expressing monolayers. This is demonstrated by multiple avenues, including the increased proliferation of $hCD4^+$ T cells, substantially higher expression of the commonly used T cell activation markers, namely CD69, CD25 and CD44, as well as elevated secretion of IL-2, a cytokine indicative of T cell activation, which is increased in CeD patients following gluten challenge (Goel, Daveson, et al., 2019; Goel, Tye-Din, et al., 2019), after stimulating the monolayers with DAPT-gliadin but not with DAPT-zein.

Interestingly, the expression of MHC class II in IECs was found to be upregulated in response to activated $CD4^+$ T cells, and this expression was positively correlated with the expression of activation markers in $hCD4^+$ T cells. In contrast, the inability of $CD4^+$ cells to proliferate and activate in the absence of MHCII-expressing monolayers supported the functionality of our model. The upregulation of CD25, known as IL-2Rα (Malek, 2008), in $hCD4^+$ T cells in the presence of gluten could be attributed to increased IL-2-mediated signaling in the co-culture system. The activation of $hCD4^+$ T cells was supported by the production of IFN-γ and IL-17A, hallmark cytokines of Th1- and Th17-type immune

responses, respectively, in the supernatant of the co-culture system exposed to gluten, which is in accordance with previous findings in patients with CeD (Goel, Daveson, et al., 2019; Monteleone et al., 2010; Nilsen et al., 1995, 1998). Other inflammatory cytokines, such as IL-6, TNF-α, IL-1α, IL-1β, and IL-15, previously implicated in CeD (Di Sabatino, Ciccocioppo, et al., 2006; Goel, Daveson, et al., 2019; Manavalan et al., 2010; Nilsen et al., 1995; Tye-Din et al., 2020), were also higher in the supernatants of co-cultures treated with DAPT-gliadin than DAPT-zein-treated group. Elevated levels of CXCL-10, CXCL-1, CXCL-5, and CCL-20 were measured in the DAPT-gliadin-treated co-cultures compared with the DAPT-zein-treated group. These chemotactic factors recruit effector/memory T cells, B cells, NK cells, DCs, and neutrophils (Sokol & Luster, 2015). Of these chemokines, the Th1-associated chemokine CXCL-10 (IFN-γ-induced protein 10 (IP-10)) was previously found to correlate with increased IFN-γ levels and to be abundantly produced by epithelial and plasma cells in the lamina propria to recruit T lymphocytes to the intestinal mucosa of patients with active CeD (Bondar et al., 2014). The serum level of CCL-20, a Th17-associated chemokine, was also found to be higher four hours after gluten challenge in CeD patients (Goel, Daveson, et al., 2019; Goel, Tye-Din, et al., 2019).

In the small intestine, $CD4^+$ T cells reside both within the lamina propria and in limited numbers in IECs in the form of IELs (R. M. Hershberg & Mayer, 2000). The co-culture system developed in this thesis focuses on evaluating the functionality and phenotype of $CD4^+$ T cells associated with those located within the lamina propria. To confirm the absence of $CD4^+$ T cells in the organoid monolayers used in the co-culture systems, I measured the levels of cytokines and chemokine released in the IFN-γ-treated

organoid monolayer-only cultures upon exposure to DAPT-gliadin. Intriguingly, the T cell-secreted cytokines, IL-2 and IL-17A, were not detectable in monolayer-only cultures treated with IFN-γ, in which $CD4^+$ T cells were absent, indicating that their release is dependent on the presence of activated T cells. In contrast, higher levels of epithelial-associated innate cytokines and chemokines, including IL-6, IL-1α, IL-1β, IL-15, CXCL-10, and CCL-20, were detected in monolayers stimulated with IFN-γ in the absence of $CD4^+$ T cells. These results confirm that $CD4^+$ T cells are not present in the monolayers, thus ruling out their involvement in cytokine and chemokine secretion, which can drive the underlying $CD4^+$ T cell activation.nMHC class II-bearing organoid monolayers exposed to DAPT-gliadin and co-cultured with $hCD4^+$ T cells showed increased expression of stress-induced markers, Qa-1 and Rae-1, compared with monolayers exposed to DAPT-zein. This finding agrees with previous studies in which the expression of human analogues of these markers were associated with cellular stress in active CeD patients (Allegretti et al., 2013; Hüe et al., 2004).

Although gluten is the main environmental driver of CeD, emerging studies in recent years have increasingly highlighted the potential role of additional co-factors in CeD pathogenesis (Caminero & Verdu, 2019)(E. F. Verdu & Schuppan, 2021)(E. F. Verdu & Caminero, 2017). This is because only a small percentage of genetically susceptible individuals who consume gluten develop CeD, with studies confirming a significant rise in CeD prevalence over the last four decades (Rubio-tapia et al., 2009)(Rubio-Tapia et al., 2012)(Lebwohl & Rubio-Tapia, 2021). Epidemiological evidence strongly supports the role of bacterial and viral infections in CeD (Bouziat et al., 2017)(Stene et al., 2006b)(E. F.

Verdu et al., 2007)(Kemppainen et al., 2017). Both pathogenic viruses and bacteria have been mechanistically implicated in CeD pathogenesis (Bouziat et al., 2018)(Bouziat et al., 2017)(Fernandez et al., 2017), with microbial factors emerging to modulate inflammation in CeD (Caminero & Verdu, 2019; E. F. Verdu & Schuppan, 2021). It has been shown that the human gastrointestinal tract harbors gluten-degrading bacteria (Caminero et al., 2014)(Caminero et al., 2015). Recent studies have suggested that duodenal opportunistic pathogens may serve as co-factors in CeD (Verdu & Schuppan, 2021)(Caminero, Meisel, et al., 2019) through mechanisms such as modification of peptide antigenicity via gluten metabolism by microbial proteases (Constante et al., 2022)(E. F. Verdu & Schuppan, 2021). For example, the metabolism of gluten by *P. aeruginosa* expressing the extracellular elastase, LasB, produces highly immunogenic gluten peptides that translocate across the epithelial barrier more effectively than gluten produced by the metabolic action of human digestive enzymes (Caminero et al., 2016). Therefore, to investigate whether bacterial metabolism influences IEC-T cell interactions, organoid monolayers in the co-culture systems were stimulated with DAPT-gliadin partially metabolized by elastase-producing WT *P. aeruginos*a PA14, a previously described duodenal pathobiont in CeD (Caminero et al., 2016), and the $CD4^+$ T cell response was evaluated. As control, organoid monolayers were treated with DAPT-gliadin, gliadin pre-digested with the *P. aeruginos*a isogenic *lasB* mutant strain lacking elastase activity ($lasB^{\Delta/\Delta}$), and the WT *P. aeruginosa* PA14 supernatant. Higher proliferation and activation phenotypes of $hCD4^+$ T cells were supported by increased secretion of IL-2 and several other pro-inflammatory cytokines, including IFN-γ, IL-6, TNF-α, IL-1α, IL-β, and IL-15, which are all implicated in CeD

with very well-known roles. These results are in accordance with the previously demonstrated *in vitro* activation of gluten-specific T cells from HLA-DQ2.5^{+} CeD patients by *P. aeruginosa*-modified gluten peptides (Caminero et al., 2016), implying that gluten modified by microbial elastase can also influence epithelial MHC class II-mediated activation of hCD4^{+} T cells. Notably, elastase in *P. aeruginosa* induces a gluten-independent pro-inflammatory immune response in the small intestine of mice via protease activated receptors (PAR)-2, leading to the overexpression of IFN-γ (Caminero, Mccarville, Galipeau, et al., 2019a), which in turn may contribute to epithelial MHC class II upregulation. These findings highlight the complex mechanisms of gluten digestion and the role of the gut microbial community in fine-tuning the gluten metabolism. This modulation, in turn, influences the immunogenicity of gluten peptides and their subsequent contribution to the pathogenesis of CeD.

Recent studies have shown that organoids derived from patients with CeD exhibited distinct phenotypic differences compared with organoids derived from healthy controls. These differences encompass areas, such as gene expression (Dieterich et al., 2020)(Pietz et al., 2017)(Porpora et al., 2022), intestinal barrier permeability function (Freire et al., 2019), and macrophage responses to gluten (Serena et al., 2019). The findings presented in this thesis advance the field by identifying a new immunological role for IECs in the MHC class II-mediated activation of CD4^{+} T cells in the context of CeD. It is possible to hypothesize that this mechanism may be key during CeD initiation, particularly in the presence of additional luminal triggers that may prime the epithelium to express stress signals or induce gluten-independent epithelial cell injury, which could lead to the release

of IFN-γ and potentially epithelial TG2. However, there is no reason that this mechanism could not be present in the established CeD and contribute to disease reactivation if gluten re-exposure occurs.

While recent studies have implicated epithelial MHC class II in the regulation of acute GvHD (Koyama et al., 2019) and bacteria-specific effector $CD4^+$ T cell responses (C. E. Heuberger et al., 2023), here, I present, for the first time, gluten-dependent $hCD4^+$ T cell activation by IECs that express celiac-associated MHC class II molecules. The findings presented in this thesis, do not suggest that the expression of MHC class II molecules in IECs replaces the function of professional APCs. Instead, I propose that the expression of MHC class II in IECs has the potential to modulate the function and phenotype of $CD4^+$ T cells that are already activated in the intestinal mucosa at the onset of the disease. I speculate that once inflammation is initiated in CeD, the antigen-presenting characteristics of professional APCs may overwhelm the capacity of IECs to activate T-cells. I propose that the intestinal epithelium is an accessory in CeD pathogenesis that in addition to transporting gluten, expressing stress-induced markers, releasing TG2, also plays an active proinflammatory role through MHC class II-mediated gluten presentation and T cell activation. I hypothesize that this intestinal epithelial immune role serves as a pro-inflammatory loop directing inflammation towards the epithelium, particularly in the context of luminal injuries or infections that have been suggested as additional co-factors in CeD pathogenesis.

In conclusion, IECs have been overlooked as functional antigen-presenting cells for gluten-specific $CD4^+$ T cells in CeD. Here, I identified a new immune role for IECs in the

activation of $CD4^+$ T cells in the context of MHC class II presentation, which could be targeted therapeutically. I hypothesize that this role may serve to localise and further increase injury to the epithelium caused by gluten-specific $CD4^+$ T cells in CeD. This model can be used to test new lines of drug development targeting this pathway and its environmental determinants. Since IECs are in first contact with dietary antigens, our results may encourage studies on the regulation of immune responses by the epithelium in other MHC class II-associated diseases.

CHAPTER 6 LIMITATIONS

LIMITATIONS

CeD, like other chronic inflammatory diseases, exhibits heterogeneity in its pathogenesis and clinical manifestations, encompassing a multifaceted range of disease associations. Most scientists agree that a single model capable of recapitulating all these features is highly unlikely. Consequently, there are ongoing efforts to improve the validity and translational potential of preclinical models, and CeD is no exception to this. It is crucial to integrate and expand our current translational research toolbox with new technologies, enabling us to address outstanding questions in the pathophysiology of the disease rather than discarding the current methodologies. With that in mind, I set out, at the beginning of my studies, to gain a deeper understanding of how the site and context of MHC class II expression by IEC affect local immune responses. My graduate work has taught me that with every new discovery, many more unanswered questions emerge. I established a humanized model that is well suited to answer the fundamental question of whether, in the absence of any other APCs, the intestinal epithelium derived from a host expressing the right MHC class II molecule could activate $CD4^+$ T cells in a gluten-dependent manner. The model provides evidence that IECs expressing DQ2.5 can under pro-inflammatory conditions activate gluten-reactive $CD4^+$ T cells *in vitro*. Nevertheless, this study has limitations. I acknowledge that this model is reductionist and may not represent the exact *in vivo* microenvironments and cellular components, as the interplay between gluten-epithelial MHC class II-T cell activation was explored using organoid monolayers from a humanized murine model. To mimic the human response more accurately, human derived organoid monolayers-human $CD4^+$ T cell co-cultures are

required. This model would require T cells to be obtained from the same patients from which organoid monolayers were obtained to ensure compatibility between TCRs and HLA molecules expressed in IECs. However, even with this approach, identifying the specific HLA molecules that present gluten peptides and mediate T cell activation remains elusive. The humanized characteristics of our *in vitro* model, namely the expression of HLA-DQ2.5 and hCD4 in the absence of other murine MHC class II molecules, render it particularly effective for investigating the interactions highlighted in this thesis, including those involving pathogen-metabolized gluten-IEC-T cell dynamics.

Another limitation of this study is that I did not define the specific IEC lineages that co-localized or expressed epithelial MHC class II. However, this would not invalidate the evidence of epithelial MHC class II expression, which was provided using several different *in vivo* and *in vitro* approaches in both human tissues and transgenic mouse models (DQ2 and DQ8). No specific IECs are specialized to act as an APCs, and it is possible that various conditions may upregulate it in more than one cell type. The key point is that the epithelium is emerging as an orchestrator of luminal antigen presentation, and the density, location, exact cell type, and functional consequences of the deterioration or alleviation of the immune response may depend on the context and location along the intestinal segment.

Lastly, all animal models have limitations, and DR3-DQ2.5, or HLA-DQ8 mice used in this study are no exception. These are transgenic mice for CeD-susceptible HLA class II alleles: HLA-DQ2.5 or DQ8, which involves the random integration of human transgenes into the mouse genome, leading to variable gene expression levels and unintended gene disruption due to the randomness of DNA insertion, which may complicate the

interpretation of experimental results. The humanized characteristics of this *in vitro* model, namely the expression of HLA-DQ2.5 and hCD4 in the absence of other murine MHC class II molecules, make this model well suited to address the question explored in this thesis and pathogen-metabolized gluten-IEC-T cell interactions.

CHAPTER 7 FUTURE DIRECTIONS

FUTURE DIRECTIONS

The findings highlighted in this thesis show that IECs upregulate MHC class II expression under inflammatory conditions and, via MHC class II-mediated pathways, can act as non-conventional APCs for the activation of underlying $CD4^+$ T cells in a gluten-dependent manner. My results also indicate activation of $CD4^+$ T cells is potentiated by the microbial elastase digestion of gluten, a previously identified co-factor in CeD. I describe the development of an organoid monolayer model derived from transgenic mice carrying CeD risk genes as a practical and relevant *in vitro* tool for mechanistic investigation of early events that occur at the epithelial level, which may occur at the onset of CeD. Although many steps in CeD pathogenesis have been well elucidated, making this disease an attractive therapeutic target, many aspects remain unclear. The model I present. In this thesis shows promise for investigating additional potential factors and mechanisms in CeD pathogenesis, which will be fundamental for engineering novel preventative and therapeutic strategies. The predictive potential of this model makes it a promising tool for the mechanistic understanding of IECs, not only as targets of tissue destruction, but also as an effector of inflammation in CeD. In that sense, the limitations mentioned in the previous sections could be addressed to gain further molecular insights into epithelial-driven pathways in CeD. For instance, I mentioned that the different strains of mice utilized in this thesis were transgenic models created by the untargeted insertion of the transgene. It would be of interest to assess the expression of MHC class II under different inflammatory conditions in recently developed HLA-DQ2.5 knock-in mice (Dewan et al., 2021).

The cellular localization of MHC class II molecules in IECs has not been confirmed, with findings showing primarily apical expression (Chiba et al., 1988)(Scott et al., 1980), or lateral and basolateral localization (Sarles et al., 1987)(Hirata et al., 1986). In the inflamed epithelium, MHC class II is relocated from multivesicular bodies (MVB) to the basolateral membrane (Sarles et al., 1987). Additionally, specific IECs lineages that express MHC class II molecules remain unclear. It is generally accepted that enterocytes express MHC class II molecules. Recent studies have suggested that two distinct subsets of epithelial cells, $Lgr5^{+}$ ISCs and Paneth cells, can express these molecules (Biton et al., 2018)(Siyuan, 2020). Using immunofluorescence staining and addressing a reviewer comment for my paper recently accepted in *Gastroenterology* (Rahmani et al., 2024), **Appendix III**, I demonstrated that MHC class II molecules do not co-express with goblet cells in biopsies obtained from active and treated patients with CeD, as well as in tissues obtained from DR3-DQ2.5 immunized and NI mice (data not shown). However, I did not determine the exact cell type in the epithelium that expresses MHC class II. Thus, future studies could identify the lineage of IECs co-localizing MHC class II and determine the identity of epithelial MHC class II using single-cell RNA sequencing or transcriptomic analysis. Additionally, such studies could assess the impact of the absence of MHC class II expression on the composition of the intestinal epithelium, potentially leading to the loss or proliferation of specific IEC lineages.

As a biomedical engineer, I am driven by the goal of creating a practical model that can effectively screen new therapeutic options. The drive to create such a model for the screening and mechanistic understanding of novel therapies is what motivates me. For

example, gluten-digesting synthesis enzymes, such as latiglutenase (IMGX003) or TAK062, both currently in clinical trials, can be evaluated in this co-culture system for mechanistic insight. After enzymatic digestion of gluten with either of these enzymes, the resulting peptides can be introduced into the co-culture system to investigate their impact on immune cell activation. Another example of therapeutics in development is the inhibition of the TG2-catalyzed deamidation and crosslinking of gluten peptides, by ZED1227 (Schuppan et al., 2021). Although active TG2 is primarily located in the lamina propria (Korponay-Szabó et al., 2004), a recent study has proposed that pathogenic TG2 released from shed enterocytes remains catalytically active and drives the interaction between TG2-specific B cells and gluten-specific T cells *in vitro* (Iversen et al., 2020b)(Iversen & Sollid, 2023). Therefore, an essential avenue for future work is to use humanized organoid monolayers expressing CeD risk genes established in this thesis to further validate the expression and functional implications of TG2 in shed enterocytes. The humanized co-culture system proposed in this thesis can be used to study the impact of ZED1227 on CD4 T cell function and phenotypic characteristics. A very recent transcriptomic analysis using human organoids showed that ZED1227 prevents gluten-induced IFN-γ response and its downstream pathways that lead to intestinal inflammation and damage (Dotsenko et al., 2024). This experiment can be performed by initially collecting shed enterocytes from organoid monolayer cultures derived from gluten-immunized and NI DR3-DQ2.5 mice, and the release of TG2 is evaluated by western blot using an anti-mouse TG2 antibody. Subsequently, organoid monolayers are incubated with gluten and ZED1227, and the expression of TG2 is measured to determine the efficacy of

the drug in inhibiting TG2. In addition, the introduction of ZED1227 into the co-culture system would have allowed the investigation of the gluten-specific D4 T cell response in the presence of a TG2 inhibitor. This study will shed light on the molecular mechanisms that drive the efficacy of ZED1227 in CeD. This model can also serve as a practical tool for evaluating various conditions that may enhance TG2 shedding by enterocytes, including gluten exposure in the presence of inflammatory signals or specific pathogens (L. Zhou et al., 2017)(Aaron & Torsten, 2019)(Milani et al., 2012)(Cui et al., 2010)(Caminero, Meisel, et al., 2019). These bacteria have previously been associated with CeD and carry deamidase- and transamidase-like activity enzymes (Kieliszek & Misiewicz, 2014). Additionally, *Streptomyces mobaraense* transglutaminase, referred to as microbial transglutaminase (mTG), has been used as an additive in the food industry; however, *in vitro* studies have shown that the degradation of gluten does not render the gluten peptide immunogenic (L. Zhou et al., 2017)(Yokoyama et al., 2004)(Kieliszek & Misiewicz, 2014). Of note, other bacterially generated TG2 enzymes have not been evaluated for their capacity to deamidate gluten in the context of CeD; thus, the humanized organoid monolayers and co-culture system presented in this thesis may serve as a suitable model for assessing the efficacy of these bacterially derived enzymes in generating immunogenic gluten and inducing an immune response. This model is an appropriate tool to facilitate further research to fully understand the interactions between bacteria and TG2 as well as to elucidate the underlying causes and consequences of TG2 activation in CeD pathogenesis.

Future studies could also utilize this humanized organoid monolayer expressing CeD risk genes to establish a co-culture system with other immune components, such as IELs,

that reside within IECs, macrophages, or DCs under defined conditions. The characterization of this humanized organoid monolayer for the expression of MHC class I molecules such as Rae-1 and Qa-1 under inflammatory conditions will facilitate the investigation of the crosstalk between IECs-IELs-$CD4^+$ T cells in response to gluten, which in turn can help identify major pathways that are modulated by such interactions in each cell type.

Another interesting avenue to explore is the study of microbe-epithelium-immune cell interactions relevant to CeD. In recent years, studies have emerged elucidating the role of bacteria as a co-factor in CeD pathogenesis (H. J. Galipeau et al., 2024)(E. F. Verdu & Schuppan, 2021)(H. J. Galipeau & Verdu, 2022). It has been shown that the microbiota can influence epithelial MHC class II expression either by direct interaction, through their metabolites, or by activating other immune cells within the mucosa (C. E. Heuberger et al., 2023)(Wosen et al., 2018)(Malik et al., 2023). Thus, subsequent studies should focus on identifying the role of CeD-associated microbes, including *E. coli, Neisseria*, and *Prevotella*, in modulating epithelial MHC class II expression. Specifically, these studies should aim to determine the mechanisms by which these microbes induce or suppress MHC class II expression in the small intestinal epithelium. The findings from these studies will offer valuable insights into dissecting the intricate interactions between gut microbial communities and the adaptive immune response in CeD. In addition to the expression of MHC class II, IECs are equipped with an array of distinct PRRs, such as TLRs molecules, enabling them to response to array of MAMPs and DAMPs signals, which facilitates the initiation of innate immune signalling and maintains the integrity of barrier functions

(Burgueño & Abreu, 2020)(Pott & Hornef, 2012). Future studies would benefit from identifying the factors that lead to increase PRRs expression in IECs. Additionally, studies should explore the impact of these receptors on immune cell interactions and cytokine production as well as their role in modulating the intestinal microbiome.

Previous studies have demonstrated that microbes can produce peptides with sequences similar to those of autoantigens that are recognized by host self-reactive T cells. This phenomenon is referred to as 'molecular mimicry' and has been linked to autoimmune disease such as type 1 diabetes and multiple sclerosis (Sethi et al., 2011)(Cole et al., 2016) (Hahn et al., 2005)(Wucherpfennig & Strominger, 1995)(Lang et al., 2002). In the context of CeD, recent studies have reported that ubiquitous bacterial peptides of *P. aeruginosa and Pseudomonas fluorescens* exhibit molecular mimicry to HLA-DQ2.5-restricted gluten epitopes, triggering an inflammatory response in gluten-reactive T cells isolated from HLA-DQ2.5 patients with CeD, independent of gluten (Petersen et al., 2020)(Ciacchi et al., 2022). This finding further supports the role of *P. aeruginosa* as a potential environmental co-factor in CeD. However, the ability of these microbially derived peptide to pass through the epithelium and present to $CD4^+$ T cells remain elusive. Thereby, the co-culture system established in this thesis is a practical tool for investigating molecular mimicry in CeD. Future studies should use this humanized system to identify specific bacterial peptides that mimic gluten and determine their impact on $CD4^+$ T cell activation and MHC class II expression.

There is great interest in next-generation microbial therapeutics, which constitute a new category of drugs, including live biotherapeutic products (LBP) and genetically

modified organisms that are used for targeted prevention or treatment of specific diseases (O'Toole et al., 2017). For instance, elafin, an anti-inflammatory serine protease inhibitor, is normally expressed throughout the gut epithelium (Shaw & Wiedow, 2011)(Motta et al., 2012) but has been shown to be decreased in patients with CeD (H. J. Galipeau et al., 2014). Elafin is an inherently unstable molecule in humans; as such, a bioengineered, bio-contained, elafin-producing *Lactobacillus rhamnosus* recombinant has recently been engineered by the P. Langella group in INRA, France. It has been shown that delivery of elafin via food-grade *Lactobacillus lactis* improved gluten-induced enteropathy in NOD-DQ8 mice (H. J. Galipeau et al., 2014), suggesting its therapeutic value for treating gluten-related disorders. The molecular mechanism underpinning elafin, delivered by the bio-contained *L. rhamnosus,* and its impact on immune cells can be evaluated in the humanized co-culture system established in this thesis.

The molecular pathways of gluten transport through the epithelial monolayer and the subsequent interaction with $CD4^+$ T cells, either through direct contact or exosome release of gluten-MHC class II complexes, remain to be determined. This information would be particularly useful for the development of therapies targeting gluten transport and blocking MHC class II associations at the epithelial level. The exact molecular dynamics of the interaction between epithelial MHC class II molecules and $CD4^+$ T cells discovered in this thesis remain unclear. I have not proven that direct IEC-$CD4^+$ T cell contact is required. Indeed, it has been shown that IECs can take up luminal antigen in small membrane vesicles referred to as exosomes (Van Niel et al., 2003). These exosomes are 30-90 nm in size and are formed by fusion of early endosomes and the MHC class II-

loading compartment. During the fusion process, the MIIC membrane invaginates inward to create exosomes that express highly immunogenic MHC class II-peptide complexes on their surfaces (Van Niel et al., 2003)(R. Hershberg et al., 2005). These exosomes are then released at the basolateral side of enterocytes and can be captured by immune cells in the LP, which promotes antigen presentation. This mechanism is likely to be enhanced during inflammation, as the expression of MHC class II molecules in IECs is augmented, particularly under the influence of IFN-γ. One approach to determine the dynamics of $CD4^+$ T cells and the intestinal epithelium in DR3-DQ2.5 mice during inflammation is to perform an adoptive transfer of GFP-labeled $CD4^+$ cells into these mice, followed by immunofluorescence staining for MHC class II and IECs as well as GFP-tagged CD4+ T cells in the intestinal tissue obtained after gluten immunization and IFN-γ-injection. This technique would allow the measurement of the distance between epithelial MHC class II and GFP-labeled $CD4^+$ T cells. Additionally, the *in vivo* dynamics of the interaction between adoptively transferred $CD4^+$ T cells into DR3-DQ2.5 mice and epithelial MHC class II can be investigated using two-photon *in vivo* live cell imaging.

Further studies are needed to investigate the molecular mechanism of gluten peptide uptake, presentation, and delivery to basolateral $CD4^+$ T cells in this model using immunoprecipitation and mass spectrometry to evaluate the peptide-MHC class II complex and single-cell RNA sequencing to identify key regulatory pathways. Additionally, imaging of fluorescently labeled gluten peptides introduced into MHC class II-expressing monolayers or intravital imaging of a transgenic mouse model carrying fluorescently

labeled MHC class II in IECs will be instrumental in confirming and visualizing these interactions.

Furthermore, organoid monolayers developed in this thesis were cultured under static micro physiological conditions. Advances in gut-on-a-chip technology, which is a microfluidic device lined with living cells under dynamic fluid flow and mechanical cues, has been shown to enhance organ-level structures, functions and tissue-specific differentiation (Bhatia & Ingber, 2014)(Shakeri et al., 2019)(Aryasomayajula et al., 2017). Integration of gut-on-a-chip technology with organoid monolayer culture provides a robust platform to more precisely control cellular and environmental interactions to mimic human physiological responses more accurately than the traditional models (Ingber, 2022)(Rahmani et al., 2019)(Jalili-Firoozinezhad et al., 2019). Extending on this work, organoid monolayers expressing CeD risk genes can be integrated with gut-on-a-chip technology, CeD-on-a-chip, providing a robust platform to more precisely control cellular and environmental interactions to mimic human physiological responses more accurately than the traditional models. This integrated system has significant implications for improving the efficacy and safety of therapeutic interventions in CeD as well as better understanding for mechanism of action (Moerkens et al., 2019)(Kasendra et al., 2018). A microfluidic device comprising two parallel channels can be utilized to conduct these experiments. The top channel is cultivated with MHC class II-bearing organoid monolayers, whereas the cellular components of interest are introduced into the lower channel. This system will facilitate the assessment of mechanisms that are typically hampered by static *in vitro* culture systems, including evaluation of the interactions

between the epithelium and circulating immune cells and their recruitment mechanism. In addition, this gut-on-a-chip technology allows the establishment of oxygen gradient, which facilitates extended co-culture of organoid monolayers with a complex living microbiome, providing a tool for the analysis of host-microorganism interactions in a more physiologically relevant context. This system allows for closer replication of the IEC response to therapeutics with the delivery of drugs and compounds across an epithelial barrier as well as better prediction of human drug pharmacokinetic parameters.

Collectively, these future studies should yield valuable insights into the functional role of antigen presentation by epithelial MHC class II under different conditions. These findings could offer a rationale for the targeted manipulation of these pathways, with the potential to enhance protective immune responses or mitigate inflammation.

CHAPTER 8 CONCLUDING REMARKS

CONCLUDING REMARKS

IECs have been overlooked as functional APCs, particularly in the context of CeD for gluten-specific $CD4^+$ T cells. The data encompassed in this thesis provides evidence for the successful establishment of a functional humanized *in vitro* epithelial model expressing the main genetic risk factors for CeD development. The events occurring in the epithelium of patients with CeD are not well understood. This new *in vitro* model expressing the main genetic risk molecules for CeD development facilitates the investigation of IEC-dietary antigen-immune cell interactions that occur during disease onset and course. A better understanding of these initial events and drivers of intestinal epithelial dysfunction is critical for the development and screening of novel therapeutics. Using this humanized organoid-derived monolayer, I identified a new immune role of IECs in the MHC class II-mediated activation of $CD4^+$ T cell. I elucidated that IFNγ-treated IEC activate $hCD4^+$ T cells in the presence of gluten peptides, and that this is exacerbated when gluten is metabolized by elastase-producing opportunistic pathogens. The results support the claim that the intestinal epithelium modulates $CD4^+$ T cell responses in CeD through antigen presentation. Thus, IECs are not just the target of tissue damage in CeD but also play a role as immune orchestrators, which could be targeted to decrease gluten-induced inflammation. The study presented in this thesis has important clinical implications, as it can be used to test new lines of drug development targeting this new IEC-mediated pathway and its environmental determinants.

The MHC class II-expressing organoid monolayer developed in this thesis constitutes a unique addition to the current preclinical models available for CeD (Pinto-

Sanchez et al., 2021). This addition to the translational toolbox will facilitate further investigations into the mechanistic understanding of current therapeutics. The model can facilitate rapid preclinical screening of novel therapeutics targeting gluten peptide modification by synthetic or microbial proteases, gluten transport, or deamidation through the intestinal epithelium.

In conclusion, I demonstrated that MHC class II-expressing IECs exposed to gluten mediate hCD4$^+$ T cell activation, indicating a previously unknown role of the intestinal epithelium in the modulation of CD4$^+$ T cell responses in CeD through antigen presentation that could be targeted therapeutically. Ultimately, the knowledge gained from this thesis may encourage further investigation into the regulation of immune responses by epithelial cells in the context of other chronic inflammatory and MHC class II-associated diseases. Lastly, this model constitutes a practical tool to investigate the drivers and triggers of IEC dysfunction as well as preclinical screening and mechanistic evaluation of novel therapies currently in development for CeD.

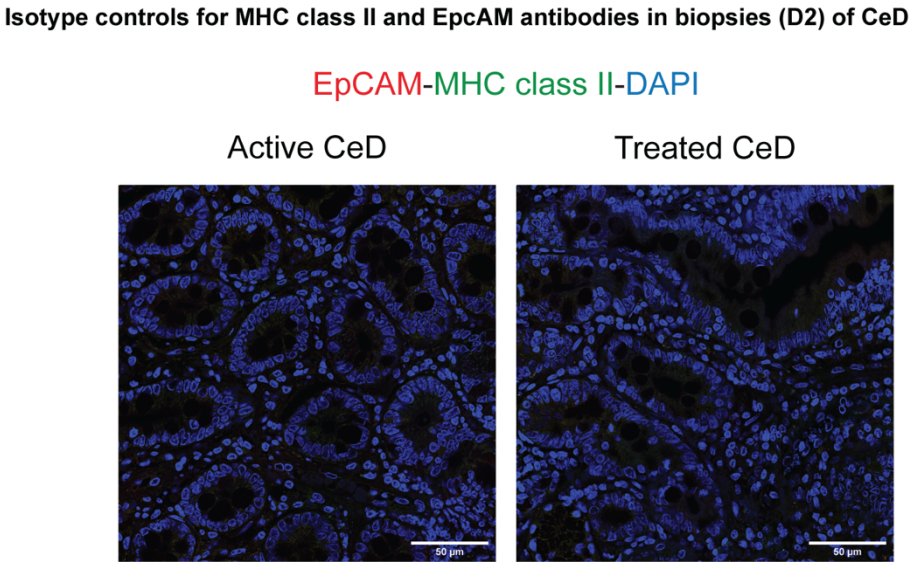
APPENDIX I SUPPLEMENTARY TABLE AND FIGURES

SUPPLEMENTARY TABLE AND FIGURES

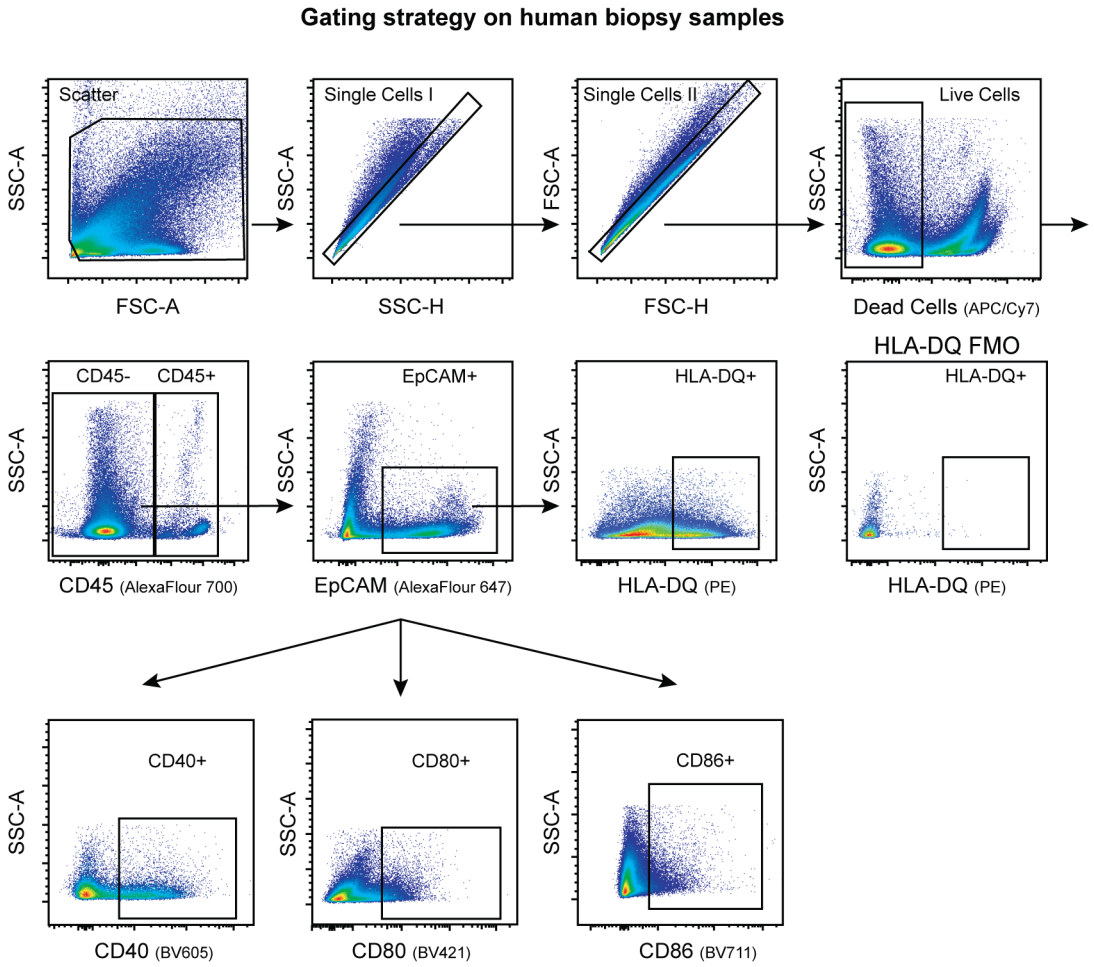
Supplementary Table 1 Clinical and demographic characteristics of patients with celiac disease (CeD) for MHCII molecule immunostaining of duodenal biopsies.

Sex	**Age**	**Diagnosis**	**Biopsy**	**Serology**
M	25	Active CeD	Marsh 3c	+TG2
F	27	Active CeD	Marsh 3a	+TG2
F	22	Active CeD	Marsh 3c	+TG2
M	35	Active CeD	Marsh 3c	+TG2
F	57	Active CeD	Marsh 3c	+TG2
F	49	Treated CeD	Marsh 0	-TG2
F	36	Treated CeD	Marsh 1	-TG2
F	52	Treated CeD	Marsh 1	-TG2
M	34	Treated CeD	Marsh 1	-TG2

F: Female, M: Male, CeD: Celiac Disease, TG2: Transglutaminase 2

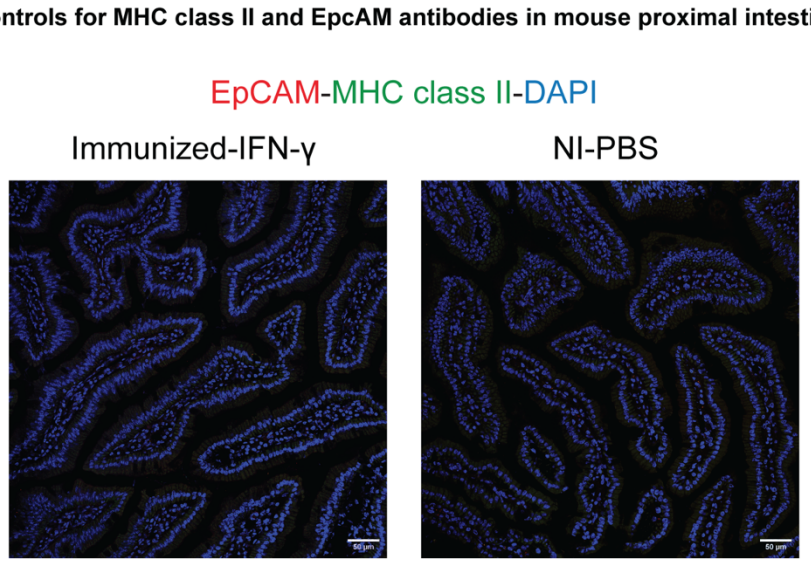


Supplementary Figure 1 Human tissue isotype control. No MHC class II and EpCAM staining we detected in duodenal biopsies obtained from. patients with active and treated CeD following staining with isotype controls from MHC class II and EpCAM antibodies.

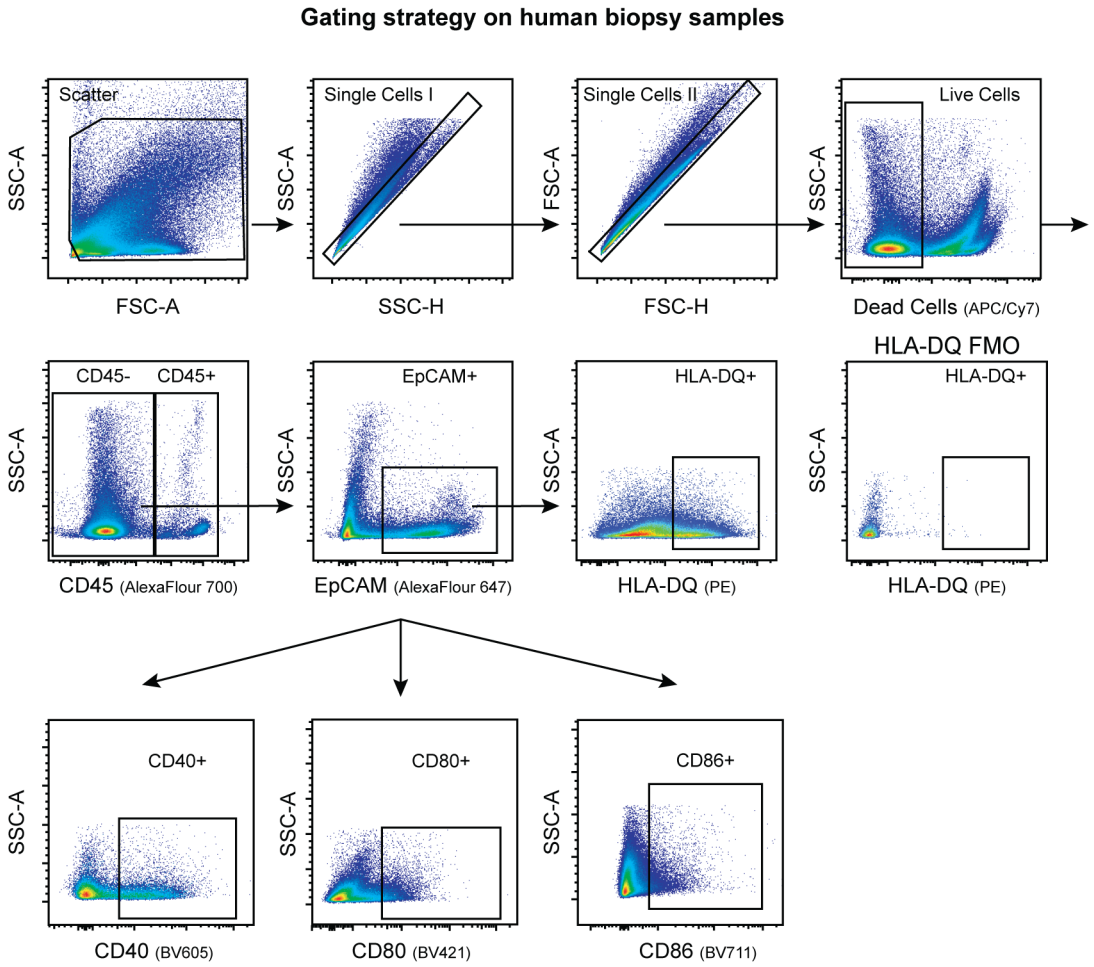


Supplementary Figure 2 Overall gating strategy for flow cytometry analysis of intestinal epithelial cells (IECs) isolated from human biopsies from the second portion of the duodenum (D2). Freshly isolated IECs from D2 biopsies of symptomatic and asymptomatic CeD patients were gated on SSC-A and FSC-A, followed by two sets of singlet isolations on SSC-A and SSC-H, as well as on FSC-A and FSC-H plots. Next. Live/dead gating was used to select viable cells. $CD45^+$ cells were excluded from live cells, followed by the selection of $EpCAM^+$ cells from $CD45^-$ cells. Subsequent gating for a specific marker (HLA-DQ, CD40, CD80, or CD86) was conducted on the $EpCAM^+$ cell population. Specifically, the $HLA\text{-}DQ^+$ cell population was identified from the live $CD45^-$

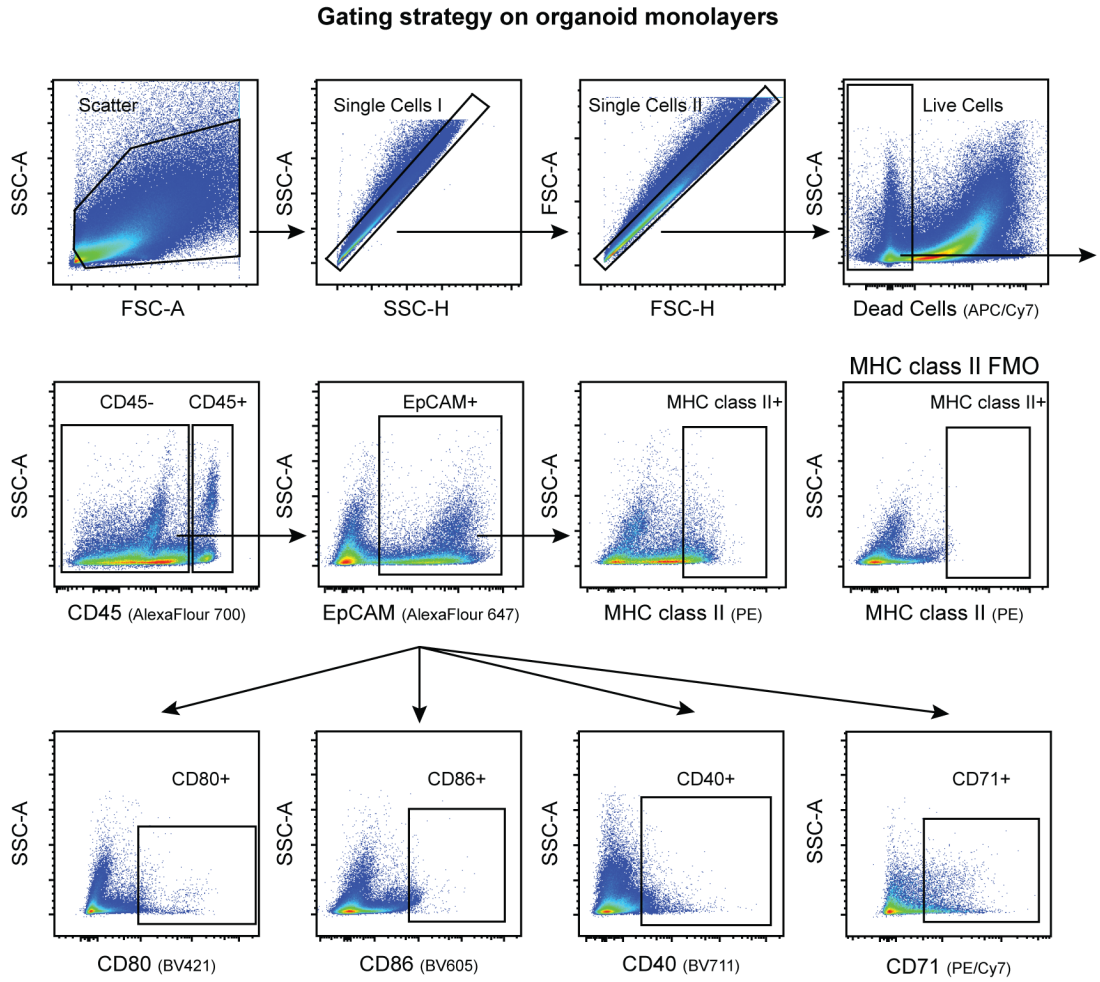
$EpCAM^+$ cells using the HLA-DQ antibody. Fluorescence minus one (FMO) control was used for every marker in the experiment.



Supplementary Figure 3 Mouse tissue isotype control. No MHC class II and EpCAM staining we detected in duodenal and proximal jejunum tissues obtained from IFN-γ-injected DR3-DQ2.5 and control mice following staining with isotype controls from MHC class II and EpCAM antibodies.

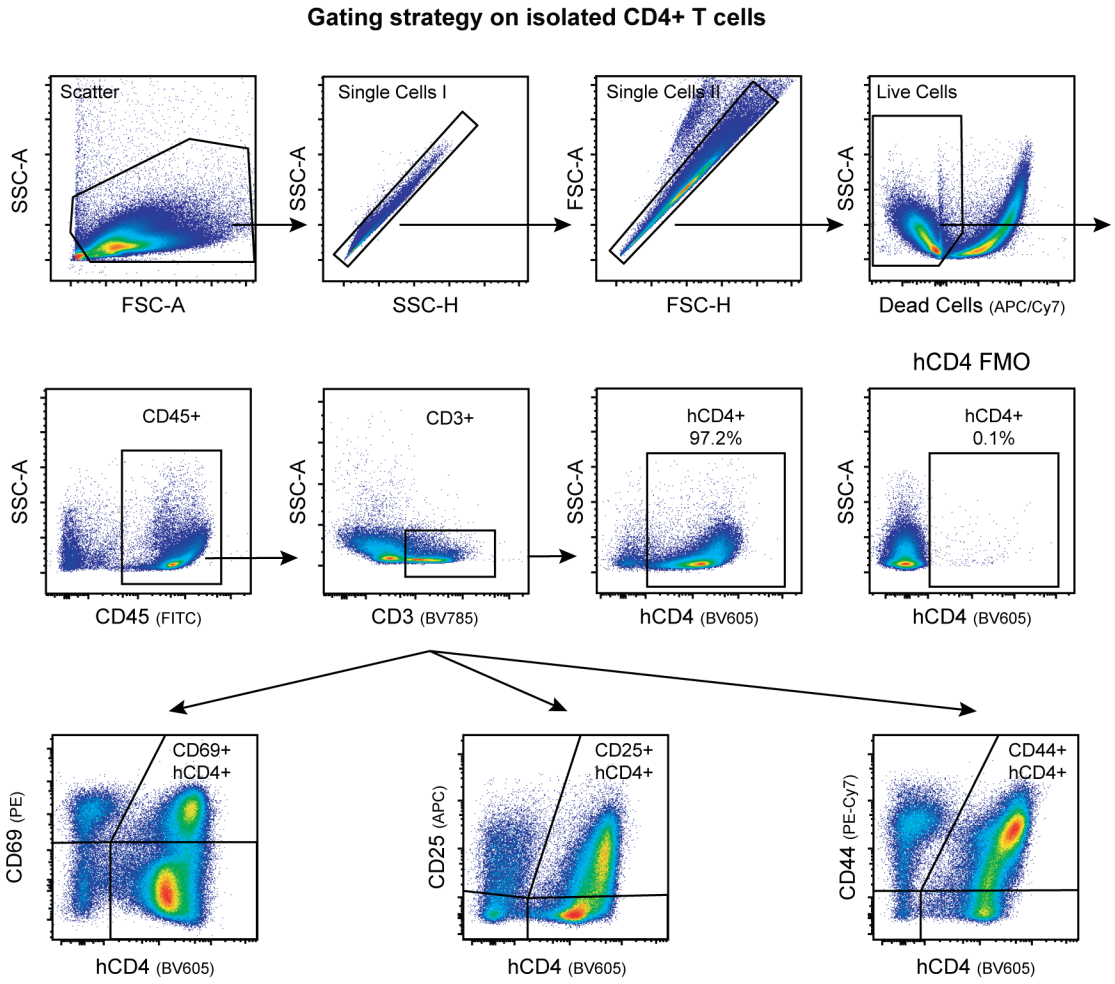


Supplementary Figure 4 Gating strategy for flow cytometry analysis of intestinal epithelial cells (IECs) isolated from murine small intestine. Freshly isolated IECs from the duodenum and proximal jejunum of mice were gated on SSC-A and FSC-A, followed by two sets of singlet isolations on SSC-A and SSC-H as well as on FSC-A and FSC-H plots. Live/dead gating was used to select the viable cells. $CD45^+$ cells were excluded from live cells, followed by the selection of $EpCAM^+$ cells from $CD45^-$ cells. Subsequent gating for a specific marker (MHC class II and Qa-1) was conducted on the $EpCAM^+$ population. The MHC class II population was selected from the $EpCAM^+$ cell population by using the HLA-DR-DP-DQ antibody. Fluorescence minus one (FMO) control was used for every marker in the experiment.



Supplementary Figure 5 Gating strategy for flow cytometry analysis of organoid monolayers derived from mouse small intestine. Organoid monolayers were gated on SSC-A and FSC-A, followed by two sets of singlet isolations on SSC-A and SSC-H, as well as on FSC-A and FSC-H plots. Next, live/dead gating was used to select viable cells. Then, $CD45^+$ cells were excluded from live cells, followed by the selection of $EpCAM^+$ cells from $CD45^-$ cells. Subsequent gating for a specific marker (MHC class II, CD80, CD86, CD40, and CD71) was conducted on the $EpCAM^+$ cell population. Specifically, the $MHCII^+$ cell population was identified from live $CD45^-EpCAM^+$ cells by using the HLA-

DR-DP-DQ antibody. Fluorescence minus one (FMO) control was used for every marker in the experiment.



Supplementary Figure 6 Gating strategy for flow cytometry analysis of hCD4$^+$ T cells isolated from gluten-immunized murine spleen. First, isolated CD4$^+$ T cells were gated on SSC-A and FSC-A, followed by two sets of singlet isolations on SSC-A and SSC-H, as well as on FSC-A and FSC-H plots. Next, live/dead gating was used to select viable cells. CD45$^+$ cells were then gated on live cells, followed by the selection of CD3$^+$ cells. Next, the hCD4$^+$ cell population was identified from CD3$^+$ cells to determine its purity. Subsequent relevant gating for a specific marker, such as CD69, CD25, and CD44 was conducted from CD45$^+$ cells for the double positive cell populations hCD4$^+$CD69$^+$,

$hCD4^+CD25^+$, and $hCD4^+CD44^+$, respectively. Fluorescence minus one (FMO) control were used for every marker in the experiment.

Appendix II: Intestinal organoids: A new paradigm for engineering intestinal epithelium *in vitro*

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Review

Intestinal organoids: A new paradigm for engineering intestinal epithelium *in vitro*

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ABSTRACT

In recent years, the advent of intestinal organoid culture systems has revolutionized *in vitro* studies of the small intestine epithelium. Intestinal organoids are derived from self-organizing and self-renewing intestinal stem cells and closely recapitulate the native intestinal epithelium. They therefore represent a more physiologically-relevant *in vitro* model than conventional cell cultures for studying intestinal development, biology and pathophysiology. Moreover, they represent a promising and unprecedented new tool in the realm of regenerative and personalized medicine. In this review, we outline the current approaches to develop intestinal organoids and describe the strategies used to induce complexity, multicellularity and modularity in organoid culture systems; this knowledge will contribute to improved biomimicry of the organoid culture system. We focus on co-culture systems and explore the convergence of organoid technology and engineering principals. Finally, we describe applications of intestinal organoids in various fields.

1. Introduction

Organoids are stem cell-originated and self-organized 3D clusters of organ-specific cells capable of maintaining the functionality, molecular and cellular heterogeneity of the originating organ, such as the brain, retina, liver or intestine [1–7]. Intestinal organoids, mini-gut, are one of the very extensively investigated organoids, derived from *ex vivo* culture of intestinal stem cells (ISCs)-expressing Lgr5 (Leu-rich repeat-containing G protein-coupled receptor 5) marker [8,9]. Intestinal organoids are capable of closely replicating the structure and cellular composition of a functional native intestinal epithelium [10]. The emergence of intestinal organoid culture systems has galvanized the *in vitro* modeling of intestinal epithelium and circumvented many of the limitations of conventional transformed cell lines, such as their inability to mirror the cellular diversity of the intestine [9,11]. The surge of the various research on intestinal organoid culture systems over the past decades has revolutionized the field of biomedical research. Intestinal organoid technology has been extensively employed in a wide range of applications, including disease modeling [12], therapeutic development [13–19], host-microbe interactions [20–22], biomolecule delivery [23,24] and intestinal biology and development stuides [25–32]. Studies have also emerged to describe the integration of genome editing technology, such as CRISPR/Cas9 [33–38] with organoids culture systems, which facilitates the genetic manipulation of organoids and transforms them into a versatile culture system. Therefore, intestinal organoid culture systems have launched a new era in the *in vitro* modeling of small intestine epithelium with promising potential applications in personalized and regenerative medicine [39–44]. This review highlights the recent advances in the development of intestinal organoid culture systems and discusses the various biology- and engineering-derived techniques used to induce more *in vivo*-like microstructure, complexity and multicellularity in intestinal organoids to make them fully physiologically-relevant culture systems.

2. Development of intestinal organoids

Organoids can be derived from two sources of stem cell, organ-restricted adult stem cells (ASCs) [10,45–47] and pluripotent stem cells (PSCs), both in the form of induced (iPSCs) and embryonic (ESCs) [48–52]. These approaches lead to the formation of defined 3D structures that reflect the villus and crypt microarchitecture of the small intestine and are capable of undergoing self-renewal and self-organization for prolong period [9,10]. To culture organoids, the main ingredients are extracellular support matrix and culture medium,

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supplemented with pro-intestinal growth factors [45,53]. The extracellular matrix (ECM) provides structural support and biochemical cues that require for the adherence, growth and differentiation of the stem cells. Additionally, the formation and growth of organoids is critically dependant on the culture medium components, which has to closely replicate signaling pathways in the *in vivo* stem cell niche, in order to maintain stem cells functions and promote their expansion and differentiation into organ's specific cell types [9,54]. The key ingredients of the intestinal organoids culture medium includes Wnt-3a (W), epidermal growth factor (EGF) (E), Noggin (N) and Rspondin 1 (R); this medium is collectively referred to as WENR medium [43,46,54,55]. Spatiotemporal addition of these growth factors into culture medium regulates the stem cell niche signaling pathways, including the Wnt, bone morphogenetic protein (BMP) and Notch, to induce ISCs expansion, proliferation and differentiation into all intestinal epithelial cell lineages [56–58]. The addition of Wnt-3a growth factor is controversial for the development of small intestine organoids; however, it is essential when growing organoids from colon. Other studies have evaluated and introduced additional components to complement ENR media to induce stem cells navigate towards particular differentiation fate [59,60]. For example, the introduction of a combination of two small molecules, such as CHIR99021 and Valproic acid [59,61,62] or LDN-193189 and CHIR99021 [63], had a synergistic impact that promoted the maintenance of Lgr5+ ISCs in a self-renewal and undifferentiated state, leading to ISC-enriched cultures. However, a differentiation phenotype can be achieved using ENR medium supplemented with the following pairs of small molecules: DAPT and CHIR99021, Valproic acid and IWP-2, or DAPT and IWP-2. These molecules coordinate with each other to induce the direct differentiation of ISCs into Paneth cells, enterocytes and secretory cell lineages, namely goblet cells and enteroendocrine cells, respectively [61,64–66]. It has also been suggested that the addition of DAPT [67] or BMP molecules [55,68,69] is sufficient to promote ISC differentiation and generate multilineage intestinal organoids. Furthermore, studies aimed at generating a more cost-effective organoid culture medium reported that Noggin protein can be replaced with LDN-193189 (LDN) [70], and that Rspondin 1 protein can be substituted with RS-246204 [71]. Although the compositions of organoid culture medium is well-defined and various papers [43,72–74] have reviewed them in details, the ingredients are sometimes altered between protocols, and a substantial amount of trial and error in the culture technique is required to determine the most effective dosage and timing to achieve the desired outcome. Morphologically, 3D intestinal organoids are composed of a closed-loop hollow lumen lined with a monolayer of intestinal epithelial cell lineage [46,53]. Initially, organoids create small cysts-like morphology, which then under optimal growth conditions grow into budding-like morphology. These budding-like microarchitectures correspond to crypt domains and are budded off the central lumen where ISCs and Paneth cells are resided [75]. On the other hand, the differentiated cell lineages of intestinal epithelium, including enterocytes, enteroendocrine and goblet cells, are lined in the villus-like domains [1,10,58,76].

The expression of other types of intestinal epithelial cells, such as M cells [77,78] and Tuft cells [66], have also explored in the intestinal organoids. The physiological functionality of intestinal organoids has also been evidenced by the presence of ion and peptide transport [35,79]. Furthermore, intestinal organoids elucidated self-renewal capacity and genetic stability over long-term term culture condition, even following cryopreservation [58,80]. Different approaches to obtaining intestinal organoids *in vitro* are described in Fig. (1); these include organ-restricted adult stem cell-derived organoids, induced Pluripotent stem cells- (PSCs)- or embryonic stem cell-derived (ESC) organoids. In the following sections, we describe each of these approaches in more detail.

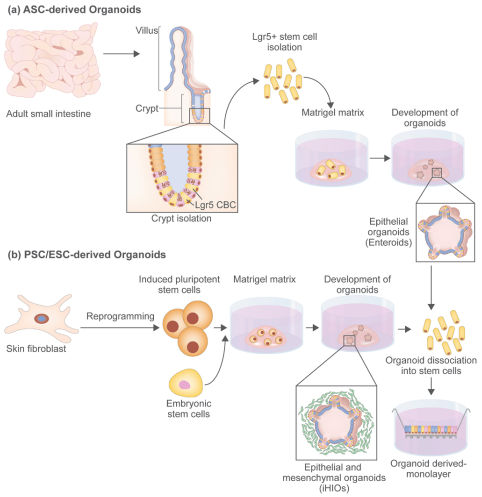

Fig. 1. An overview of current approaches to develop intestinal organoids *in vitro*. There are two major strategies: **(a)** Adult stem cell-derived organoids, also referred to as, enteroids, **(b)** Pluripotent stem cell- or embryonic stem cell-derived organoids, also referred to as, human intestinal organoids (HIOs).

2.1. 3D adult stem cell-derived intestinal organoids (enteroids)

Adult stem cell-derived organoids are generated by harvesting stem cells containing crypts or isolating single Lgr5-expressed ISCs from human [51] or mouse small intestine or colon tissue [81–83]. ASC-derived organoids reproduce the intestinal epithelial layer and, as such, are referred to as enteroids or colonoids, depending on whether they originate from the small intestine or colon [84]. Sato et al. [10,45] was the first group to report the formation of intestinal organoids via isolating single Lgr5-expressed stem cells from mouse intestinal crypt tissues and embedded them in a Matrigel. Despite the close interaction between the stem cells and subepithelial myofibroblasts *in vivo*, the self-renewing Lgr5+ stem cells were able to spontaneously proliferate and differentiate in the absence of any stromal and mesenchymal cells. The enteroids replicated the cellular composition of intestinal epithelium and showed a capacity to maintain region-specific identity depending on the origin of the tissue [85,86]. Location-specific physiological activity was also observed in enteroids in an analysis of their functional absorptive and secretory capabilities; for instance, goblet cells secreted mucin, which covered the apical surface of the enteroids [47]. Moreover, studies have adopted human and murine enteroid culture models to successfully establish enteroids from a wide range of species, including chicken [87–89], monkey [88,90], pig [88,91], bovine [88,90], rat [92], dog [88,93], cat, horse and sheep [88]. These enteroid culture systems showed the ability to maintain the genetic and biologic characteristics of the original animals and therefore represent *in vitro* models of the species of interest. For the purpose of this review paper, we focus solely on intestinal organoids derived from human and mouse models.

ASC-derived organoids have limitations, such as the absence of mesenchymal cells. These cells, which include myofibroblasts, endothelial cells, and smooth muscle cells, promote signaling pathway activity and the proliferation of cells by supplying growth factors to the culture medium. Therefore, to support the growth and proliferation of enteroids, a well-defined cocktail of growth factors, namely WENR, must be exogenously supplied to the enteroid culture.

Commercially available recombinant growth factors, mainly including Wnt3a, R-Spondin 1 and Noggin, are costly to obtain, especially for large-scale cultures. Consequently, Miyoshi et al. [94] pioneered a novel methodology to facilitate more cost-effective methods of culturing enteroids by generating a cell line capable of supplying WRN into the culture media. The conditioned media obtained from this cell line allowed the successful growth and differentiation of enteroids. Subsequently, other studies have adopted similar techniques to create enteroids derived from human intestinal biopsies, potentially paving the path towards patient-specific therapies and personalized medicine [26,121].

To investigate the impact of mesenchymal cells on the development of enteroids, Ootani et al. [97] developed a growth-factor independent methodology. In this technique, tissue obtained from the neonatal mouse small intestine that contained stromal fragments was embedded in a type I collagen gel at the air-liquid interface. This approach yielded co-cultures of epithelial-mesenchymal enteroids that formed an expanding sphere-like configuration. The presence of stromal cells closely recapitulated the microenvironment of an ISCs niche that was capable of producing the required growth factors, thereby eliminating the need for the exogenous addition of those growth factors. However, this technique was hampered by the inability to expand and passage the culture system. Other studies [98] have also adopted this air-liquid interface culture technique to develop mouse derived enteroids; however, no study has described the use of this approach to develop human derived enteroids.

The role of intestinal sub-epithelial myofibroblasts (ISEMFs) in the *in vitro* and *in vivo* growth and differentiation of human enteroids was also investigated [99]. ISEMFs were found to be essential because they supplied growth factors to the epithelium and facilitated cross-talk between the epithelium and mesenchymal cells. The long-term preservation of the subcutaneously implanted ISEMF-supported enteroids was also confirmed in a mouse model. Yin et al. [61] explored this problem and identified two small molecules capable of activating signaling pathways that could establish a Lgr5-riched enteroid culture system. In this system, ISCs were maintained in their undifferentiated and self-renewing state, which led to a substantial increase in ISCs number. Moreover, Mustata et al. [100] reported that enteroids derived from mouse fetal intestinal epithelium at different developmental stages formed into spheroids. These fetal spheroids presented poorly differentiated intestinal characteristics with a hollow cystic-like morphology as opposed to budding-like morphology observed in organoids derived from adult intestine. These fetal spheroids expressed low levels of Lgr5-expressing cells compare to organoids; however, they showed a self-renewal capacity, an indefinite nurturing capability and a potential to develop into organoids containing differentiated cell types. These findings provided further insights into the different step of small intestine development.

2.2. 3D embryonic stem cell- and induced-pluripotent stem cell-derived organoids (HIOs)

An alternative approach to generating human intestinal organoids involves the direct differentiation of ESCs or iPSCs into 3D intestinal organoids [101–106]. The development of this culture system does not depend on the availability of the small intestinal tissue as this type of organoids are derived from iPSCs or ESCs. Spence et al. [50] were the first group to establish a methodology to generate organoids from human iPSCs. These organoids are also referred to as human intestinal organoids (HIOs) or induced human intestinal organoid (iHIOs) cultures. The development of these organoids follows steps similar to those used in ASC-derived organoids but with slight variations in culture medium compositions [49,107]. HIOs form 3D sphere-like structures and thus generate the full range of functionality and characteristics of the small intestinal epithelium within a period of one to three months [49,50]. HIOs recapitulate a fetal intestine phenotype, whereas enteroids exhibit a more adult intestine phenotype [48,106]. HIOs also lack the segmental specificity observed in enteroids [75]. However, HIOs possess mesenchymal cells, which are juxtaposed and in close contact with the epithelial layer, which are lacking in enteroids [50,52]. The combination of epithelial and adjacent mesenchymal cells more accurately recapitulates the native intestine microenvironment, which plays a crucial role in epithelium growth, differentiation and functionality [108]. Despite the lengthy culture period, HIOs are similar to enteroids in that they contain heterogenous lineages of differentiated intestinal cells [21,109,110] and closely recapitulate *in vivo* intestinal developmental processes [28,107].

Despite the fact that HIOs display a fetal intestinal phenotype in early-stage passages *in vitro*, HIOs were able to transform into more mature IECs upon transplantation *in vivo*. Watson et al. [52] established the first *in vivo* transplantable HIOs and evaluated the effect of an *in vivo* environment on HIOs maturation. The generated HIOs were embedded in type I collagen gel and engrafted underneath the kidney capsule of mice. Upon transplantation, the mouse vasculature infiltrated the HIOs and established a network of peripheral capillaries that allowed the HIOs to develop a more mature phenotype. Additionally, the mesenchymal cells in the HIOs differentiated into more mature cell types, including smooth muscle cells and sub-epithelial myofibroblasts, and contributed to the successful integration of the transplanted HIOs to the host tissue. This finding further supported the resemblance between HIOs formation processes and the *in vivo* long-term processes involved in intestinal development [28,52].

The functionality, permeability and absorptive properties of HIOs have also been confirmed [52,106]. HIO culture systems could therefore accelerate personalized medicine approaches by allowing the creation of biopsy-free individual organoids that demonstrate specific phenotypes associated with genetic variations.

Moreover, Tsai et al. [111] revealed the mechanisms that modulate regional specificity in intestinal organoids. They manipulated the length of exposure to different growth factors and found that this controlled the specific gene expression patterns that led to regional specification. This approach led to the successful development of region-specific hESC-derived intestinal organoids, such as ileum-like or duodenum-like organoids, that were capable of expressing and maintaining the regional identity both *in vitro* and upon transplantation *in vivo*. Moreover, HIOs have also been successfully transplanted into injured colon tissues, inducing tissue regeneration. Additionally, the implanted HIOs maintained the characteristics of native small intestinal tissue, including the development of villi and the presence of Paneth cells. These findings demonstrated that intestinal organoids are intrinsically capable of being phenotypically stable and preserving organ-specificity [112,113]. Overall, these studies indicate the potential of human-derived enteroids and HIOs to advance translational applications, regenerative medicine and personalized treatments.

All currently available HIO models form heterogeneous organoid cultures with various sizes, cell numbers, and morphologies. Because the yield of viable organoids has also been reported to be low, with only 13% of pre-organoid spheroids capable of developing into mature intestinal organoids, there is a need to enhance the efficiency of HIO culture conditions [114]. To address this problem, Arora et al. [114] applied process engineering principles to construct an automated micropipette that could sort cells based on their morphological features. Using this robust methodology, this group was able to sort organoids in the early stage of development to optimize the organoid culture conditions and thereby create more space and an improved nutrient supply for the rest of the organoids. These changes increased the yield of homogenous and high-quality organoids.

2.3. 2D intestinal organoid-derived monolayers

A major limitation of organoids is their 3D closed cyst-like morphology and the inaccessibility of the apical-luminal surface of the

epithelium, which is exposed only on the inside of the organoid. This creates restrictions specifically related to modeling intestinal epithelium-pathogen interactions. Additionally, embedding 3D organoids in a gel matrix would localizes them at different depths within the matrix, thus limiting the uniformity of their access to the media and the researchers' ability to achieve proper real-time imaging [115]. Consequently, establishing 2D organoid monolayers could potentially mitigate these shortcomings while maintaining the functional and phenotypical characteristics of 3D organoids and native intestinal epithelium [116].

Recent efforts have led to the generation of 2D organoid monolayers from 3D organoids containing all the differentiated cells of the intestinal epithelium. Moon et al. [117] were the first group to develop an enteroid monolayer on layer of gelatin gel. After they were initially grown as 3D organoids, the structures were coaxed to form a monolayer by dissociating their 3D configuration into single-cell suspension, which were then plated on ECM-coated porous membranes or well plates to form 2D monolayer culture system. In this system, the apical surface of the cells faces outward, while the basolateral section is bonded to the coated surface. Hence, this organoid monolayer culture system makes both the basolateral and the apical surfaces of the organoids accessible. When grown in the presence of pro-intestinal growth factors, the organoid monolayers further expanded into a polarized monolayer expressing all the differentiated cell types of the intestinal epithelium. Scott et al. [67] subsequently established a culture system for growing a fully confluent mouse enteroid monolayers on a thin layer of type I collagen gel in the presence of ISEMFs. Whether a more differentiated monolayer or a more self-renewable monolayer formed was controlled by the addition of specific growth factors into the culture medium. Furthermore, when the dissociated monolayers were embedded into Matrigel, they were able to form 3D enteroids.

It was recently reported that the self-renewing characteristics of organoid monolayers can be enhanced by manipulating the stiffness of the ECM [118,119]. For example, applying a thick layer of collagen hydrogel induced long-term proliferation in colonoid monolayers, an effect that was attributed to the use of hydrogel with biochemical components and stiffness similar to those of the native ECM. The formation of a self-renewable enteroid monolayers have also been reported on a layer of type I collagen, Matrigel gel [68–70] or feeder cell layer [120] that led to the formation of confluent enteroid monolayers expressing all of the differentiated cell lineages of intestinal epithelium.

Interestingly, it was found that the monolayer underwent self-patterning, with stem and proliferative cells located at the periphery and differentiated cells maintained at the center [118]. Following this approach, VanDussen et al. [121] successfully developed enteroid monolayers from biopsied tissues obtained from different sections of the intestines of healthy individuals or patients with various pathologies. They concluded that this powerful culture system can be immediately utilized for patient-based assays.

Recently, Tong et al. [65] reported a sandwich configuration of ECM to develop enteroid monolayers, which they referred to as the 'Bolstering Lgr5 Transformational (BLT) Sandwich' culture systems. In this system, enteroid monolayers were sandwiched between a layer of a type IV collagen-coated on the surface of a porous membrane and a layer of type I collagen gel that overlaid the monolayer. The enteroid monolayers formed via BLT culture system were enriched in Lgr5-expressing ISCs population, in contrast to monolayers developed on a gel layer solely composed Matrigel, type IV collagen and type I collagen. In a recent study, Throne et al. [69] revealed that enteroid monolayers can be directly cultured on a layer of Matrigel, eliminating the initial step of generating 3D organoids. Additionally, they identified spatially defined regions within enteroid monolayers that closely mimicked the cellular composition of crypts and villi domains.

One of the main characteristics of the 2D enteroids monolayer culture systems is that they offer feasible access to both the apical and basolateral surfaces of the intestinal epithelium and they are therefore more amenable to investigations of host-pathogen interactions, high-throughput quantifications and functional analyses [22,120]. Additionally, enteroid monolayers facilitate the incorporation of intestinal organoids into engineering-based cell culture systems. As described here, various studies have recently been conducted to establish enteroid-derived monolayers; however, HIO-derived monolayers have not been extensively studied because the presence of the mesenchymal layer hampered the formation of a neat and accessible epithelial monolayer.

3. Biology-derived approaches: intestinal organoid Co-Culture systems

The microenvironment of the intestine is a very dynamic, complex and multicellular system that requires continuous cross-talk between the intestinal epithelium, immune system, enteric microbiota, and intestinal metabolites. Hence, intestinal organoid culture systems still require further maturation and cellular complexity to fully replicate the native intestinal epithelium. To introduce cellular complexity to organoid culture systems and recapitulate interactions between intestine epithelium and its microenvironment, studies have investigated the impact of intestinal metabolites, the microbiota, immune cells and nutrient constituents on intestinal organoid proliferation and differentiation. The absorption of nutrients and other dietary constituents into the body is the main function of the small intestine epithelium. For example, Cai et al. [122] utilized enteroids as an *ex vivo* model to examine the impact of dietary nutrients, such as Vitamin C, caffeic acid and curcumin, on the growth and differentiation of intestinal organoids. One of the limitations of conventional intestinal organoid culture systems is that they lack immune, vascular and nervous systems [15]. Therefore, co-culturing intestinal organoids with intestinal metabolites and other cell types, including microbial, immune and enteric nerve cells, would introduce more cellular diversity and modularity to *in vitro* culture models, rendering them a physiologically-relevant models for investigating the physiology and pathophysiology of the intestine. A summary of the various approaches used to induce multicellularity, complexity and mechanical cues in intestinal organoids is provided in Fig. (2) and Table (1).

3.1. Intestinal organoid and enteric pathogen interactions

Studies have demonstrated that enteric microbes play a fundamental roles in the pathogenesis of a broad range of intestinal immune-mediated diseases and systemic disorders [123]. Investigating the interactions between the intestinal epithelium and enteric pathogens has been hampered by the absence of a model that properly mimics the intestinal epithelium *in vitro*. Therefore, studies have developed to utilize intestinal organoids as pre-clinical *ex vivo* model systems that allow more accurate investigations of host-microbe interactions [124]. Furthermore, the intestinal epithelial layer plays a critical role in maintaining intestinal homeostasis and defending against enteric pathogens, which utilize the intestinal epithelium as a platform for attachment, invasion and replication, triggering small intestine infection and disease.

Indeed, intestinal organoids have been employed in studies investigating the pathophysiology of human viral gastroenteritis [125–127]. Worldwide, both human rotavirus (HRV) and human norovirus (HuNoV) are known to be leading causes of gastroenteritis and diarrhea [128,129]. Studies in which HIOs were co-cultured with rotavirus revealed that the virus targets human IECs, predominantly enterocytes and enteroendocrine cells, as well as mesenchymal cell lineages [130]. Enteroids also showed swelling when cultured in the presence of rotavirus, as a result of pathophysiological response caused by chloride secretion. This finding supported the hypothesis that rotaviruses activate the enteric nervous system by promoting the secretion of serotonin from enteroendocrine cells [131]. In addition, studies have

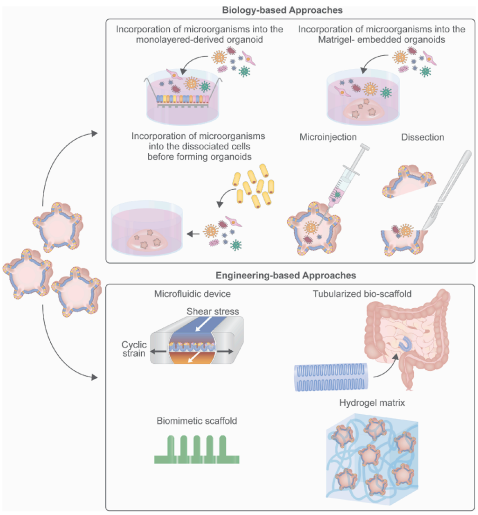

Fig. 2. Schematic representing biology-based approaches and engineering-based techniques to introduce complexity, multicellularity and mechanical cues to intestinal organoid culture systems to improve their recapitulation of the *in vivo* intestinal tissue.

indicated that enteroids and HIOs could be exploited as an *ex vivo* platform to culture and differentiate pathogenic rotaviruses isolated from different patients to identify genetic differences among hosts regarding susceptibility and resistance to infection [130]. The innate immune response initiated by the epithelium could be one factor that contributes to differences in host susceptibility. It has been reported that infected enteroids induced a strong innate immune response by activating interferon-γ (IFN-γ) transcriptional pathways and promoting the expression of IFN-stimulating genes (ISGs). This group further suggested that the exogenous addition of type I-IFN (IFN-α and β) was able to restrict rotavirus replication [132]. Similar results were reported by Hakim et al. [133]; they found that HRV stimulated higher levels release of IFN-γ than IFN- α and IFN-β. It was further observed that all three subsets of IFNs induced higher expression levels of antiviral ISGs. Moreover, HRV robustly replicated within human-derived enteroids at a higher rate compared to laboratory-derived rotavirus strains, supporting the notion that rotaviruses exhibit host specificity characteristics [131,132]. This finding was also confirmed by Yin et al. [128,134], who reported that human-derived enteroids were more vulnerable than mouse-derived enteroids to infection by HRV. This group further assessed the efficacy of antiviral therapies, such as ribavirin and IFN-α, on this system, and the results confirmed that these therapies successfully prevented virus replication in human-derived enteroids. Hence, intestinal organoid culture systems represent a powerful tool to address the previous challenges, such as the ability of HRV to properly replicate in immortalized cell lines.

The use of 2D enteroid monolayers cultivated on the membranes of Transwell plates has been extensively applied as an alternative to 3D organoid models, which pose some challenges related to the induction of cellular complexity in organoids and the ability to study host-enteric pathogen interactions. One of the major challenges is that these organoids prevent pathogens from accessing the luminal epithelium lineage, which is the major site of infection. This shortcoming has been addressed in some studies by applying an invasive microinjection procedure as a means to introduce enteric pathogens to the lumen of 3D organoids [135]. Conversely, organoid monolayer cultures allow non-invasive access to the polarized intestinal epithelium lineage. Although

Table 1
Summary of various approaches for co-culturing intestinal organoids with microorganisms.

Method	Organoid Origin	Microorganisms Type	Reference
Micro-Injection	hiPSCs	*Clostridium difficile*	[153–155]
	hiPSCs	*Salmonella enterica serovar Typhimurium*	[139]
	Mouse enteroids	*Salmonella enterica serovar Typhimurium*	[141]
	hiPSCs	*Escherichia coli* O157:H7	[149]
	Human enteroids	*Cryptosporidium parvum* (*C. parvum*)	[156]
	Human enteroids	Lactobacillus rhamnosus GG (LGG)	[157]
Monolayer-derived Organoids	Human enteroids	Enterohaemorrhagic *Escherichia coli*	[148]
		Enterohaemorrhagic *Escherichia coli*	[26]
		Enteroaggregative *Escherichia coli*	[138]
		Enteropathogenic *Escherichia coli*	[77]
		Noroviruse	[20]
		Salmonella enterica serovar Typhimurium	[158]
		Cholera enterotoxin/Rotaviruse	[159]
		Enterovirus	[152]
		Shigella flexneri	[160]
Inoculation of Dissociated Cells	Human enteroids	Rotavirus	[131–133]
	hiPSCs	Rotavirus	[130]
	Mouse enteroids	Bacterial compounds: MAMPs	[161]
	Mouse enteroids	*Salmonella enteric serovar Typhimurium*	[140]
	Human/mouse enteroids	Retrovirus	[128,134,162]
	Mouse enteroids	*Lactobacillus reuteri* D8	[151]

hiPSCs: Human Induced Pluripotent stem cells hASCs: Human Adult Stem Cells.

the organoid monolayers approach is not suitable for evaluating long-term host-pathogen interaction, it is considered suitable for short-term examination because it is less time-consuming, more reproducible and more controllable than *in vivo* models [136].

Human noroviruses (HuNoV), another leading cause of gastroenteritis, cannot be cultivated *in vitro* in the presence of 2D or 3D transformed cell lines, such as Caco-2 cells or INT-407 cells, or in animal models [137]. Human enteroids, however, supported HuNoV replication and infection as well as viral particle production [138]. Norovirus displayed a great tendency to replicate in and infect enterocytes derived from different segments of the small intestine [138]. Additionally, it was observed that some strains of HuNoV were able to replicate only in the presence of bile. Together, studies in which HIOs or enteroids were co-cultured with enteric viruses have indicated that this culture system could represent a vital platform for studying viral infection of formerly noncultivatable pathogens, an opportunity that was not previously attainable *in vitro*.

Intestinal enteroids and organoids have also been successfully co-cultured with bacteria, such as *Salmonella enterica serovar Typhimurium*, a bacteria that leads to gastroenteritis in humans [139]. Zhang et al. [140] colonized mouse enteroids with *Salmonella enterica serovar Typhimurium* and successfully demonstrated a *Salmonella*-induced inflammatory response *in vitro*; this response manifest as a reduction in enteroid growth and epithelial tight junction integrity. Additionally, *Salmonella*-infected enteroids exhibited a down-regulation of $Lgr5^+$ stem cell expression, lower level of activation of inflammatory signaling pathways and up-regulation of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and IFN-γ, compared to non-infected enteroids. In this study, the author suggested that the cultures showed a complex response to infection even in the absence of classical immune cells. Interestingly, the presence of a gradient of α-defensins, an antimicrobial peptide secreted by Paneth cells, in the lumen of 3D enteroids hampered the growth of *Salmonella enterica* for 20 h following infection [141]. Mouse enteroids lack human genetic specificity; thus, studies have adopted HIOs and human enteroids, as an intestinal epithelium models to more accurately investigate host-pathogen interactions. Forbester et al. [139] reported that HIOs can serve as a useful model to study *Salmonella* infection. Micro-injecting *Salmonella* into the lumen of HIOs disrupted the integrity of the intestinal barrier and resulted in invasion into the epithelium. The *Salmonella* adhered to the apical surface of the HIO epithelium. The expression of pro-inflammatory cytokines, goblet cells and enteroendocrine cells were upregulated, while the expression of $Lgr5^+$ cells were down-regulated.

Furthermore, the expression of other IEC types has also been explored in organoids culture systems. For example, Microfold (M) cells are highly sophisticated IECs that are located in the gut-associated lymphoid tissue (GALT) and lymphoid follicles of the Peyer's patches. These cells regulate the mucosal immune response via transferring microorganisms and antigens to the underlying lymphoid tissue [142]. Thus, studies have investigated the conditions and mechanisms of M cells differentiation in intestinal organoid with the aim of introducing more physiological compositions of IECs into organoid culture systems [78,143,144]. Rouch et al. [77] reported the formation of a human enteroid monolayers that contained functional intestinal M cells. When the M cell-rich monolayers were infected with *Salmonella* Typhimurium, the bacteria showed a tendency to preferentially infect and induce trans-differentiation in M cells. The functionality of the M cells was also confirmed via their ability to endocytose both microparticles and *Salmonella* Typhimurium. Studies have also emerged to explore the expression of tuft cells in intestinal organoid and elucidate the critical role epithelial tuft cells play in promoting mucosal immunity to helminth infection [145–147]. Therefore, the expression of M cells and tuft cells not only introduced more physiological multicellularity in the resulting organoids, but their expression also had a great impact on host-pathogen interactions and the intestinal immunity-related studies due to the key role they play in supporting the mucosal immunity in response to intestinal pathogens. These studies further evidenced the fundamental role of intestinal organoids in investigating the function of specific IECs.

A biobank of human enteroids obtained from patients has also been generated, which is a great tool for modeling disease and developing pergolide therapy. For example, VanDussen et al. [121] developed human enteroids' biobank to investigate *Escherichia coli* (*E. coli*) infection. The adherence patterns were observed after enteroid monolayers were exposed to different strains of pathogenic *E. coli,* including enteropathogenic, enteroaggregative and enterohaemorrhagic *E. coli* (EPEC, EAggEC and EHEC, respectively). This binding affinity was not detected in immortalized cell lines, such as HeLa cells. In addition, the enteroids derived from different sections of the intestine, such as ileal or rectal sections, presented different levels of adherence to *E. coli* strains, confirming the existence of region-specific adherence factors on the enteroids [47]. The attachment of EHEC, a main disease-causing food borne *E. coli*, to human colonoid monolayers was also assessed [148]. During the early phase of infection, EHEC preferentially colonized the mucus-producing goblet cells and ultimately causing damage to the brush borders. Karve et al. [149] established a co-culture system composed of human enteroids, neutrophils and *E. coli* O157:H7 to investigate bacterial pathogenesis. They observed that pathogenic *E. coli*, in contrast to its commensal counterpart, rapidly replicated within the lumen of enteroids. The inflammatory response associated with neutrophils recruitment was also upregulated. In a recent study, human enteroids were obtained from different sections of the human small intestine and colon to confirm the presence of segment-specific patterns in the physical contacts between IEC and EAggEC [150]. Another study established a co-culture system composed of mouse enteroids, lamina propria lymphocytes (LPLs) and *Lactobacillus reuteri* D8 to evaluate the protective impact of these components on the intestinal mucosa. The presence of LPLs markedly increased the expression of $Lgr5^+$-expressing ISCs and Paneth cells in enteroids. Additionally, the presence of L. *reuteri* D8 not only enhanced the growth of intestinal enteroids, but also ameliorated the damage caused to the enteroids following stimulation with TNF-α and led to ISC regeneration [151]. Recently, Senger et al. [152] generated human fetal-derived enterospheres (FEnS), adult-derived enterospheres (AEnS) and monolayers composed of each to develop a preclinical model of necrotizing enterocolitis (NEC). They applied these models to understand the biology and pathogenesis of the disease, which occurs in premature infants. They showed that the FEnS mimicked fetal intestinal development and based on their developmental characteristics categorized them into early and late FEnS groups, with the latter resembling AEnS characteristics. These culture systems allowed evaluation of the genetic pathways that are involved in NEC pathogenesis. The responsiveness and epithelium function were evaluated in both FEnS and AEnS monolayers following exposure to lipopolysaccharide and commensal *E. coli*; in the late FEnS monolayers, the gene expression of major inflammatory cytokines, such as TNF-α and CXCL8/18, were upregulated, whereas the early FEnS monolayers did not show this pattern.

Furthermore, the 3D architecture of the intestinal organoids properly replicated the oxygen gradient and hypoxia observed within the native intestine, both of which are lacking in conventional 2D cell culture models. Obligate anaerobic bacteria, such as *Clostridium difficile* (*C. difficile*), survive upon cultivation within the lumen of HIOs. Using this co-culture system, studies further reported that these bacteria were capable of interrupting intestinal barrier function by decreasing its polarity and reducing mucous production [134–136]. These studies elucidate the potential of intestinal organoids for investigating anaerobic bacteria, which are abundant in the intestinal microbiome.

The different techniques that have been utilized to induce physiologically-relevant cellular complexity through cocultures of various enteric pathogens and intestinal organoids are summarized in Table 1. Collectively, the intestinal organoids displayed promising potential for improving our understanding of the molecular mechanisms underlying

IEC infections. Studies have applied intestinal organoids to explore the interactions between host intestinal epithelium, enteric pathogens and gut microbiota. Therefore, intestinal organoids offer a sophisticated and physiologically relevant *ex vivo* model of intestinal epithelium for advancing our understanding of host-enteric pathogen interactions and facilitating further discoveries of antimicrobial therapeutics.

3.2. Intestinal organoid and immune cell interactions

The small intestine possesses a very dynamic microenvironment in which there is constant cross-talk between the immune system, intestinal epithelium and microbiome. The physiological relevance of organoids culture system was further improved by co-culturing organoids with immune cells. Thus, developing strategies to co-culture these cellular components will offer a great platform for investigating their complex interactions; obtaining such a platform is vital to broadening our current understanding of intestinal physiology and disease pathogenesis.

Farin et al. [163] evaluated the impact of IFN-γ on mouse enteroids. They found that in the presence of IFN-γ, Paneth cells underwent degranulation followed by a significant deterioration in the growth of enteroids. This finding clarified the crucial role played by Paneth cells in maintaining the ISCs niche. In the healthy intestine, Paneth cells were quickly replaced; however, constant exposure to IFN-γ led to progressive cell death and promoted inflammation. In addition, exposure of mouse enteroids to TNF-α led to the disruption of the enteroids epithelium, thus exposing the lumen contents to immune cells and enhancing inflammation [44]. Another study was conducted on enteroids to determine the role of IECs in innate immune responses [164]. Higher expression of toll-like receptors (TLR) and a selection of cytokines (TNF-α and IFN-γ) was detected in IECs upon TLR agonist stimulation, suggesting that IECs play a critical role in maintaining mucosal homeostasis and microbial sensing.

Innate cellular responses have also been modeled *in vitro* by co-culturing HIOs with neutrophils followed by further exposure to Shiga toxin-producing *E. coli* O157:H7. This system resulted in the release of cytokines, reduced epithelial barrier function and enhanced stress response induced by the presence of reactive oxygen species (ROS) [149]. Furthermore, Lindemans et al. [165] demonstrated the impact of innate lymphoid cells (ILCs) on reinstating intestinal epithelial barrier functions and integrity after intestinal tissue damage. The authors concluded that interleukin (IL)-22, mainly released by ILCs, contribute to mediating epithelial regeneration, miniating ISC niche and enhancing enteroid growth and proliferation after tissue injury. Subsequently, other studies illustrated that various cytokines have a positive impact on promoting the growth, survival and functional activities of mouse and human enteroids. HIOs required *in vivo* implantation to mature and replicated adult intestine; however, Jung et al. [166] recently revealed that the *in vitro* maturation of HIOs can be achieved by co-culturing them with human T lymphocytes. They identified that IL-2 was a key contributor to the maturation of HIOs, leading to enhanced intestine-specific functional activities and expression of comparable degrees of mature intestinal markers.

In another study, Nozaki et al. [167] co-cultured mouse enteroids with mouse-isolated intraepithelial lymphocytes (IELs). They reported that both conventional and unconventional IELs were able to survive, proliferate, and maintain their functional activity in the lumen of enteroids when cultured in the presence of medium containing a mixture of the cytokines IL-15, IL-2, and IL-7. This study revealed that direct cell-to-cell interactions with IECs allowed the IELs to survive and expand. Additionally, by utilizing this co-culture system, they further illustrated that IELs were able to maintain a high level of migratory behavior in addition to their spatio-temporal interactions with IECs. Time-lapse imaging confirmed that the IELs remained highly motile and were constantly moving into and out of the enteroids. This finding was in contrast to the prevailing view that IELs are unable to circulate, and that the intestinal epithelium is their final destination. In a recent study, enteroids and IELs were co-cultured, and the results suggested that IL-15 regulates the motility of γδ-T-IELs, a major subset of unconventional IELs, within the intestinal mucosa [168]. Moreover, co-cultures of enteroids with macrophages have also been explored, with the results showing that this system further improved enteroid maturation and epithelial barrier integrity [169]. The co-culture system was then exposed to enteropathogenic and enterotoxigenic E. *coli*, resulting in an orchestrated response initiated by intraepithelial macrophage projections, followed by proper phagocytosis, which led to the stabilization of barrier functions. Hahn et al. [170] adopted a similar system in which macrophages were co-cultured with enteroids to create an organoid-based epithelial to mesenchymal transition (OEMT) system that could potentially be utilized as an intestinal fibrosis model. The cytokines released by macrophages induced the manifestation of mesenchymal phenotypes in the enteroid cultures treated with transforming growth factor-β1 (TGF-β1). Additionally, exposure to TNF-α and TGF-β synergistically promoted the proliferation of mesenchymal cells and induced epithelial to mesenchymal transition (EMT) in the enteroids. These studies support the valuable role of integrating the immune system into intestinal organoid culture systems to improve our understanding of the signaling pathways that ignite the innate immune response upon exposure to pathogenic infection.

3.3. Intestinal organoid and neural cell interactions

The enteric nervous system (ENS) plays a crucial role in promoting epithelial permeability, secretion, fluid exchange, blood flow and mobility [171]. The ENS consists of a complex array of autonomous neurons organized in two main plexi, the myenteric and submucosal plexi, both of which are modulated by the central nervous system. These networks of neurons and glia are spread throughout the small intestine, where they regulate key intestinal epithelial functions [125,172]. Workman et al. [171] recently developed functional ENS-containing HIOs. Their system integrated human vagal neural crest cells (NCCs) with HIOs via mechanical aggregation to successfully establish a functional ENS within HIOs (HIOs-ENS). Following the incorporation of ENS progenitor cells with HIOs, the proliferative NCCs migrated into the mesenchyme region of the HIOs, where they self-organized and differentiated into the neuronal and glial cell types of the ENS and exhibited neuronal activities, such as modulating waves of propagating contractions. Furthermore, upon transplantation of the HIOs-ENS into mice, they matured into ganglionic fiber structures resembling the myenteric and submucosal plexus. The engrafted organoids also contained functional interstitial cells of Cajal (ICCs) and an electrochemical coupling agent that modulated the peristaltic-like contractions. This ENS-containing HIO model could potentially to be used as an *in vitro* functional intestinal model to investigate the intestinal motility disorders and to explore ENS-intestinal epithelial interactions with immune cells, enteric pathogens and the microbiome in addition to investigating the intestinal motility disorders. Recently, Kaelberer et al. [173] co-cultured mouse enteroids with nodose neurons to mimic neural circuit synapses *in vitro*. Using this co-culture system, the authors revealed that enteroendocrine cells synapse with vagal neurons, which facilitated fast sensory transduction from the intestinal luminal to the brain. This finding suggested an *in vitro* model for the gut-brain neural circuit in which the intestinal lumen is connected to the brainstem via one synapse so that brain can readily sense intestinal stimulation.

4. Engineering-derived approaches

To improve the physiological relevance of intestinal organoids, there are still other microenvironmental factors, including topological, mechanical and biophysical cues that must be incorporated into organoid culture systems for them to fully recapitulate the native intestinal microenvironment.

Table 2
Engineering-derived techniques to enhance complexity and mechanical cues of the intestinal organoid culture systems.

Technique	A) Microfluidic Channels	B) Hydrogel Matrices	C) Biomimetic Scaffold	D) Tissue-Engineered Small Intestine
Scaffold Material	PDMS- based Porous Membrane	RGD functionalized polyethylene glycol (PEG) hydrogels [238,239] Four-arm PEG hydrogels with maleimide groups (PEG-4MAL) [240] Recombinant engineered ECM (eECM) [237] Fibrin-laminin hydrogel functionalized with RGD [242]	PDMS	Decellularized small intestine [245,246,250–253] PGA/PLLA scaffold [41,246–248,254]
Organoid and ECM Source	HIOs on Matrigle gel [225] Human enteroids on Matrigel and type I collagen gel [227]	Mouse enteroids embedded in synthesized hydrogel [238,239] HIOs embedded in synthesized hydrogel [240] Mouse enteroids at the air-liquid interface on the eECM [98] Mouse and human enteroids embedded in synthesized hydrogel [242]	Human enteroids on cross-linked type I collagen hydrogel [119]	HIOs/enteroids on an acellular intestinal scaffold
Organoid Morphology	2D organoid monolayer transform into 3D villi-crypt structure under perfusion	3D organoid	2D organoid monolayer	2D organoid monolayer
Advantages	- Definitive crypt-villus structure in monolayer - Small reagent amount - Dynamic microenvironment and vascularization - Accessible lumen - Controlled biochemical and biomechanical cues - Controlled spatial organization - Compartmentalization and co-culturing	- Definitive crypt-villus structure in 3D - Controlled biochemical biomechanical cues - Controlled spatial organization - Biocompatible, tunable matrix - Ability to manipulate the mechanical strength of matrix	- Generate preferred microstructure - Access to the lumen of organoids - Spatial control over ECM arrangements - Reproducibility - Ability to fabricate multi component and microstructure scaffold	- Naturally-derived scaffold - Successful implantation and integration into native intestine - Reconstruct whole native small intestine - Reduce the need for ECM coating of the substrate - Long-term culture - Dynamic microenvironment via perfusion and vascularization
Disadvantages	- Short-term culture - Relatively complex fabrication process - Low mechanical strength of the matrix	- Relatively complex fabrication process - Static microenvironment	- Short-term culture - Static microenvironment	- Low mechanical strength of the matrix - Restrict permeability and diffusion studies

The intestinal organoids that have so far been discussed was developed based on biological-derived approaches that have relied solely on the biochemical cues and the intrinsic nature of stem cells to undergo self-renewal and self-organization. However, the impact of mechanical and topological cues on intestinal cell fate and differentiation needs to be further characterized. Organoids lack a proper crypt-villi axial microstructure similar to that observed *in vivo* and fail to provide appropriate compartmentalization for epithelial, proliferative and stem cells [10,45,53]. Therefore, the emergence of engineering-derived approaches [174,175] not only complements biology-based techniques, but also opens new opportunities to investigate complex physiological and pathophysiological processes *in vitro*. Some engineering-derived techniques [176] have combined biochemical and mechanical cues to recreate an organ-like cellular microenvironment that contains well-defined components, including a matrix, tissue microstructures and vasculature.

The integration of organoid culture systems and biomaterial-based microfabrication techniques that have been introduced in recent years could potentially bridge the gap between *in vitro* organ models and their *in vivo* counterparts. The convergence of organoid science with engineering technology offers unprecedented platforms for accurately engineering 3D intestinal epithelia *in vitro*. A summary of the different engineering-based techniques that have introduced mechanical cues and complexity to intestinal organoids is shown in Fig. (2) and Table (2).

4.1. Engineering biomimetic-based scaffold

The small intestine epithelium is highly structured and exhibits a very distinct crypt-villus topography. The villi structures are lined with a monolayer of differentiated intestinal epithelial cells that extrude into the lumen, whereas the crypt region harbors Paneth and ISCs. The topological, biochemical and mechanical cues induced by this defined morphology greatly influence intestinal cell fates and differentiation [57]. This concept is being extensively investigated in intestinal immortalized cell lines that have been cultured on 3D microfabricated scaffolds [177–181]. Over the past few decades, various microfabrication techniques have been exploited to process biomaterials and develop them into 3D scaffolds so that they properly recapitulate the micro- and nano-structures of the intestinal microenvironment [181–183].

Micropatterned cell culture models were previously developed to mimic stem cell spatial patterning [184,185] and have been adopted to cultivate intestinal organoid. For example, Uchida et al. [186] applied a micropatterned culture system to develop HIOs under xenogeneic-free culture conditions, thus inducing stochastic differentiation. The micropatterned culture model was composed of a circle-patterned glass surface coated with the polyethylene glycol (PEG) micropatterning substrate. The author observed that the intestinal organoids self-organized into spatially segregated clusters within a patterned surface and exhibited a more identical size distribution. The presence of functional smooth muscle cells and ENS was identified along the mesenchymal layer, and they were found to induce contractions and peristalsis-like movements in organoids upon exposure to histamine and atropine.

The ECM components in addition to biochemical gradients and ECM biomechanical stiffness exerted a substantial impact on biological processes within a tissue microenvironment [187]. To examine the impact of the ECM gradient on intestine ISCs growth and differentiation, Wang et al. [119,136], constructed a device composed of microfabricated Polydimethylsiloxane (PDMS)-based stamps patterned with an array of rounded-top pillars and microwells corresponding to villi and crypt structures, respectively, as shown in Fig. (3a). The cross-linked collagen hydrogel was then micromolded onto the surface of the stamps to generate a micropatterned collagen scaffold, on which the human enteroid monolayers was cultivated. Notably, the spatio-temporal addition of growth factors onto the micropatterned scaffold facilitated the proper migration and differentiation of intestinal cell lineages. It was showed that stem and proliferative cells resided within the microwells, while differentiated cells lined the villi-like structures. This micropatterned scaffold provided an effective approach that allowed for gradients to be introduced into growth media across enteroids so that the impacts of these gradients on cell fate navigation could be further examined. This study offered a promising model for emulating the key features of intestinal topology that are missing from organoid culture systems.

Moreover, PDMS/polystyrene-based microraft arrays (MRAs) coupled with culture chambers containing 2500–5000 microwells as media reservoirs have been employed as a high-throughput technique to facilitate the rapid isolation of single ISCs and their co-culture with Paneth cells embedded in a Matrigel matrix [188]. This technique supported long-term ISCs maintenance and their growth into enteroids, in addition to revealing that direct contact between Paneth cells and ISCs is necessary to induce the rapid formation of enteroids. In another study, intestinal organoids were utilized as an *ex vivo* preclinical model to evaluate the interactions and toxicity of Bismuth telluride (Bi_2Te_3) nanowires in a biological system. It was proposed that these engineered nanowires can be applied for the construction of medical devices and biological sensors [189]. Therefore, incorporating biomimetic scaffolds and engineering techniques into organoid culture systems elucidate great potential for enhancing the physiological relevance of the organoid culture system.

4.2. Engineering microfluidic-based scaffold

Microfluidic-based devices have demonstrated promising potential for enhancing the field of biomedical research. Microfluidic technology has been developed and established over the past decades and are now being extensively applied as a platform for cell culturing and organ-on-a-chip modeling [190–192]. Microfluidic-based devices facilitate cell and ECM microenvironment pattering, allowing for the development of miniaturized macroscopic cell culture systems in which a small amount of fluid is perfused, thus enabling precise control of cell placement and signaling gradients [193–196]. Microfluidic devices offer intricate spatial control of cells, substrates, and biophysical and other biochemical cues because they facilitate compartmentalization and allow the regulation of fluid flow parameters [192,197]. The extensive studies performed using microfluidic organ-on-a-chip systems demonstrate the potential of this model to serve as a platform for creating biochemical gradients [198,199], sorting cells [200], manipulating spatial cell patterning [201,202] and analyzing various critical factors one-at-a-time [203,204]. Furthermore, these systems also provide a tool for drug screening [204–207], disease modeling [208–210,212] and co-culturing cells with bacteria, viruses and immune cells [12,213]. Microfluidic devices are relatively easy to fabricate and handle [214] and have a high rate of reproducibility, great optical clarity and excellent resolution [215,216]. In an effort to microengineer a physiologically biomimicking platform, microfluidic devices have been integrated with intestinal organoid so that the culture systems more closely recapitulate the native intestinal microenvironment *in vitro*.

A microfluidic device composed of PDMS pillars was utilized to evaluate enteroid swelling and morphological changes in cultures under continues perfusion. The device was composed of an array of PDMS-based pillars incorporated into a microfluidic channel to capture and immobilize enteroids, as depicted in Fig. (3b). This design facilitated real-time tracking and allowed volume changes in enteroids to be monitored upon exposure to osmotic challenge [217]. Recently, Pajoumshariati et al. [218] engineered a microfluidic-based cell-encapsulation device to investigate interactions between intestinal organoids and Peyer's patch immune cells. This group encapsulated immune cells and mouse enteroids within separate alginate-gelatin microgels and placed them in the interconnected compartments within the microfluidic device; these compartments facilitated intercellular signaling

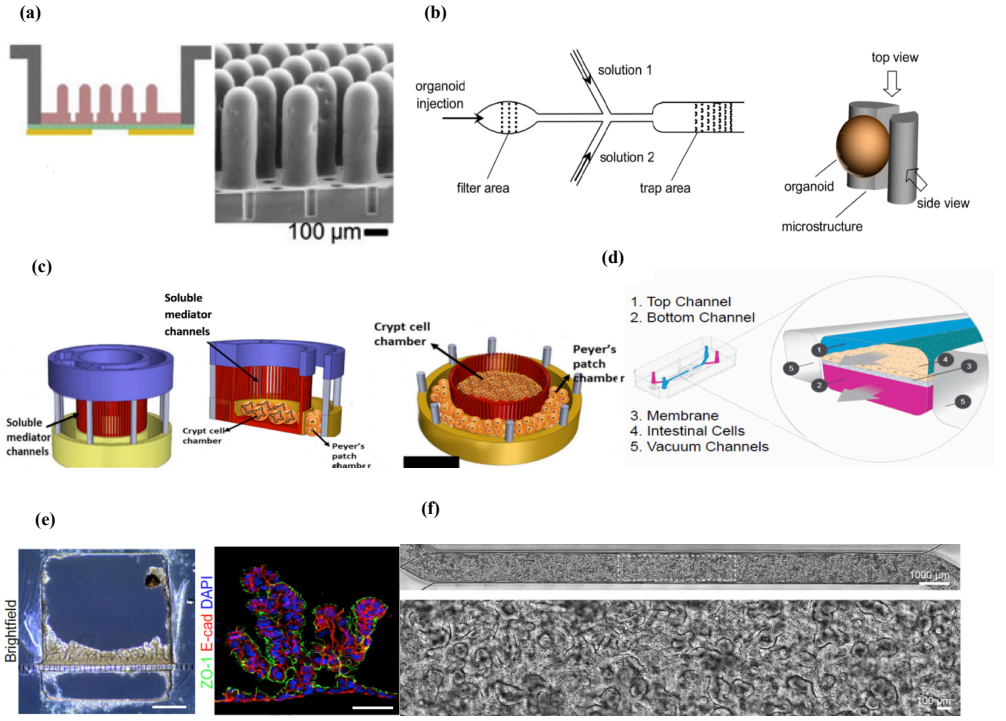

Fig. 3. Different emerging engineering-based strategies to create a scaffold for the development of intestinal organoids, **(a)** Biomimetic scaffold of crypt-villus microstructure. Reprinted from Ref. [119] with permission from Elsevier, **(b)** microfluidic device micro-channel patterned with PDMS pillars. Reprinted from Ref. [217] with permission from AIP publishing, **(c)** Droplet-based microfluidic device for co-culture of enteroids and Peyer's patch cell-encapsulated microgels. Reprinted with permission from Ref. [218]. Copyright (2018) American Chemical Society, **(d)** hiPSC-derived Organoid-on-a-chip device. Reprinted from Ref. [225] with permission from Elsevier, **(e)** Represents bright field and fluorescent images of 3D villus-crypt structure in cross section of chips, **(f)** Bright-filed image of growing monolayer-derived organoid on a chip. (e–f) Reprinted from Ref. [227] with permission from Springer Nature.

and transport, as displayed in Fig. (3c). Co-culturing the enteroids with immune cells, with which they had direct cell-to-cell contact, greatly enhanced the growth, proliferation and differentiation of the encapsulated enteroids. This study provided insight into the potential of using droplet-based microfluidic devices to co-culture enteroids with the aim of assessing the impact of other cellular components and environmental factors on the IECs.

4.3. Engineering intestinal organoid-on-a-chip

Intestinal organoids, despite their tremendous potential for improving our understanding of native intestinal epithelium in healthy and disease states, have their own limitations. First and foremost, adopting an enclosed-lumen shape is non-physiological in the sense that the secreted materials and shed apoptotic cells accumulate within the contained luminal space, whereas in the human intestine, they are removed via peristalsis and luminal flow. Additionally, the lack of access to the apical surface of the cells makes it challenging to conduct transport studies or to expose the epithelia to commensal microbes or pathogenic organisms for periods longer than one day. Finally, these co-culture systems lack a native tissue-tissue interface, a vascular compartment and immune cells, all of which are key contributors to both normal and pathological intestinal physiology.

To overcome these obstacles, the 'human gut-on-chip' technology was developed; a 3D biomimetic system based on microfluidic technology [215,219,220]. This chip recapitulated organ-level functions, while maintaining the native cellular microenvironment by manipulating mechanical forces, biochemical cues, fluid flow, and cell-cell and cell-matrix interactions [186,188]. Therefore, studies have emerged to integrate intestinal organoid cell culture systems with gut-on-a-chip and, more ideally, human organ-on-chip technologies [221,223,224] to engineer a more robust *in vitro* model to explore human biology and pathophysiology.

Very recently, Workman et al. [225] micro-engineered an intestinal organoid-on-a-chip by incorporating HIOs into a small microfabricated chip. This was accomplished through a multistep approach. The chip was composed of two parallel hollow microchambers separated by a porous ECM-coated membrane. The HIOs monolayers were then cultivated onto the apical surface of a Matrigel-coated membrane, as presented in Fig. (3d). These chips formed integrated systems in which living human cells were exposed to an engineered microenvironment that can be precisely controlled and manipulated to emulate human intestinal physiological and pathological states. The HIO monolayers developed into a confluent and polarized layer that contained all the IECs lineages, as shown in Fig. (3e). Constant exposure of the HIO monolayers to fluid flow and mechanical shear stress induced the

formation of 3D villus-crypt microstructures that projected into the lumen of the microchannel, as depicted in Fig. (3f). The permeability of the monolayers was evaluated via introducing external stimuli, such as different cytokines and signaling factors. Following the same methodology, Kasendra et al. [227] established a very similar organoid-on-chip device. In their study, the human enteroids were cultured on the apical surface of an ECM-coated (Matrigel-type I collagen) PDMS-based membrane. Endothelial cells were seeded onto the lower channel to mimic a neighboring vasculature. The chip was able to replicate normal intestinal functions, as confirmed by experiments exploring its permeability, absorptive capacity and secretory functions.

Therefore, the integration between microfluidic device technologies and organoid culture systems can further improve the development and function of intestinal organoids by the spatiotemporal regulation of microenvironmental cues [226]. This technology has shown promising potential for contributing to the realms of personalized medicine, development and disease modeling.

4.4. Engineering synthetic extracellular matrix

The ability of the ECM to maintain the ISCs niche and regulate cellular behavior plays a crucial role in organoid culture systems. The ECM supplies a surface for ISCs to attach to and regulates signaling pathways to stimulate proliferation, migration and differentiation of cells along the villus-crypt axis [228]. Currently, the majority of organoid culture systems rely on Matrigel as a matrix for cells to develop into fully differentiated and functional 3D organoids. However, Matrigel comprises a mixture of poorly identified components that vary from batch-to-batch, and this limits the reproductivity and consistency of results obtained using developed organoids. Importantly, Matrigel is a tumor-derived matrix and is therefore not suitable for clinical translational applications [229]. Collagen has also been successfully applied as an ECM alternative to Matrigel that can be used both *in vitro* and *in vivo* for the expansion and differentiation of various intestinal organoid [97,230–232]. Peng et al. [234] demonstrated that 3D enteroids achieved long-term proliferation and multilineage differentiation when grown in a type I collagen gel. Furthermore, type I collagen foam was also found to be a feasible delivery system for carrying encapsulated ISCs to the intestinal mucosa. However, the translation of these results to human applications remains hampered. Additionally, the gelation kinetics and the stiffness of the matrix are restricted and cannot be precisely controlled when using Matrigel or collagen. To address these limitations, studies have applied biomaterial technologies to fabricate synthetic microenvironments, predominantly in the form of bioengineered hydrogels, to support the growth, proliferation and differentiation of intestinal organoids [235]. These 3D hydrogels allow controllable stiffness, are composed of well-defined components and exhibit structural stability that closely replicates the structural, mechanical and biochemical properties and cues of native ECM; these models have thus accelerated the optimization of culture conditions [235,236]. Overall, the introduction of the biomaterial-based matrices into organoid culture systems has promoted more controlled studies of cell-ECM and cell-cell interactions.

The effects of spatial geometry and the mechanical properties of the culture microenvironment on intestinal organoid growth and differentiation were reported by DiMarco et al. [98]. They observed reproducible contractile behaviors within mouse enteroids that were cultured at the air-liquid interface on a type I collagen matrix; these contractions are an essential functional component of the normal small intestine that was not expressed under conventional submerged culture conditions. Improving the functionality of intestinal organoids contributes to the ability to modulate the spatial biomechanics and geometric properties of the matrix and surrounding culture microenvironment. This group further developed an elastin-based recombinantly engineered ECM (eECM) composed of cell-adhesive domains. The efficiency of organoids formations with proper configurations was augmented when embedded in eECM matrix, contributing to the simultaneous controlling over the biochemical and biomechanical properties of the matrix. They authors concluded that the yield of intestinal organoid formation can be profoundly improved by enhancing cell adhesion and reducing the mechanical stiffness of the matrix [237].

Gjorevski et al. [238,239] were the first group to integrate biomaterial technologies with organoid culture systems to establish a series of synthetic hydrogels. This engineered platform served as a mechanically dynamic and biologically controllable matrix to culture mouse and human enteroids. They engineered enzymatically cross-linked PEG hydrogels that exhibited controllable stiffness, and this led to an increase in the expansion of enteroids. It was suggested that soft and hydrolyzable PEG-based hydrogels enriched with laminin-111 induced a budding-like morphology and facilitated the differentiation of enteroids. However, a stiff and nondegradable PEG hydrogel that lacked lamini-111 promoted the expansion of ISCs into sphere-like structures. Recently, Cruz-Acuña et al. [240,241] reported on the development of a synthetic hydrogel that supported the growth, proliferation and differentiation of HIOs. These engineered hydrogels were composed of four-armed, maleimide-terminated PEG hydrogels (PEG-4MAL) that were cross-linked with degradable peptides and accessorized with naturally occurring Arg-Gly-Asp (RGD), which acted as a bioadhesive peptide. The modular structure of this hydrogel was established to obtain an injectable delivery system that could transfer encapsulated HIOs to mouse colonic mucosal wounds, as displayed in Fig. (4a). Upon injection, the hydrogel polymerized *in situ*, facilitated the local engraftment of organoids onto the injured site and promoted the differentiation of the organoids into the native tissue, thereby accelerating the wound healing process. However, in this study, the differentiation of HIO spheroids into functional intestinal epithelium was achieved only after implantation into mice. In a recent study, Broguiere et al. [242] reported that PEG hydrogels formed as described by Ref. [240] was solely supported by the formation of HIOs and were not suitable for the proliferation of enteroids. To address this shortcoming, Broguiere et al. [242] engineered well-defined fibrin-based hydrogels supplemented with laminin-111 and functionalized with RGD domains. The biochemical and physical properties of these fibrin-laminin hydrogels supported long-term growth, proliferation and differentiation of murine- and human-derived intestinal organoids, as well as pancreas- and liver-derived organoids. This group further concluded that in comparison to PEG-based hydrogels, fibrin-laminin hydrogels were

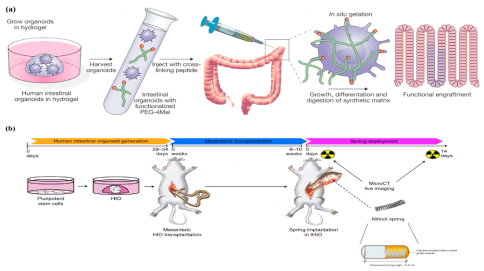

Fig. 4. Schematic representing **(a)** synthetic and modular PEG-4MAL-based matrix capable of improving the growth and expansion of HIOs *in vitro*. This matrix was then served as an injectable delivery system to implant HIOs *in vivo* and exhibited functional engraftment. Reprinted from Ref. [241] with permission from Springer Nature, **(b)** transplantation of HIOs into the mesentery of mice and allowing it to grow for 8–10 weeks. In the second step, a compressed nitinol spring embedded within a double-coated capsule was incorporated inside the lumen of growing HIOs *in vivo*. MicroCT was used to monitor the linearly deployment of the spring *in vivo*. Reprinted from Ref. [255] with permission from Springer Nature.

suitable for a wider range of applications and can be used to culture organoids obtained from different tissues.

Therefore, merging engineered biomaterial-based ECMs with intestinal organoids has been demonstrated to be an approach that shows great potential for generating a controlled and modular platform that closely mimics the native ECM-tuned microenvironment. Engineered ECM illustrates promising potential to support the sustained differentiation and growth of organoids because it can provide a matrix that can control the environmental cues that are presented at each stage of organoid development, while also circumventing the high costs associated with Matrigel. The modular properties of these synthetic hydrogels facilitate the *in vivo* delivery of intestinal organoids or ISCs to advance, thus advancing regenerative therapy for intestinal diseases.

4.5. Engineering organoid-derived intestinal grafts: tissue-engineered small intestine

Tissue engineering of the small intestine shows great potential as a favorable treatment for different intestinal diseases, such as short bowel syndrome (SBS). Two main components are essential to develop tissue-engineered small intestine (TESI): a cell source and a scaffold for cell cultivation [243]. Studies have confirmed that intestinal organoids derived from both adult and fetal mouse small intestine as well as the human colon can be used as a cell source for TESI [112]. Additionally, it is crucial that the scaffold supports the growth, proliferation, differentiation and vascular ingrowth of the intestinal organoids, in addition to being suitable for implantation without causing acute inflammation or a chronic immune response. The available scaffolds that can be used to develop intestinal organoids have been categorized into allogenic or xenogeneic tissues, such as decellularized small intestinal tissue, biological scaffolds and synthetically engineered scaffolds [244]. Levin et al. [245] described an approach for establishing TESI through the *in vivo* transplantation of enteroids onto a decellularized scaffold. The authors reported that the mouse omentum was capable of acting as a living bioreactor that could supply both nutrients and a vascular network to promote the growth and expansion of enteroids into small intestinal epithelium. This group seeded human postnatal enteroids onto a decellularized porcine scaffold, and then evaluated the ability of the scaffold to offer instructive cues to facilitate the differentiation of cells and the generation of a transplantable TESI. In another study, Finkbeiner et al. [246] employed HIOs to recellularize two different collagen-coated scaffolds: an acellular porcine intestinal scaffold and a synthetic biodegradable tubular polyglycolic/poly L-lactic acid (PGA/PLLA) scaffolds. Although HIOs seeded onto the acellular matrix, the recellularized intestine did not thrive upon implantation *in vivo*. The HIO-populated PGA/PLLA scaffold, in contrast, developed into intestinal tissues that were almost indistinguishable from the native intestine upon transplantation *in vivo*. Subsequently, other groups [41,247,248] also confirmed that enteroid-populated tabularized PGA/PLLA scaffolds have the capacity to develop viable, vascularized and well-differentiated TESI. This Upon transplantation into mice, these TESIs developed smooth muscle cells and ISEMFs with cellular and functional characteristics similar to those of the native intestine. In another study, Shaffiey et al. [249] observed that the presence of ISC niche components, such as ISEMFs, macrophages and probiotic bacteria such as *Lactobacillus rhamnosus*, can significantly improve the differentiation and expansion of enteroids seeded onto a tubular polylactic-*co*-glycolic acid (PLGA) scaffold into donor-like epithelium. Additionally, following implantation of this tubular TESI into mouse and dog models, the remarkable regeneration of colonic mucosal, through integration with the host tissue and inducing angiogenesis, was observed.

Schweinlin et al. [250] further integrated shear stress and subepithelial mesenchymal cells to TESIs to add cellular complexity and improve epithelium barrier function. To create a more *in vivo*-like microenvironment, this group designed a bioreactor facilitating the growth of the intestinal tissue at the interface between two flowing fluids. This group introduced enteroids co-cultured with fibroblast cells onto a decellularized small intestine graft. They demonstrated the construction of a more physiologically relevant model of TESI, in which fibroblasts migrated into the scaffold and induced higher expression of differentiated epithelial cells by secreting growth factors that control epithelial behavior and signaling pathways. Furthermore, it was reported that the recellularization techniques used to repopulate the acellular scaffold can impact the expansion and proliferation of seeded enteroids. A homogeneous enteroid monolayers that achieved high-yield cell viability and differentiation was produced when the enteroid culture was cultivated onto a decellularized intestine scaffold via an airbrush at a low pressure [253]. A transplantable vascularized intestinal graft was engineered by Kitano et al. [251]. They developed a perfusable intestine model on a vascularized natural scaffold by recellularizing an acellular rat small intestinal matrix with an HIO monolayers and then re-endothelializing the vasculature with human umbilical venous endothelial cells (HUVECs). Upon transplanting the grafts into rat, the successful integration with the native intestine was observed, which promoted the maturation of the regenerated epithelium.

The impact of mechanical cues on the growth and maturation of HIOs was very recently been investigated by Polling et al. [255]. Their group engineered a novel technique to induce an *in situ* intraluminal uniaxial mechanical strain onto transplanted HIOs. A mechanical cue was generated by a compressed nitinol spring within a gelatin double-coated capsule, which was then incorporated inside the lumen of the transplanted HIOs. The detailed procedure is summarized in Fig. (4b). In contrast to conventionally transplanted HIOs that did not receive mechanical force, the mechanically manipulated HIOs exhibited significantly more advanced functional, structural and morphological maturation, as well as a stronger resemblance to the native human intestine. The authors concluded that this approach is a practical technique with the potential to be translated into larger-scale animals and could lead the way towards a more mature, human-sized engineered small intestine that could be transplanted. Recently, it was revelated that wrapping an enteroid-seeded PGA scaffold with a vascularized membrane led to the formation of the higher-quality TESI than were formed by scaffolds implanted into the abdominal wall or injected into the subcutaneous space [256]. Collectively, these studies bridge the gap between tissue regeneration and whole organ transplantation, with promising capabilities to bioengineer a fully functional small intestine. The TESI shows great potential to accelerate advancements in regenerative and personalized medicine, in addition to its potentially decisive impact on intestinal transplantation and shortcomings of available intestine donors.

5. Application of intestinal organoids

Intestinal organoids have been established as robust *ex vivo* and relevant preclinical models with great potential to be utilized as predictor of clinical and therapeutic outcomes because of their capability to closely replicate *in vivo* physiology [5,26,31]. Moreover, organoids allow scientists to study certain epigenetic factors that affect both normal and abnormal development. For example, Cao et al. [103] developed intestinal organoids derived from a colorectal cancer (CRC)-prone mouse model and screened them for the presence of epigenetically active compounds, such as histone deacetylase (HDAC) inhibitors, Sirtuin modulators, and methyltransferase inhibitors. Intestinal organoids also offer a model to study the homeostasis of the intestine, including the role of the ISC niche in stem cell self-renewal and differentiation [58,257].

The ground-breaking development of intestinal organoid culture systems derived from patients, disease-prone animal models, iPSCs or ECSs present opportunities for *in vitro* human disease modeling [258,259]. Intestinal diseases are challenging to treat and widespread;

hence, they demand proper *in vitro* models to investigate disease pathogenesis and develop novel and effective therapeutics. Infectious diseases [20,130,131,137,139,260], cystic fibrosis (CF) [35,261,262], cancer [33,263–269] and inflammatory bowel diseases (IBDs) [170,270–275] are all examples of intestinal diseases that have been modeled *in vitro* using intestinal organoids. CF is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) transporter that results in the accumulation of thick secretions in the gastrointestinal (GI) and pulmonary tracts [261]. Beyond using intestinal organoids derived from patients and the CF mouse models used to explore this disease *in vitro*, Schwank et al. [35] applied CRISPR-Cas9 genome editing technology to improve CF symptoms and repair the CFTR locus in an organoid model of CF. IBD is caused by chronic inflammation of the GI tract caused by a combination of genetic, immunological, microbial, and environmental factors. While there is no direct model for this condition, studies have adapted intestinal organoids to model various aspects of this disease, including cell death, bacterial infection, and inflammation, each of which has improved our understanding of the pathogenesis of IBD [44,154,157,276].

Furthermore, intestinal cancer organoids have been used to model the pathophysiology of GI malignancies [277]. CRC organoids have been generated from patient-derived biopsies, CRC mouse models, and wild-type organoids in which mutations have been induced by gene editing technologies, such as CRISPR-Cas9 [33,34,36,278–280]. Moreover, biobanks have been established that consist of patient-derived enteroids that can be cryopreserved for long periods of time and passaged and propagated indefinitely while maintaining the histopathological characteristics and the genetic profile of the disease and parental tissue [51,263,281,282]. These living biobanks facilitate various downstream applications and offer the opportunity to simulate disease characteristics *ex vivo*.

Intestinal organoid disease models offer a physiologically relevant systems that can be used to investigate drug discovery, drug toxicity, bioavailability and tissue responsivity [283]. CF organoids have been utilized to evaluate the impact of various drugs, suggesting that organoids show promise for reflecting the potential outcomes of particular therapies. For instance, Dekkers et al. [284] examined the response of the CFTR transporter to the drugs VX-770 and VX-809 in intestinal organoid systems and led to findings that were consistent with the published clinical data on these drugs. In another study, Lorenzi et al. [285] exposed intestinal organoids with mutated tumor suppressor gene to 5-fluorouracil (5-FU), a chemotherapy drug, to study drug resistance. Recently, Vlachogiannis et al. [264] conducted phenotypic and genotypic profiling of intestinal organoids obtained from a patient-derived CRC biobank in which they confirmed that there was a high level of resemblance between the parental patient tumor and the developed organoids. The organoids were subsequently used as an *ex vivo* model to determine the response to anticancer drugs. The results showed that the organoids response to chemotherapeutic agents were comparable to those observed in patients. Additionally, the responses of patient-derived CRC organoids to combinatorial anticancer drugs were very similar to the results obtained when these organoids were transplanted into mice. While these studies suggest that organoids show potential to be used as predictive models to explore drug efficacy in preclinical settings, the therapeutic application of intestinal organoids remains in its infancy, and more extensive research is needed to confirm how accurately the outcomes from drug testing on organoids reflect the effectiveness of a drug in patients. Thus, intestinal organoids can possibly serve to compliment or replace the current *in vitro* models used for drug evaluation.

In addition to the promise they show for basic scientific discovery, disease modeling and therapeutic development, intestinal organoids have also been established as promising tools in the field of cell-based therapy [286,287] and drug delivery [23]. Organoids can act as alternative source for cells or for whole intestine or colon transplantation [244,288,289]. Intestinal organoid transplantation has been performed in mice, in which it was shown to contribute to colon repair following injury [112,290].

Overall, intestinal organoids have become a promising technology with applications in a wide range of fields. In addition to representing an *in vitro* model that closely mimics disease characteristics observed in humans, intestinal organoids also offer a physiologically relevant platform for engineering novel and more effective therapeutics.

6. Conclusion and future perspectives

Intestinal organoids are self-organizing multicellular culture systems derived from self-renewing ISCs that closely recapitulate the cellular composition, structure and function of the native intestine *in vitro*. The ability of intestinal organoids to maintain the host's genetic profile *in vitro* offers an unparalleled opportunity for modeling intestinal diseases. Patient-derived organoids may provide unique perspectives into the development of personalized therapies. These models can be utilized in proof-of-concept studies that investigate a range of biologically and physiologically important interactions, including host-pathogen cross-talk and complex microbe-diet-host interactions. Integrating intestinal organoids with additional cell types, such as endothelial cells, enteric nerves, immune cells, myofibroblasts and microbiota, will introduce *in vivo*-like multicellularity and complexity and will further improve the physiological relevance of the intestinal organoid culture systems. In addition, exploring the synergy between organoid science and engineering techniques will complement organoid culture systems by introducing mechanical, physical and topological cues. The flexibility with which organoids can incorporate various engineering-derived systems as well as the feasibility with which complexity can be introduced or withdrawn make organoids a very sophisticated technology. Overall, the evolution of complex intestinal organoid cultures has paved the path to the development of an integrated and translational *in vitro* model of the native intestine, which will contribute to increasing our understanding of intestinal development, biology, physiology and pathophysiology. They also show promising potential for opening new opportunities for personalized and regenerative medicine.

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Appendix III: Gluten-Dependent Activation of $CD4^+$ T Cells by MHC Class II-Expressing Epithelium

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Gluten-Dependent Activation of CD4$^+$ T Cells by MHC Class II–Expressing Epithelium

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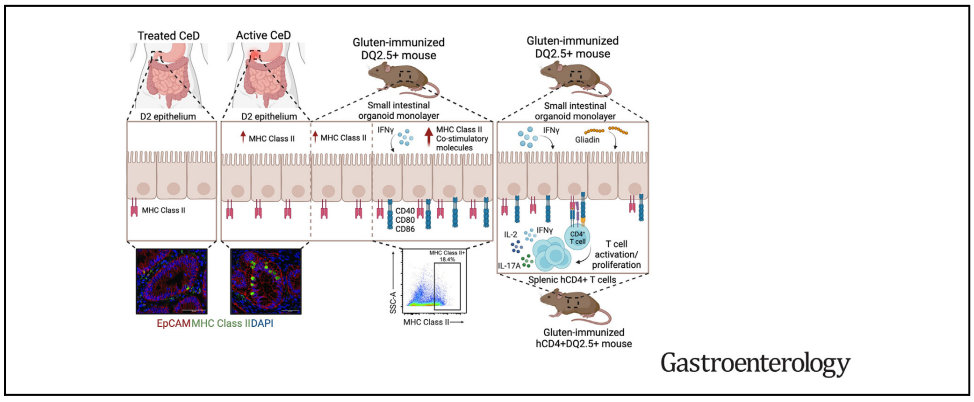

BACKGROUND & AIMS: Intestinal epithelial cell (IEC) damage is a hallmark of celiac disease (CeD); however, its role in gluten-dependent T-cell activation is unknown. We investigated IEC-gluten-T-cell interactions in organoid monolayers expressing human major histocompatibility complex class II (HLA-DQ2.5), which facilitates gluten antigen recognition by CD4$^+$ T cells in CeD. **METHODS:** Epithelial major histocompatibility complex class II (MHCII) was determined in active and treated CeD, and in nonimmunized and gluten-immunized DR3-DQ2.5 transgenic mice, lacking mouse MHCII molecules. Organoid monolayers from DR3-DQ2.5 mice were treated with or without interferon (IFN)-γ, and MHCII expression was evaluated by flow cytometry. Organoid monolayers and CD4$^+$ T-cell co-cultures were incubated with gluten, predigested, or not by elastase-producing *Pseudomonas aeruginosa* or its *lasB* mutant. T-cell function was assessed based on proliferation, expression of activation markers, and cytokine release in the co-culture supernatants. **RESULTS:** Patients with active CeD and gluten-immunized DR3-DQ2.5 mice demonstrated epithelial MHCII expression. Organoid monolayers derived from gluten-immunized DR3-DQ2.5 mice expressed MHCII, which was upregulated by IFN-γ. In organoid monolayer T-cell co-cultures, gluten increased the proliferation of CD4$^+$ T cells, expression of T-cell activation markers, and the release of interleukin-2, IFN-γ, and interleukin-15 in co-culture supernatants. Gluten metabolized by *P aeruginosa*, but not the *lasB* mutant, enhanced CD4$^+$ T-cell proliferation and activation. **CONCLUSIONS:** Gluten antigens are efficiently presented by MHCII-expressing IECs, resulting in the activation of gluten-specific CD4$^+$ T cells, which is enhanced by gluten predigestion with microbial elastase. Therapeutics directed at IECs may offer a novel approach for modulating both adaptive and innate immunity in patients with CeD.

Keywords: Celiac Disease; Organoid Monolayers; MHC Class II; T-Cell Activation; Gluten; Microbial Metabolism.

Celiac disease (CeD) has a global prevalence of approximately 1% and is an immune-mediated systemic condition precipitated by gluten in genetically predisposed individuals.[1] CeD is characterized by gluten-dependent small intestinal villous atrophy, crypt hyperplasia, intraepithelial lymphocytosis,[2] gluten-dependent autoantibodies specific for transglutaminase 2 (TG2)[3] and a persistent adaptive immune response directed preferentially against deamidated gluten peptides.[4] Specific major histocompatibility complex (MHC) class II (MHCII) genes are necessary, but insufficient for CeD, and co-factors have been implicated.[5] Ninety percent of patients with CeD carry HLA-DQA1*05 and HLA-DQB1*02, which encode the protein HLA-DQ2.5, expressed by professional antigen-presenting cells (APCs), such as B cells and dendritic cells. HLA-DQ2.5 allows the presentation of a distinct set of deamidated, protease-resistant gluten peptides recognized by specific $CD4^+$ T cells found in HLA-DQ2.5$^+$ patients with CeD.[6] Patients negative for HLA-DQ2.5 are usually positive for HLA-DQ8,[7] which can also present a characteristic set of deamidated gluten peptides.

Gluten encompasses prolamin proteins in wheat, rye, and barley, of which wheat gliadins are the best-characterized fraction.[8,9] Prolamins are partially digested in the human gut by host[10] and microbial proteases,[11] allowing immunogenic, protease-resistant gluten peptides to undergo transcytosis mediated by secretory immunoglobulin (Ig)A linked to CD71 to cross the epithelium.[12] Selective deamidation mediated by TG2[13] enhances the avidity of immunogenic gluten peptides for binding to HLA-DQ2.5 molecules.[14,15]

IECs play key roles in CeD pathogenesis by expressing stress-induced markers secondary to inflammation and infections,[16–18] and by releasing TG2 into the gut lumen to generate TG2-gluten complexes.[19] A recent study concluded that TG2- and gliadin-specific B cells and plasma cells are the preferred APCs for gluten in the lamina propria.[20,21] IECs express MHCII, and proinflammatory stimuli can upregulate MHCII in IECs[22,23]; however, the functional consequences of this expression in CeD have never been demonstrated. Here, we investigated the epithelial expression of CeD-associated MHCII in patients with CeD and in gluten-immunized mice that lack all murine MHCII molecules and transgenically express human DR3-DQ2.5. We further dissected the conditions leading to the gluten-dependent activation of $CD4^+$ T cells.

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Intestinal epithelial cells express major histocompatibility complex class II molecules, but evidence of their capacity to present gluten peptides to activate $CD4^+$ T cells in the context of celiac disease is lacking.

NEW FINDINGS

Patients with active celiac disease show enhanced epithelial major histocompatibility complex class II expression in the duodenum. Gluten stimulation of interferon-γ–treated organoid monolayers from immunized DR3-DQ2.5 mice activates T cells expressing the human CD4 receptor.

LIMITATIONS

The mechanism of gluten peptide- major histocompatibility complex class II and $CD4^+$ T-cell interactions remain to be determined.

CLINICAL RESEARCH RELEVANCE

Identification of the immune role of intestinal epithelial cells in activating $CD4^+$ T cells, which is central to celiac disease pathogenesis, will open new lines of drug development targeting this pathway and its environmental determinants.

BASIC RESEARCH RELEVANCE

Intestinal epithelial cells have been overlooked as functional antigen-presenting cells for gluten-specific $CD4^+$ T cells and may serve to localize and further increase injury to the epithelium caused by gluten-specific $CD4^+$ T cells in celiac disease.

Materials and Methods

Human Samples

Second-part duodenal (D2) biopsy samples were obtained during endoscopy from 5 active (elevated TG2 serology and histology reporting villous atrophy) and 4 treated (>2 years on gluten-free diet [GFD]) patients with CeD attending the Hospital Bonorino Udaondo, Argentina (Supplementary Table 1). D2 biopsies from 3 patients with CeD diagnosed by TG2 serology and histology (2F, symptomatic, mean age: 34; 1F, asymptomatic, 46 years old) were obtained at the McMaster University Celiac Disease Clinic and used for IEC isolation and flow cytometry. Patients had no concomitant autoimmune or chronic inflammatory diseases, such as type 1 diabetes or inflammatory bowel disease. This study was approved by the Research Ethics Committee of the Hospital Bonorino Udaondo (code #6005) or the Hamilton Integrated Research Ethics Board (HiREB #15311).

Mice

DR3-DQ2.5 mice,[24] lacking all murine MHCII and expressing only human HLA-DQ2.5 and DR3, were used to culture organoid monolayers. DR3-DQ2.5-hCD4[25] mice were used to isolate $CD4^+$ T cells and in some experiments, to culture organoid monolayers. HLA-DQ8 mice, which express only human DQ8,[24] were used to culture the monolayers. Mice were 8 to 12 weeks old and fed a GFD (Envigo Teklad, TD. 05620) for 2 generations

Abbreviations used in this paper: **APC, antigen-presenting cell; CeD, celiac disease; D2, second-part duodenal; DAPT, deamidated pepsin-trypsin-digested; IEC, intestinal epithelial cell; IEL, intraepithelial lymphocyte; IFN-γ, interferon-γ; IL, interleukin; Ig, immunoglobulin; MHCII, major histocompatibility complex class II; NI, nonimmunized; NK, natural killer; TG2, transglutaminase 2; TNF, tumor necrosis factor; WT, wild type.**

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and throughout the experiments, which were approved by the McMaster University Animal Care Committee and McMaster Animal Research Ethics Board (performed under Animal Utilization Protocol #210930). See Supplementary Methods for details.

Mouse Immunization

Mice were immunized using a validated protocol.[26] Briefly, mice were gavaged with pepsin-trypsin-digested gliadin (PT-gliadin) and cholera toxin once a week for 3 weeks, followed by gavages with gliadin 3 times a week for 3 weeks. Non-immunized (NI) mice received cholera toxin alone during the immunization phase and vehicle alone during the challenge phase. Naïve mice that did not receive any treatments were used as additional controls in some experiments. Analysis of anti-gliadin and anti-TG2 antibodies, small intestinal histology (villus height-to-crypt depth ratios and $CD3^+$ intraepithelial lymphocyte [IEL] counts), and gene expression were conducted as previously described.[26,27] In some experiments, gluten-immunized mice were intraperitoneally injected with 1×10^5 U of recombinant mouse (rm)IFN-γ (R&D Systems; 485-MI) (gluten-immunized + IFN-γ-injected). See Supplementary Methods for details.

IEC Isolation

IECs were freshly isolated from human biopsies or from the duodenum and proximal jejunum of the mice. See Supplementary Methods for details.

Organoid Monolayer Cultures

Organoid monolayers were developed from the duodenum and proximal jejunum of mice as previously described.[28,29] In some experiments, organoid monolayers were stimulated with 10 ng/mL IFN-γ (R&D Systems, 485-MI). See Supplementary Methods for details.

Immunofluorescence Staining

MHCII expression was assessed by immunofluorescence staining of human biopsies and sections of the duodenum and proximal jejunum of mice using an HLA-DR-DQ antibody (LS Bio, B5329). Immunofluorescence was also used to assess the cellular composition and polarity of murine organoid monolayers. See Supplementary Methods for details.

Gene Expression

To measure the expression of *Cd74* and *Ciita*, two MHCII-related genes, total RNA was extracted from isolated IECs and organoid monolayers using the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. Complementary DNA was assayed using SsoFast EvaGreen mix (Bio-Rad) on a CFX Real-Time PCR System. See Supplementary Methods for details.

Organoid Monolayer-$CD4^+$ T-Cell Co-cultures

Organoid monolayers were derived from gluten-immunized DR3-DQ2.5, or in some experiments from gluten-immunized+IFN-γ-injected DR3-DQ2.5-hCD4 mice and treated with IFN-γ. The IFN-γ–containing medium was then removed, and the monolayers were treated with deamidated pepsin-trypsin-digested (DAPT)-gliadin, or wild-type (WT) *Pseudomonas aeruginosa* PA14–digested DAPT-gliadin. Controls were DAPT-zein, media alone, *P aeruginosa* PA14 supernatant, or DAPT-gliadin digested with an isogenic *P aeruginosa* nonfunctional *lasB* mutant ($lasB^{\triangle/\triangle}$). $CD4^+$ T cells were isolated from the spleens of gluten-immunized DR3-DQ2.5-hCD4 mice, labeled with CellTrace Violet, and introduced into the basolateral side of organoid monolayers. After 4 days, $CD4^+$ T-cell proliferation and activation were assessed using flow cytometry. See Supplementary Methods for details.

Flow Cytometry Analysis

IECs, organoid monolayers, or $CD4^+$ T cells were stained using commercially available antibodies. HLA-DQ expression in human samples was assessed using an HLA-DQ antibody (NBP3-08720; R&D Systems). MHCII expression in murine IECs or organoid monolayers was evaluated using an HLA-DR-DP-DQ antibody (Invitrogen; MA1-80678). MHCII expression in organoid monolayers from HLA-DQ8 mice was measured using an HLA-DQ antibody (BioLegend, 318104). Cytokine and chemokine levels in the supernatants were measured using a multiplex LEGENDplex immunoassay kit (BioLegend). See Supplementary Methods for details.

Statistical Analysis

The GraphPad Prism (version 9.0 for Mac IOS) was used for analysis. Data are shown as dot plots, where each dot represents an individual mouse or human and are presented as the mean ± standard error of the mean (SEM). Data distribution was assessed using the Shapiro-Wilk test and analyzed using a one-way analysis of variance test or unpaired Student *t* test, as appropriate. Tukey's post hoc test for multiple comparisons was used, where applicable. A P value $<.05$ was considered statistically significant. Samples in which technical issues were encountered were excluded from analysis. See Supplementary Methods for details.

Results

Patients With Active CeD Have a Higher Expression of MHCII in IECs

We determined the effect of CeD activity on IEC MHCII expression by evaluating its expression in D2-obtained biopsies (Figure 1*A*). Sections from both active and treated patients revealed MHCII immunostaining in the lamina propria. MHCII expression was prominently observed in IECs from patients with active CeD compared with treated CeD who achieved a Marsh ≤ 1 (Figure 1*B*), suggesting that increased inflammation is associated with higher epithelial MHCII expression. No staining was detected in isotype controls (data not shown). Using flow cytometry, IECs from patients with CeD demonstrated expression of the HLA-DQ isotype of MHCII (Figure 1*C*; Supplementary Figure 1), as well as CD40, CD80, and CD86 (Supplementary Figure 2*A–C*). Altogether, these data reveal that in CeD, the epithelium exhibits characteristics of APCs.

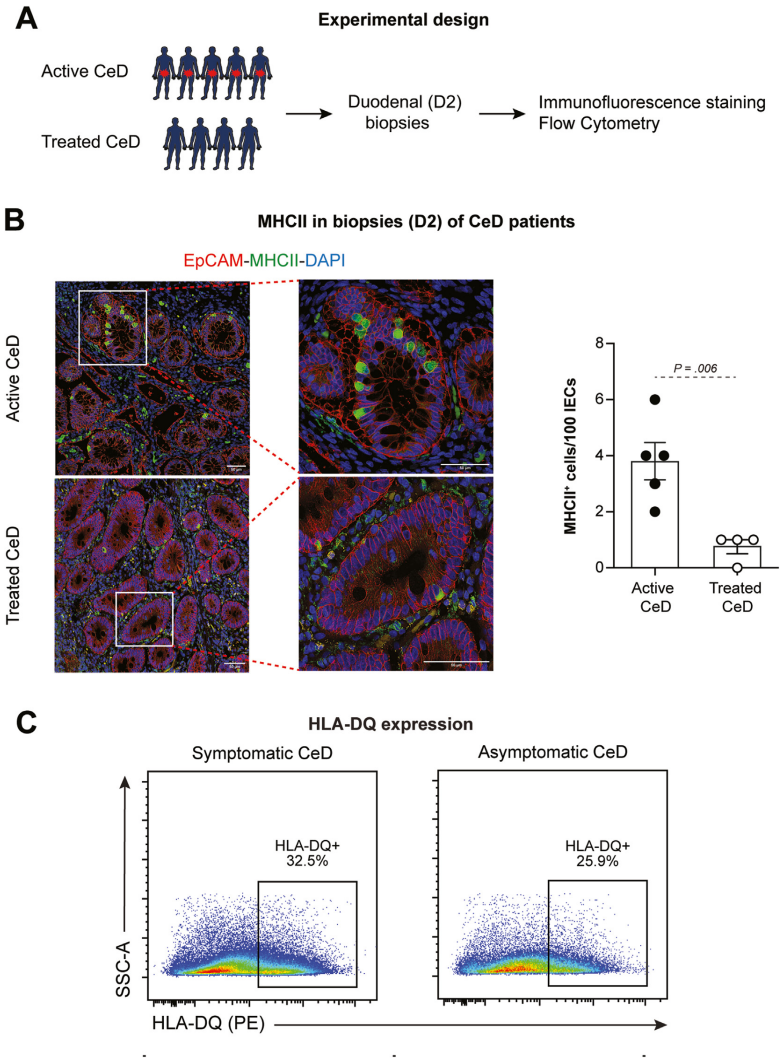


Figure 1. CeD activity demonstrates higher expression of MHCII molecules in IECs. (*A*) Duodenal biopsies (D2) were obtained from patients with active (n = 5) and treated CeD (n = 4), and MHCII or HLA-DQ expression was assessed by immunostaining or flow cytometry. (*B*) Representative immunofluorescence staining of D2 biopsies for $MHCII^+$ ($EpCAM^+$: *red*; $MHCII^+$: *green*; 4′,6-diamidino-2-phenylindole [DAPI]: *blue*; *scale bar*, 50 μm), and quantification of $MHCII^+$ cells per 100 IECs in D2 biopsies. (*C*) Percentage of HLA-DQ^+ cells, gated on live $CD45^-EpCAM^+$ cells using the HLA-DQ antibody from D2 biopsies from symptomatic and asymptomatic patients with CeD, assessed by flow cytometry. Representative dot plots are shown. Data are presented as mean ± SEM, with each *dot* representing an individual patient with CeD. *P* value was determined using a two-tailed unpaired Student *t* test.

Gluten Immunization Induces MHCII Expression in DR3-DQ2.5 Mice

To dissect the functional consequences of MHCII expression in IECs of patients with CeD, we used transgenic mice carrying the human CeD risk gene, DR3-DQ2.5, and evaluated small intestinal inflammation after gluten immunization (Figure 2*A*). Gluten-immunized mice had higher serum anti-TG2 IgG (Figure 2*B*), higher small intestinal anti-gliadin IgA (Figure 2C), reduced small intestinal villus-to-crypt ratios (Figure 2*D*), and higher $CD3^+$ IEL counts (Figure 2*E*) than NI mice. Gene expression analysis in the small intestine revealed 22 genes that were differentially expressed between gluten-immunized and NI mice (Figure 2*F*). Genes related to innate immune function, such as *Rhoa, Tollip*, and MAP kinases (MAPK), including *Mapk3*, *Mapk1,* and *Map3k1*, were overexpressed in gluten-immunized mice. The expression of other inflammation-related genes, such as *Il15, Il1a, Tlr3,* and *Cfb*, was higher in gluten-immunized than NI mice (Figure 2*F*). Gluten-immunized mice also had higher IEC MHCII expression, as assessed by immunofluorescence staining (Figure 2*G*) and flow cytometry (Figure 2*H*; Supplementary Figure 3), compared with NI mice. No staining was detected in isotype controls (data not shown). To investigate whether induced inflammation in vivo leads to stronger small intestinal MHCII expression, gluten-immunized mice were injected with IFN-γ. Expression of epithelial MHCII and *Cd74* mRNA, which encodes an invariant chain that facilitates the assembly and trafficking of MHCII,[30] were higher in gluten-immunized+IFN-γ-injected mice compared with controls, which displayed lamina propria $MHCII^+$ cells only (Figure 3*A*–*C*).

Qa-1, a stress-induced non-classical MHC class I (MHCI) marker and a murine homolog of HLA-E that is increased in patients with CeD,[31] was higher in gluten-immunized than in NI mice (Supplementary Figures 3 and 4). Therefore, gluten-immunized DR3-DQ2.5 mice develop small intestinal inflammation, which is associated with the upregulation of MHCII expression in IECs.

Gluten Immunization and IFN-γ Induce MHCII and Costimulatory Molecule Expression in Organoid Monolayers

We developed organoid monolayers from the duodenum and proximal jejunum of naïve mice and verified the expression of the main intestinal epithelial lineage markers. E-cadherin staining confirmed the presence of adherens junctions, indicating epithelial polarity. The monolayers from both DR3-DQ2.5 (Figure 4*A*) and HLA-DQ8 (Supplementary Figure 5*A*) mice were populated with $Mucin2^+$ goblet cells, $Chromogranin\ A^+$ enteroendocrine cells, $Villin1^+$ enterocytes, and $Lysozyme^+$ Paneth cells.

We then used organoid monolayers from gluten-immunized, NI and naïve DR3-DQ2.5 mice to investigate the expression of epithelial MHCII (Figure 4*B*). Organoid monolayers from gluten-immunized mice showed enhanced expression of MHCII molecules compared with monolayers from NI or naïve mice (Figure 4*C*; Supplementary Figure 6).

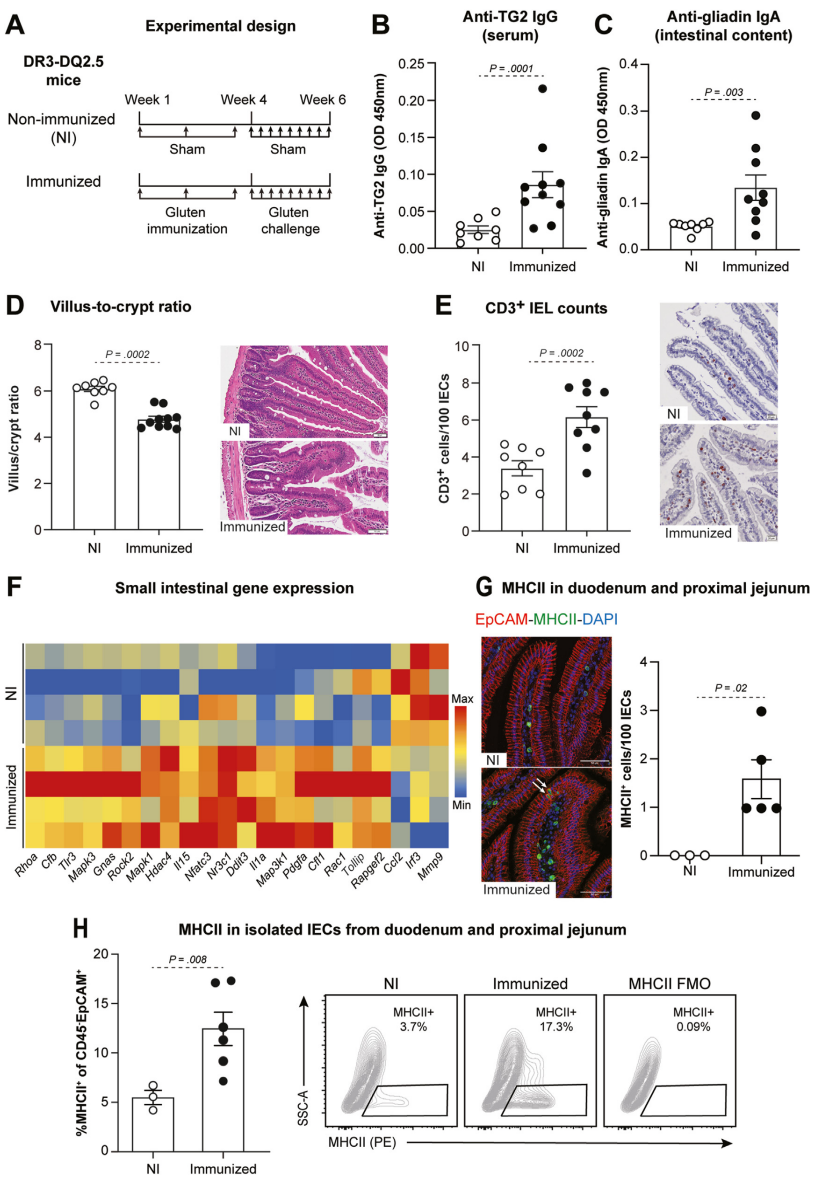
We then determined the effect of IFN-γ, a cytokine secreted by gluten-specific $CD4^+$ T cells in CeD, on the expression of MHCII by IECs. In vitro IFN-γ treatment increased the expression of MHCII in organoid monolayers from NI mice, and this expression was further upregulated in monolayers from gluten-immunized DR3-DQ2.5 (Figure 4*C*) and HLA-DQ8 mice (Supplementary Figure 5*B* and *C*). Monolayers from WT (C57BL/6) mice did not show any MHCII expression, indicating that the human MHCII antibody did not cross-react with murine MHCII molecules (Supplementary Figure 7). Monolayers from gluten-immunized+IFN-γ-injected mice had higher expression of MHCII compared with monolayers from control mice, which was further upregulated by in vitro treatment with IFN-γ (Figure 5*A* and *B*). Expression of *Cd74* and *Ciita*, the main transcription factor regulating MHCII expression,[30] was higher in monolayers from gluten-immunized+IFN-γ-injected mice, and further increased after IFN-γ stimulation in vitro compared with controls (Figure 5*C*).

We next investigated the expression of the classical costimulatory molecules CD80, CD86, and CD40, which are required, in addition to antigens bound to MHCII, to activate $CD4^+$ T cells (Supplementary Figure 6). The expression of CD40 was higher in organoid monolayers from gluten-immunized mice compared with monolayers from NI or naïve mice (Figure 4*D*). IFN-γ treatment increased the expression of CD40, CD86, and CD80 in organoid monolayers from gluten-immunized, but not from NI or naïve mice (Figure 4*D*–*F*). Similar results were obtained using organoid monolayers from gluten-immunized HLA-DQ8 mice (Supplementary Figure 5*D*–*F*). These results indicate that the expression of costimulatory molecules requires several inflammatory stimuli, such as those provided by in vivo gluten immunization and a permissive IFN-γ milieu.

We also assessed the expression of Qa-1 and CD71, a receptor implicated in transepithelial gluten peptide transport,[12] in monolayers from DR3-DQ2.5 mice. Organoid monolayers from gluten-immunized mice stimulated with IFN-γ had higher CD71 and Qa-1 expression compared with monolayers from gluten-immunized mice treated with media, or monolayers from NI or naïve mice (Figure 4*G*; Supplementary Figure 8). Taken together, these data demonstrate that under induced in vivo or in vitro inflammatory conditions, organoid monolayers from DQ2.5 or DQ8 mice express MHCII and markers involved in IEL activation and transepithelial transport.

MHCII Organoid Monolayers Activate $hCD4^+$ T Cells in a Gluten-Dependent Manner

The immune response in CeD is $CD4^+$ T-cell dependent and HLA-DQ restricted.[24] To investigate epithelial MHCII-$CD4^+$ T-cell interactions, we used mice that, in addition to DQ2.5, carry functional human CD4 receptor on T cells (DR3-DQ2.5-hCD4).[25] We established a co-culture system using MHCII-expressing organoid monolayers from gluten-immunized DR3-DQ2.5 mice and splenic $CD4^+$ T cells from gluten-immunized DR3-DQ2.5-hCD4 mice. IFN-γ–treated MHCII-expressing organoid monolayers were


A
Experimental design
DR3-DQ2.5 mice
Week 1
Week 4
Week 6
Non-immunized (NI)
Sham
Sham
Immunized
Gluten immunization
Gluten challenge
B
Anti-TG2 IgG (serum)
P = .0001
Anti-TG2 IgG (OD 450nm)
NI
Immunized
C
Anti-gliadin IgA (intestinal content)
P = .003
Anti-gliadin IgA (OD 450nm)
NI
Immunized
D
Villus-to-crypt ratio
P = .0002
Villus/crypt ratio
NI
Immunized
NI
Immunized
E
CD3+ IEL counts
P = .0002
CD3+ cells/100 IECs
NI
Immunized
NI
Immunized
F
Small intestinal gene expression
NI
Immunized
Max
Min
Rhoa
Cfb
Tlr3
Mapk3
Gnas
Rock2
Mapk1
Hdac4
Il15
Nfatc3
Nr3c1
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Map3k1
Pdgfa
Cfl1
Rac1
Tollip
Rapgef2
Ccl2
Irf3
Mmp9
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MHCII in duodenum and proximal jejunum
EpCAM-MHCII-DAPI
NI
Immunized
P = .02
MHCII+ cells/100 IECs
NI
Immunized
H
MHCII in isolated IECs from duodenum and proximal jejunum
P = .008
%MHCII+ of CD45-EpCAM+
NI
Immunized
NI
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MHCII+ 3.7%
MHCII+ 17.3%
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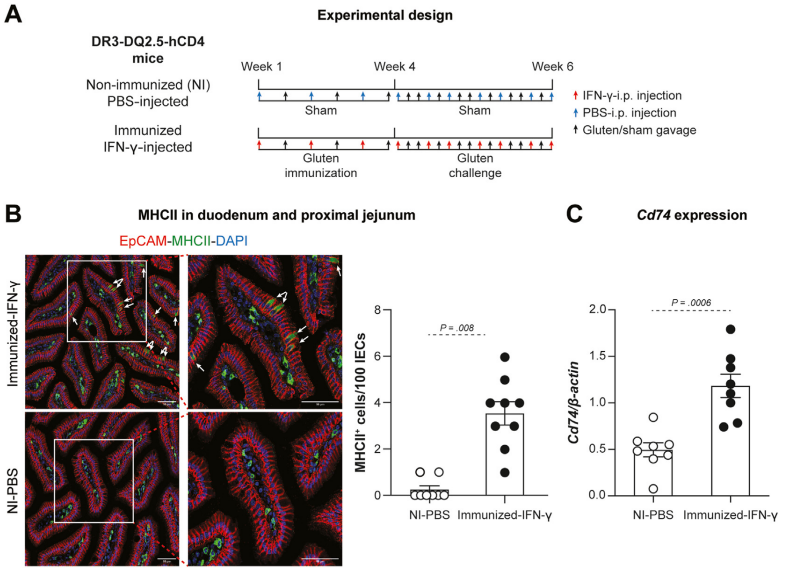


Figure 3. IFN-γ injection and gluten immunization enhance epithelial MHCII expression in DR3-DQ2.5-hCD4 mice. (*A*) NI DR3-DQ2.5-hCD4 mice received sham gavages and were injected with phosphate-buffered saline (NI-PBS; *open dots*). Gluten-immunized mice received PT-gliadin and cholera toxin, followed by gluten challenges. Gluten-immunized mice received intraperitoneal (i.p.) injections of rmIFN-γ throughout the experiment (immunized-IFN-γ; *black dots*). (*B*) Representative immunofluorescence staining of the duodenum and proximal jejunum for MHCII$^+$ (*white arrows*; EpCAM$^+$: *red*; MHCII$^+$: *green*; 4′,6-diamidino-2-phenylindole [DAPI]: *blue*; *scale bar*, 50 μm). Quantification of MHCII$^+$ cells per 100 IECs from NI-PBS (n = 8) and immunized-IFN-γ (n = 9) mice. (*C*) *Cd74* mRNA expression relative to the housekeeping gene *β-actin* in IECs isolated from NI-PBS (n = 8) and immunized-IFN-γ (n = 8) mice. Data are presented as mean ± SEM. Each *dot* represents an individual mouse. All *P* values were determined using a two-tailed unpaired Student *t* test.

apically stimulated with DAPT-gliadin, DAPT-zein, or media before introducing hCD4$^+$ T cells into the basolateral side of the organoid monolayers (Figure 6*A*). The proliferation of hCD4$^+$ T cells, which required the presence of the monolayer (Supplementary Figure 9*A* and *B*), increased by 2.5-fold in response to DAPT-gliadin stimulation compared with DAPT-zein treatment (Figure 6*B*). Concomitantly, hCD4$^+$ T cells co-cultured with DAPT-gliadin–treated monolayers, but not in the absence of monolayers (Supplementary Figure 9*C*–*E*), exhibited an activated phenotype with higher expression of known T-cell activation markers, including CD69, CD25, and CD44, compared with hCD4$^+$ T cells from DAPT-zein–treated monolayers (Figure 6*C*–*E*; Supplementary Figure 10). In addition, T-cell

Figure 2. Gluten immunization induces inflammation and MHCII expression in DR3-DQ2.5 mice. (*A*) NI DR3-DQ2.5 mice received sham gavages and were used as controls (*open dots*). Gluten-immunized mice received PT-gliadin and cholera toxin, followed by gluten challenges (*black dots*). (*B*) Serum anti-TG2 IgG levels in NI (n = 8) and immunized (n = 10) mice. (*C*) Anti-gliadin IgA levels in the intestinal contents in NI (n = 8) and immunized (n = 9) mice. (*D*) Quantification of the small intestinal villus height-to-crypt depth ratios in NI (n = 8) and immunized (n = 10) mice. Representative hematoxylin and eosin (H&E)-stained small intestinal sections are shown. *Scale bar*, 50 μm. (*E*) Quantification of CD3$^+$ IELs per 100 IECs from NI (n = 8), and immunized (n = 9) mice. Representative CD3$^+$ stained sections of the duodenum and proximal jejunum, where CD3$^+$ IELs are stained in *red*, are shown. *Scale bar*, 20 μm. (*F*) Heatmap of significantly altered genes in the small intestinal tissues of NI (n = 4), and immunized (n = 4) mice. (*G*) Representative immunofluorescence staining of the duodenum and proximal jejunum for MHCII$^+$ (*white arrows*; EpCAM$^+$: *red*; MHCII$^+$: *green*; 4′,6-diamidino-2-phenylindole [DAPI]: *blue*; *scale bar*, 50 μm). Quantification of MHCII$^+$ cells per 100 IECs from NI (n = 3) and immunized (n = 5) mice. (*H*) Percentage of MHCII-expressing cells gated on live CD45$^-$EpCAM$^+$ cells from IECs isolated from the duodenum and proximal jejunum of NI (n = 3) and immunized (n = 6) DR3-DQ2.5 mice. Representative dot plots showing MHCII$^+$ cells, gated on live CD45$^-$EpCAM$^+$ cells using an HLA-DR-DP-DQ antibody. Data are presented as mean ± SEM, with each *dot* representing an individual mouse. All *P* values were determined using a two-tailed unpaired Student *t* test. One sample from the immunized group in (*C*) and (*E*) was removed from the analysis because of technical issues. In the heatmap, *columns* represent each gene, and *rows* indicate individual mice. The *color* indicates the degree of expression.

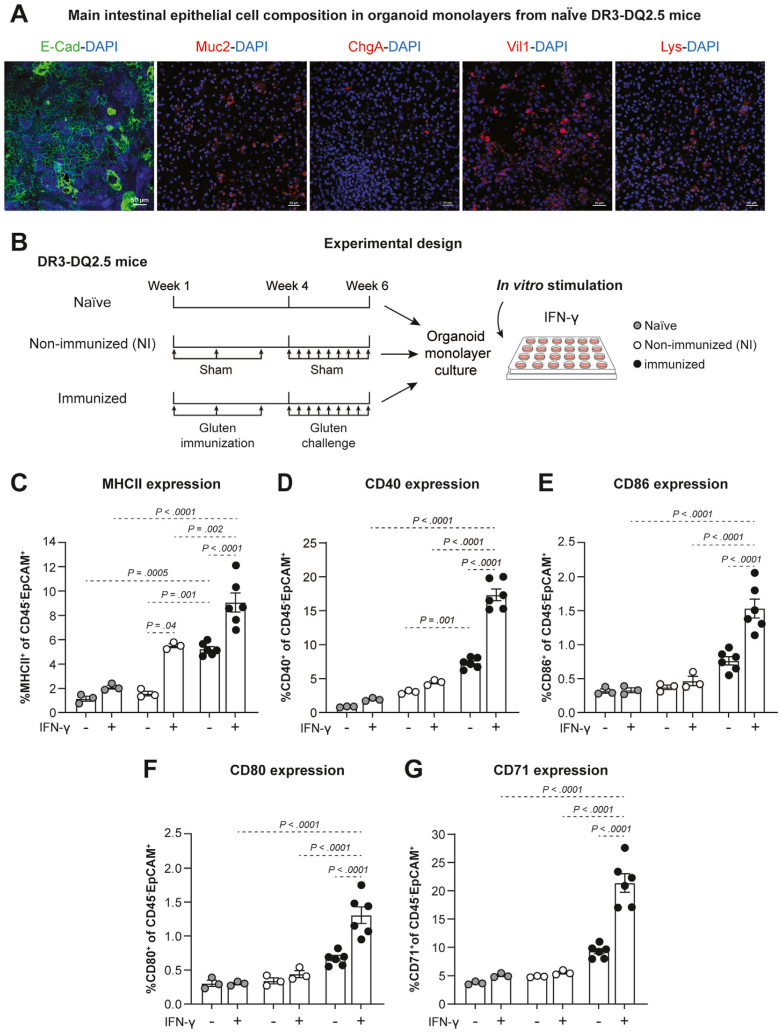


Figure 4. Gluten immunization and IFN-γ induce MHCII expression in organoid monolayers from DR3-DQ2.5 mice. (*A*) Representative immunofluorescence staining of organoid monolayers from naïve DR3-DQ2.5 mice for E-cadherin$^+$ (E-Cad$^+$: *green*; 4′,6-diamidino-2-phenylindole [DAPI]: *blue*); Mucin2$^+$ (Muc2$^+$: *red*; DAPI: *blue*); Chromogranin A$^+$ (ChgA$^+$: *red*; DAPI: *blue*); Villin1$^+$ (Vil1$^+$: *red*; DAPI: *blue*); and Lysozyme$^+$ (Lys$^+$: *red*; DAPI: *blue*). *Scale bar*, 50 μm. (*B*) Naïve DR3-DQ2.5 mice received no treatment and were used as controls (n = 3; *gray dots*). NI DR3-DQ2.5 mice received sham immunization and challenge and were used as controls (n = 3; *open dots*). Gluten-immunized DR3-DQ2.5 mice received PT-gliadin and cholera toxin, followed by gluten challenges (n = 6; *black dots*). Organoid monolayers were then stimulated in vitro with or without IFN-γ. (*C*–*G*) Percentage of (*C*) MHCII-expressing cells using an HLA-DR-DP-DQ antibody, (*D*) CD40-expressing cells, (*E*) CD86-expressing cells, (*F*) CD80-expressing cells, or (*G*) CD71-expressing cells, gated on live CD45$^-$EpCAM$^+$ cells from organoid monolayers from naïve, NI, and immunized DR3-DQ2.5 mice stimulated in vitro with or without IFN-γ. Data are presented as mean ± SEM. Each *dot* represents an individual mouse. All *P* values were determined using one-way analysis of variance with Tukey's post hoc test for multiple comparisons.

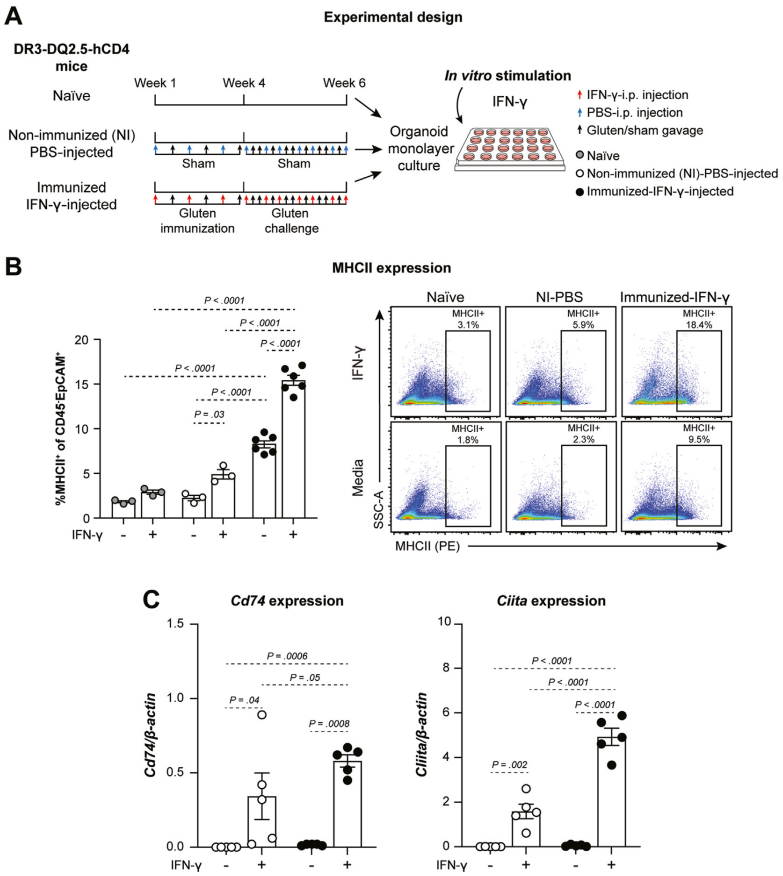
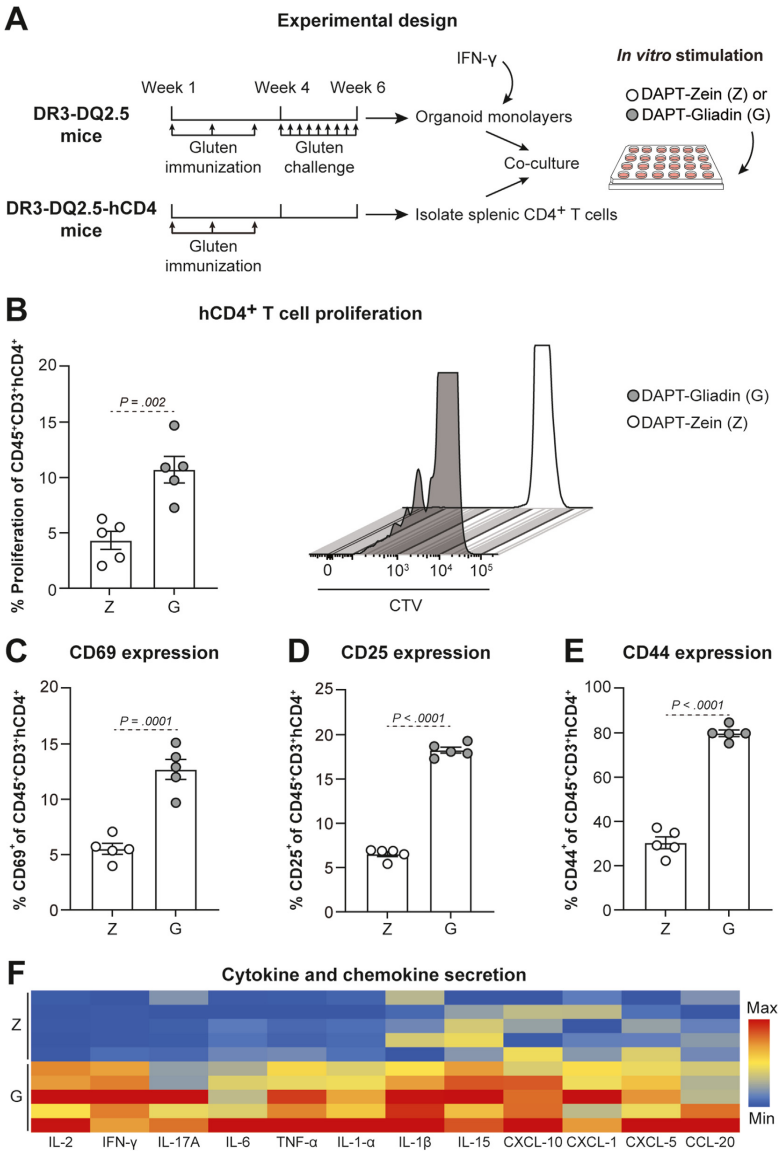


Figure 5. Expression of MHCII in organoid monolayers from gluten-immunized+IFN-γ-injected DR3-DQ2.5-hCD4 mice. (*A*) Organoid monolayers were developed from naïve (no treatment; n = 3; *gray dots*), NI+phosphate-buffered saline–injected (NI-PBS; n = 3; *open dots*), and gluten-immunized+IFN-γ-injected (immunized-IFN-γ; n = 6; *black dots*) DR3-DQ2.5-hCD4 mice. The organoid monolayers were then stimulated with or without IFN-γ. (*B*) Percentage of MHCII$^+$ expressing cells from organoid monolayers, gated on live CD45$^-$EpCAM$^+$ cells using an HLA-DR-DP-DQ antibody. Representative dot plots showing MHCII$^+$ cells, gated on live CD45$^-$EpCAM$^+$ cells using an HLA-DR-DP-DQ antibody. (*C*) *Cd74* and *Ciita* mRNA expression relative to the housekeeping gene *β-actin* in organoid monolayers from NI-PBS (n = 5; *open dots*) and immunized-IFN-γ (n = 5; *black dots*) treated in vitro with or without IFN-γ. Data are presented as mean ± SEM. Each *dot* represents an individual mouse. All *P* values were determined using one-way analysis of variance with Tukey's post hoc test for multiple comparisons.

activation markers were positively correlated with monolayer MHCII expression (Supplementary Figure 11).

Stimulation of monolayers with DAPT-gliadin, but not DAPT-zein, increased the secretion of several cytokines and chemokines in the co-culture supernatant (Figure 6*F*). The DAPT-gliadin–stimulated monolayers yielded a 37-fold elevation in interleukin (IL)-2 compared with DAPT-zein–treated monolayers (Supplementary Figure 12*A*). In accordance with clinical studies showing elevated gluten-mediated serum cytokines in patients with CeD,[32] we found a 28-fold and 4-fold increase in the secretion of IFN-γ (Supplementary Figure 12*B*) and IL-17A (Supplementary Figure 12*C*), respectively, after treatment of the monolayers with DAPT-gliadin compared with DAPT-zein. Notably, the T-cell–secreted cytokines IL-2 and IL-17A were not detectable in monolayer-only cultures, in which CD4$^+$ T cells were absent (Supplementary Figure 13).

The concentrations of proinflammatory cytokines, such as IL-6, tumor necrosis factor (TNF)-α, IL-1α, and IL-1β, were elevated in response to DAPT-gliadin treatment of co-


A
Experimental design
IFN-γ
In vitro stimulation
Week 1
Week 4
Week 6
DR3-DQ2.5 mice
Gluten immunization
Gluten challenge
Organoid monolayers
DAPT-Zein (Z) or
DAPT-Gliadin (G)
Co-culture
DR3-DQ2.5-hCD4 mice
Isolate splenic CD4+ T cells
Gluten immunization
B
hCD4+ T cell proliferation
% Proliferation of CD45+CD3+hCD4+
P = .002
Z
G
DAPT-Gliadin (G)
DAPT-Zein (Z)
CTV
C
CD69 expression
% CD69+ of CD45+CD3+hCD4+
P = .0001
D
CD25 expression
% CD25+ of CD45+CD3+hCD4+
P < .0001
E
CD44 expression
% CD44+ of CD45+CD3+hCD4+
P < .0001
F
Cytokine and chemokine secretion
Max
Min
IL-2
IFN-γ
IL-17A
IL-6
TNF-α
IL-1-α
IL-1β
IL-15
CXCL-10
CXCL-1
CXCL-5
CCL-20

culture compared with DAPT-zein treatment (Supplementary Figure 12*D*–*G*). In addition, an increase in IL-15, a major cytokine in the pathogenesis of CeD,[31] was observed on exposure to DAPT-gliadin vs DAPT-zein (Supplementary Figure 12*H*). The secretion of chemokines known to participate in immune and inflammatory cell recruitment and migration,[33,34] such as C-X-C motif chemokine ligand (CXCL)-10, CXCL-1, CXCL-5, and C-C motif chemokine ligand (CCL)-20, were higher in the supernatants from DAPT-gliadin–treated monolayers than in the supernatants from DAPT-zein–stimulated monolayers (Supplementary Figure 12*I*–*L*). When monolayers were stimulated with IFN-γ in the absence of $CD4^+$ T cells, higher levels of epithelial-associated innate cytokines and chemokines, including IL-6, IL-1α, IL-1β, IL-15, CXCL-10, and CCL-20 were detected (Supplementary Figure 13).

Finally, the expression of the stress-induced markers, Qa-1 and Rae-1, a ligand for NKG2D activating natural killer (NK) cell receptors, was higher in co-cultures treated with DAPT-gliadin (Supplementary Figure 14*A*–*C*).

Collectively, our data show that epithelial monolayers expressing MHCII induce the proliferation and activation of underlying $hCD4^+$ T cells in a gluten-dependent manner, leading to increased secretion of a panel of CeD-associated cytokines and chemokines.

Modulation of $hCD4^+$ T-Cell Activation by Opportunistic Pathogen-Derived Elastase

Microbial factors have emerged as modulators of inflammation in CeD.[5,35] We previously showed that gluten metabolized by bacterial elastase had increased immunogenicity.[36] To investigate whether bacterial metabolism influences IEC–T-cell interactions, organoid monolayer-$CD4^+$ T-cell co-cultures were stimulated with gliadin predigested with elastase-producing WT *P aeruginosa* PA14. DAPT-gliadin, gliadin predigested with the *P aeruginosa* isogenic *lasB* mutant strain lacking elastase activity, referred to as $lasB^{\triangle/\triangle}$, and the WT *P aeruginosa* PA14 supernatant were used as controls (Figure 7*A*). Compared with co-cultures treated with DAPT-gliadin predigested with $lasB^{\triangle/\triangle}$, DAPT-gliadin, or WT *P aeruginosa* PA14 supernatant, treatment with DAPT-gliadin predigested with WT *P aeruginosa* PA14 led to 1.7-, 1.4-, and 2.6-fold increases in $hCD4^+$ T-cell proliferation, respectively (Figure 7*B*). Increased expression of the activation markers CD69, CD25, and CD44 (Figure 7*C*–*E*) in $hCD4^+$ T cells confirmed a more robust activated phenotype of these cells in co-cultures stimulated with DAPT-gliadin predigested with WT *P aeruginosa* PA14 vs controls. This was associated with elevated levels of a panel of cytokines and chemokines (Figure 7*F*), including a 5.4-, 3.7-, and 676-fold increase in IL-2 levels in the supernatant when the organoid monolayers were treated with DAPT-gliadin predigested with WT *P aeruginosa* PA14 compared with DAPT-gliadin predigested with $lasB^{\triangle/\triangle}$, DAPT-gliadin, or WT *P aeruginosa* PA14 supernatant, respectively (Supplementary Figure 15*A*). The secretion of IFN-γ (Supplementary Figure 15*B*) and IL-17A (Supplementary Figure 15*C*) was the highest when organoid monolayers were treated with DAPT-gliadin predigested with WT *P aeruginosa* PA14 vs controls. In addition, organoid monolayers treated with DAPT-gliadin predigested with WT *P aeruginosa* PA14 exhibited elevated release of the proinflammatory cytokines IL-6, TNF-α, IL-1α, IL-1β, and IL-15 compared with controls (Supplementary Figure 15*D*–*H*). Stimulation of organoid monolayers with DAPT-gliadin predigested with WT *P aeruginosa* PA14 led to higher production of CXCL-10, CXCL-1, CXCL-5, and CCL-20 compared with controls (Supplementary Figure 15*I*–*L*). Taken together, these results demonstrate that MHCII-expressing organoid monolayers enable more robust proliferation and activation of the underlying $hCD4^+$ T cells when gliadin is partially metabolized by bacterial elastase.

Discussion

IECs express MHCII molecules in the small intestine,[37–39] implying that they possess an essential prerequisite for functioning as APCs. The functional consequences of this expression remain controversial, with some studies associating it with inflammation and others with immunosuppressive activities and epithelial differentiation or renewal.[40–43] These conflicting results are likely contextual, suggesting that a complete understanding will require investigation of specific diseases. IECs are central to the pathogenesis of CeD; however, whether they are involved in the presentation of gluten-derived peptides to MHCII-restricted $CD4^+$ T cells has never been demonstrated. Here, we show that IECs from patients with CeD have higher epithelial MHCII expression compared with IECs from patients compliant with a GFD that achieved histological remission. Using humanized organoid monolayers, we

Figure 6. MHCII-expressing organoid monolayers induce a gluten-dependent $CD4^+$ T-cell response. (*A*) Organoid monolayers were co-cultured with splenic $CD4^+$ T cells in the presence of DAPT-zein (Z; *open dots*) or DAPT-gliadin (G; *gray dots*). Organoid monolayers from gluten-immunized DR3-DQ2.5 mice (n = 5) were further stimulated with IFN-γ for 24 hours before co-culture. Splenic $CD4^+$ T cells were isolated from gluten-immunized DR3-DQ2.5-hCD4 mice (n = 5). (*B*) *Left panel*, percentage of proliferating CellTrace Violet (CTV)-labeled $hCD4^+$ T cells, gated on live $CD45^+CD3^+hCD4^+$ cells that were co-cultured with organoid monolayers in the presence of DAPT-zein (Z) or DAPT-gliadin (G). Right panel, representative histograms of CTV-labeled $hCD4^+$ T cells gated on live $CD45^+CD3^+hCD4^+$ T cells. (*C*–*E*) Percentage of (*C*) CD69-expressing cells, (*D*) CD25-expressing cells, or (*E*) CD44-expressing cells gated on live $CD45^+CD3^+hCD4^+$ T cells that were co-cultured with organoid monolayers in the presence of DAPT-zein (Z) or DAPT-gliadin (G). (*F*) Heatmap of cytokines and chemokines in co-culture supernatants that were significantly different between (DAPT)-zein (Z) and DAPT-gliadin (G). Data are presented as mean ± SEM. Each *dot* represents an individual mouse. All *P* values were determined using a two-tailed unpaired Student *t* test. In the heatmap, *columns* represent each cytokine or chemokine, and *rows* indicate individual mice. The *color* indicates the degree of expression.

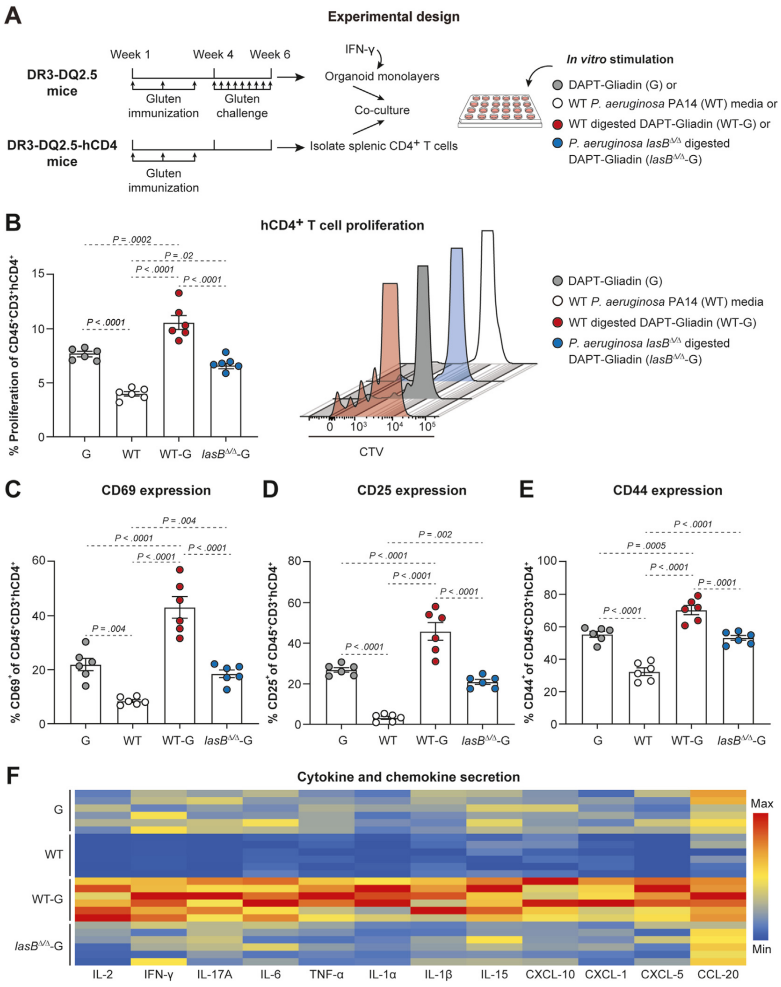


Figure 7. Gluten metabolized by opportunistic pathogen-derived elastase induces a robust $CD4^+$ T-cell response. (*A*) Organoid monolayers were co-cultured with splenic $CD4^+$ T cells in the presence of DAPT-gliadin (G; *gray dots*), WT *Pseudomonas aeruginosa* PA14 (WT; *open dots*) supernatant, WT digested DAPT-gliadin (WT-G; *red dots*), or *P aeruginosa* $lasB^{\Delta/\Delta}$ digested DAPT-gliadin ($lasB^{\Delta/\Delta}$-G; *blue dots*). Organoid monolayers derived from gluten-immunized DR3-DQ2.5 mice (n = 6) were stimulated with IFN-γ for 24 hours before co-culture. Splenic $CD4^+$ T cells were isolated from gluten-immunized DR3-DQ2.5-hCD4 mice (n = 6). (*B*) *Left panel*, percentage of proliferating CTV-labeled $hCD4^+$ T cells gated on live $CD45^+CD3^+hCD4^+$ cells that were co-cultured with organoid monolayers in the presence of DAPT-gliadin (G), *P aeruginosa* PA14 (WT) supernatant, WT digested DAPT-gliadin (WT-G), or *P aeruginosa* $lasB^{\Delta/\Delta}$ digested DAPT-gliadin ($lasB^{\Delta/\Delta}$-G). *Right panel*, representative histograms of CTV-labeled $hCD4^+$ T cells gated on live $CD45^+CD3^+hCD4^+$ T cells. (*C–E*) Percentage of (*C*) CD69-expressing cells, (*D*) CD25-expressing cells or (*E*) CD44-expressing cells, gated on live $CD45^+CD3^+hCD4^+$ T cells that were co-cultured with organoid monolayers in the presence of DAPT-gliadin (G), *P aeruginosa* PA14 (WT) supernatant, WT digested DAPT-gliadin (WT-G), or *P aeruginosa* $lasB^{\Delta/\Delta}$ digested DAPT-gliadin ($lasB^{\Delta/\Delta}$-G). (*F*) Heatmap of cytokines and chemokines that were significantly different between co-cultures stimulated with (DAPT)-gliadin (G), wild-type *P aeruginosa* PA14 (WT) supernatant, WT digested DAPT-gliadin (WT-G), and *P aeruginosa* $lasB^{\Delta/\Delta}$ digested DAPT-gliadin ($lasB^{\Delta/\Delta}$-G). Data are presented as mean ± SEM. Each *dot* represents an individual mouse. All *P* values were determined using one-way analysis of variance with Tukey's post hoc test for multiple comparisons. In the heatmap, *columns* represent each cytokine or chemokine, and *rows* indicate individual mice. The *color* indicates the degree of expression.

demonstrated that IECs mediate gluten presentation in the context of MHCII molecules and activate hCD4$^+$ T cells. Finally, predigestion of gluten by elastase-producing opportunistic pathogens, known to metabolize gluten peptides,[36] further modulated hCD4$^+$ T-cell activation in our model.

Approximately 90% of the CeD population carries the HLA-DQ2.5 allele,[6] and the remaining patients express HLA-DQ2.2 and/or -DQ8.[44] Gluten peptides are presented to CD4$^+$ T cells preferentially via these HLA-DQ molecules expressed in professional APCs such as dendritic cells, macrophages, and B cells.[21,45,46] Although MHCII expression and function are generally restricted to professional APCs, several studies have shown that non-hematopoietic cells, including IECs, can express MHCII.[23,37,38,40] More importantly, the expression of MHCII in enterocytes of patients with CeD has been previously reported.[22,39] Despite this, the role of epithelial MHCII in gluten presentation and T-cell activation in CeD has been overlooked. Thus, we first determined that patients with active CeD have enhanced duodenal epithelial MHCII expression compared with patients on a GFD in histological remission, implying that CeD activity is associated with upregulation of epithelial MHCII. We then confirmed the presence of CeD-associated MHCII, HLA-DQ, in IECs isolated from biopsies of patients with CeD using flow cytometry. These results suggest that the intestinal epithelium has the capacity for MHCII-mediated gluten antigen presentation and T-cell activation during CeD activity. To further investigate gluten-epithelial MHCII-CD4$^+$ T-cell interactions we used humanized organoid monolayers from mice carrying HLA-DQ2.5, the genotype expressed in *cis* or *trans* by most patients with CeD.[6,8,47] Gluten-immunized DR3-DQ2.5 mice developed proximal small intestinal inflammation and proinflammatory gene expression, including *Il15,* which encodes a key cytokine in CeD.[48] Gluten immunization also enhanced the expression of MHCII in isolated IECs from DR3-DQ2.5 mice, suggesting that in vivo inflammation upregulates MHCII, and this is recapitulated in organoid monolayers. This is supported by our data in gluten-immunized mice also injected with IFN-γ, which further upregulated the expression of epithelial MHCII and associated genes (*Cd74*). This is consistent with previous studies indicating that IECs express IFN-γ receptors, and that IFN-γ is a potent inducer of IEC MHCII upregulation,[23,43,49,50] suggesting that in vivo inflammation can prime the intestinal epithelium to further upregulate MHCII. The epithelial expression of costimulatory molecules and CD71 required inflammatory stimuli of in vivo gluten immunization in addition to in vitro stimulation with IFN-γ. Our model recapitulates findings that CD71, a transferrin receptor involved in gluten peptide transport across the intestinal epithelium, is overexpressed in patients with active but not potential CeD.[12]

A key finding of this study is that MHCII-bearing organoid monolayers are functional and can activate CD4$^+$ T cells in a gluten-dependent manner. This is demonstrated by multiple avenues, including the increased proliferation of hCD4$^+$ T cells, higher expression of T-cell activation markers, which correlated with monolayer MHCII expression, and elevated secretion of IL-2, a cytokine indicative of T-cell activation[51] that is increased in patients with CeD following gluten challenge.[32,34] The inability of CD4$^+$ cells to proliferate and activate in the absence of MHCII-expressing monolayers supports the functionality of our model, whereas the absence of IL-2 and IL-17A in monolayer-only cultures indicates that their release is dependent on the presence of activated T cells. The upregulation of CD25 (IL-2Rα[51]) on hCD4$^+$ T cells in the presence of gluten could be attributed to increased IL-2–mediated signaling in the co-culture system. The production of IFN-γ in the supernatant of co-culture exposed to gluten is in accordance with previous observations in patients with CeD.[34,52] Other inflammatory cytokines, such as IL-6, TNF-α, IL-1α, IL-1β, and IL-15, previously implicated in CeD,[34,53–55] were higher in the supernatants of co-cultures treated with gluten and monolayer-only cultures treated with IFN-γ. We also detected an increase in CXCL-10, CXCL-1, CXCL-5, and CCL-20 levels in gluten-treated co-cultures, which are chemotactic for effector/memory T cells, B cells, NK cells, dendritic cells, and neutrophils.[56] The Th1-associated chemokine CXCL-10 (IFN-γ-induced protein 10) was previously found to correlate with increased IFN-γ levels and is produced by epithelial and plasma cells in the lamina propria of patients with active CeD.[57] Serum CCL-20, a Th17-associated chemokine, was also found to be higher 4 hours after gluten challenge in patients with CeD.[32,34] Finally, MHCII-bearing organoid monolayers exposed to gluten and co-cultured with hCD4$^+$ T cells showed increased expression of the stress-induced markers, Qa-1 and Rae-1. This agrees with studies in which the expression of human analogs of these markers was associated with cellular stress in patients with active CeD.[16,17] Although recent studies have implicated epithelial MHCII in the regulation of acute graft-vs-host disease[43] and bacteria-specific effector CD4$^+$ T-cell responses,[49] we show for the first time, gluten-dependent hCD4$^+$ T-cell activation by IECs that express celiac-associated MHCII molecules.

Duodenal opportunistic pathogens are emerging as cofactors in CeD.[5] One mechanism involves modification of peptide antigenicity by the metabolism of gluten by microbial proteases.[5,58] Here, we investigated whether gluten partially metabolized by elastase-producing *P aeruginos*a PA14, a previously described duodenal pathobiont in CeD,[36] modified hCD4$^+$ T-cell activation. We observed higher proliferation and activation phenotypes of hCD4$^+$ T cells with increased secretion of IL-2 and several other proinflammatory cytokines. These results are in accordance with the previously demonstrated in vitro activation of gluten-specific T cells from patients with CeD by *P aeruginosa*–modified gluten peptides,[36] implying that gluten modified by microbial elastase can also influence epithelial MHCII-mediated activation of hCD4$^+$ T cells.

Recent studies using organoids from patients with CeD have reported phenotypic differences between CeD and controls, including gene expression,[59] permeability function,[60] and macrophage responses to gluten.[61] Our results advance the field by identifying a new immunological role for IECs in activating CD4$^+$ T cells in the context of CeD.

Nevertheless, our study has limitations. We provide evidence of higher MHCII expression in duodenal biopsies of patients with active CeD. However, gluten-IEC MHCII-T-cell activation was explored in organoid monolayers from a humanized murine model. Human organoid monolayer-$CD4^+$ T-cell co-cultures would require T cells to be isolated from the same patient to restrict T-cell receptors to HLA molecules expressed by IECs. Even in this case, we would not be able to determine which HLA molecules present gluten peptides that mediate T-cell activation. The humanized characteristics of our in vitro model, namely the expression of HLA-DQ2.5 and hCD4 in the absence of other murine MHCII molecules, make this model well suited to address the question explored in this study and pathogen-metabolized gluten-IEC-T-cell interactions. The mechanism of MHCII-$CD4^+$ T-cell interaction remains to be determined, but it could involve gluten-MHCII exosome release by IECs.[62] Further investigations are required to determine whether the mechanisms described here contribute to CeD onset, its maintenance, or both.

In conclusion, we demonstrated that MHCII-expressing IECs exposed to gluten mediate $hCD4^+$ T-cell activation, indicating a previously unknown role for the intestinal epithelium in the modulation of $CD4^+$ T-cell responses in CeD through antigen presentation that could be targeted therapeutically. Because IECs are in first contact with dietary antigens, our results may encourage the study of the regulation of immune responses by the epithelium in other MHCII-associated diseases.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2024.07.008.

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Data Availability
NanoString raw data has been deposited in the Gene Expression Omnibus (GEO) database under accession number GSE219286.

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