Investigating coeliac disease patient transglutaminase 2-specific B cells in the duodenal bulb

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Tiivistelmä

Tutkimuksen tausta ja tavoitteet: Keliakia on gluteenin laukaisema sairaus, jolle on tyypillistä autoimmuunivaste transglutaminaasi 2 (TG2)-entsyymiä kohtaan. Keliakiassa TG2-spesifiset plasmasolut erittävät immunoglobuliiniluokan (Ig) A autovasta-aineita TG2:ta vastaan. Näiden solujen on esitetty saavan alkunsa IgD- ja IgM-positiivisista B-soluista. B-solujen on oletettu yleisesti kehittyvän imukeräsissä, jolle on tyypillistä Pax5-transkriptiotekijän ja aktivaatio-indusoidun sytidiinideaminaasin (AID) ilmentäminen. Tuoreen hypoteesin mukaan TG2-spesifisten B-solujen kehitys saattaa kuitenkin tapahtua imukerästen ulkopuolella suoliston limakalvolla. Suurin osa keliakiapotilasmateriaalista koostuu ohutsuolibiopsinäytteistä. Ne sisältävät vähän imukeräsiä, mikä ei mahdollista B-solujen kehityksen tutkimista. Imukeräsiä sisältävää imukudosta on kuitenkin runsaasti duodenaalisessa bulbuksessa. Työn tarkoituksena oli tutkia, onko keliakiapotilaiden TG2-spesifisiä B-soluja imukeräsissä vai niiden ulkopuolella ainutlaatuisen suuren, keliaakikoista ja terveistä kontrolleista kerätyn duodenaalisen bulbusbiopsinäytesarjan avulla.

Tutkimusmenetelmät: Ensimmäisessä vaiheessa käytiin läpi 211 suomalaista ja 268 romanialaista duodenaalista bulbusbiopsinäytettä, joista seulottiin imukudoksellisia imukeräsiä todennäköisimmin sisältävät näytteet, mukaan lukien eristetyt imukeräset (ILF) ja Peyerin levyt (PP). Toisessa vaiheessa TG2-spesifisiä B-soluja havainnointiin immunofluoresenssivärjäyksen avulla. Värjäysprotokollassa käytettiin biotinyloitua rekombinantti-TG2:ta laimennoksella 1:100 ja vasta-aineita IgA:ta, IgD:tä, IgM:ää, AID:tä ja Pax5:ttä vastaan kolokalisaation havaitsemiseksi.

Tutkimustulokset: Kaikista 479 seulotusta näytteestä 14 % sisälsi ILF-rakenteellista imukudosta ja 2,7 % PP-rakenteellista imukudosta. Immunofluoresenssivärjäyksen optimoinnissa primäärivasta-ainelaimennoksiksi valittiin 1:25 IgD:lle ja IgM:lle, 1:100 AID:lle ja 1:250 Pax5:lle. Lopulliseen värjäykseen valittiin neljä hoitamatonta keliaakikkonäytettä ja kolme kontrollinäytettä. Keliaakikoissa havaittiin yhteensä kolme TG2-IgA-, neljä TG2-IgD- ja neljä TG2-AID-tuplapositiivista solua, jotka eivät olleet osana mitään tunnistettavaa imukudosta. TG2-IgM- ja TG2-Pax5-tuplapositiivisia soluja ei havaittu keliaakikoissa. Kahdessa kontrollissa havaittiin yksittäisiä IgD-, IgM- ja Pax5-positiivisia, TG2-spesifisiä B-soluja.

Johtopäätökset: Keliakiapotilaiden duodenaalisista bulbusbiopsinäytteistä löydettiin yksittäisiä TG2-spesifisiä B-soluja, jotka ilmentivät IgD:tä tai AID:tä ja jotka eivät olleet osana mitään tunnistettavaa imukudosrakennetta. Tulosten mukaan duodenaalisen bulbuksen limakalvolla saattaa siis kehittyä pieni määrä TG2-spesifisiä soluja imukerästen ulkopuolella.

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Abstract

Background and aims: Coeliac disease (CD) is a gluten-induced disorder, which is characterized by an autoimmune response against enzyme transglutaminase 2 (TG2). In CD, TG2-specific plasma cells secrete immunoglobulin (Ig) class A autoantibodies against TG2. These cells are assumed to develop from IgD and IgM positive B cells. The common development of B cells is suggested to occur in lymphoid follicles and is characterized by the expression of transcription factor Pax5 and activation-induced cytidine deaminase (AID). However, a recent hypothesis suggests that the development of TG2-specific B cells may occur at extra-follicular sites of the intestinal mucosa. The majority of CD-patient material consists of small intestine biopsies, which are devoid of lymphoid follicles, and therefore is insufficient for studying the B cell differentiation. However, there is an abundance of lymphoid tissues that contain lymphoid follicles in the duodenal bulb. The aim of this thesis was to investigate whether CD-patient TG2-specific B cells exist in lymphoid follicles or at extra-follicular sites using a uniquely large collection of duodenal bulb biopsies from CD-patients and healthy controls.

Methods: The first phase was to screen 211 Finnish and 268 Romanian duodenal bulb biopsies to identify specimens most likely to contain lymphoid tissues with lymphoid follicles, including isolated lymphoid follicles (ILFs) and Peyer's patches (PPs). The second phase was to detect TG2-specific B cells using immunofluorescence staining. The staining protocol consisted of biotinylated recombinant TG2 at a dilution of 1:100 and co-localization using antibodies against IgA, IgD, IgM, AID, and Pax5.

Results: Out of all the 479 screened specimens, 14% contained structures resembling ILFs and 2.7% contained structures resembling PPs. The chosen primary antibody dilutions in the optimized immunofluorescence staining were 1:25 for IgD and IgM, 1:100 for AID, and 1:250 for Pax5. Four specimens from untreated CD-patients and three specimens from non-CD controls were chosen for the final staining. In CD-patients, a total of three TG2-IgA double positive cells, four TG2-IgD double positive cells, and four TG2-AID double positive cells were identified that were no part of any recognizable lymphoid tissue. No TG2-IgM or TG2-Pax5 double positive cells were detected in CD-patients. Single IgD, IgM, and Pax5 positive TG2-specific B cells were detected in two controls.

Conclusions: Single TG2-specific B cells expressing IgD or AID were identified that were no part of any recognizable lymphoid tissue structure in CD-patient duodenal bulb biopsies. Therefore, the results suggest that a low number of TG2-specific B cells may develop at extra-follicular sites in the duodenal bulb mucosa.

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Petri Purola

Table of Contents

Abbreviations

AID	activation-induced cytidine deaminase
APC	antigen-presenting cell
BCR	B cell receptor
BSA	bovine serum albumin
CD	coeliac disease
CLP	common lymphoid progenitor
CSR	class-switch recombination
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DGP	deamidated gliadin peptide
E2A	E box binding protein 2A
EBF1	early B cell factor 1
EmA	endomysial antibody
FITC	fluorescein isothiocyanate
GALT	gut-associated lymphoid tissue
GFD	gluten-free diet
HLA	human leukocyte antigen
HSC	hematopoietic stem cell
IEL	intraepithelial lymphocyte
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILF	isolated lymphoid follicle
LP	lamina propria
Lti	lymphoid tissue inducer
Lto	lymphoid tissue organizing
MHC	major histocompatibility complex
MICA	major histocompatibility class I polypeptide-related sequence A
NK	natural killer
NKG	natural killer cell group
PBS	phosphate-buffered saline
PP	Peyer's patch
rTG2	recombinant transglutaminase 2
SHM	somatic hypermutation
TCR	T cell receptor
TG2	transglutaminase 2
TNF	tumor necrosis factor
T_{reg}	$CD4^{+}CD25^{+}$ regulatory T cell
TRITC	tetramethylrhodamine isothiocyanate
V(D)J	variable, diversity, joining

1 Introduction

Coeliac disease (CD) is an autoimmune-mediated disorder induced by gluten in genetically susceptible individuals (Stamnaes and Sollid 2015). It affects approximately 1 in 100 people in Europe and the United States (Singh et al. 2018). The consumption of gluten induces an abnormal immune response coupled with gastrointestinal symptoms due to structural changes in the intestine (Lindfors et al. 2019). At the moment, the only treatment is gluten-free diet. Therefore, there is a significant need to study CD and find new treatment options.

$CD4^+$ T cells recognize gluten peptides deamidated by the enzyme transglutaminase 2 (TG2), which is located in the small intestine and various other organs (Nurminskaya and Belkin 2012). It has been hypothesized that TG2 and deamidated gluten peptides form complexes, which allow gluten-reactive T cells to provide help to TG2-specific B cells (Mesin et al. 2012). In CD, differentiated TG2-targeting B cells, known as TG2-specific plasma cells, produce anti-TG2 autoantibodies that are predominantly immunoglobulin (Ig) class A and present in serum and various tissues (Stamnaes and Sollid 2015).

According to the current knowledge, the development of B cells takes place in lymphoid tissues, which contain lymphoid follicles (Mesin et al. 2012). In lymphoid follicles, early phase B cells express antigen-specific IgD and IgM on their surface, and upon encountering with an antigen, a differentiation to plasma cells occurs along with a class-switch to IgA (Di Niro et al. 2012). The diagnostic biopsies for CD are normally taken from the duodenum or jejunum, and generally do not contain lymphoid tissues with lymphoid follicles. Therefore, most of the CD-patient material is insufficient for studying B cell development in CD.

There is an abundance of lymphoid tissues that contain lymphoid follicles in the duodenal bulb, which is a small area between the stomach and duodenum (See Figure 2). The CD-researchers at the Tampere Center for Child Health Research have a uniquely large collection of frozen duodenal bulb biopsies from CD-patients and non-CD controls. Therefore, it is now possible for the first time to investigate the development of human TG2-specific B cells in this specific location. The aim of this thesis was to investigate if early phase TG2-specific B cells can be found from the duodenal bulb using immunofluorescence staining. In addition to IgD and IgM, early phase B cells express transcription factor Pax5 and activation-induced cytidine deaminase, which were observed with immunofluorescence by using corresponding antibodies. The focus of this thesis is the immunology of CD, CD-specific antibodies, and the development of CD-specific B cells.

2 Literature Review

2.1 Overview of coeliac disease

Coeliac disease (CD) is a chronic autoimmune-mediated enteropathy elicited by dietary gluten protein in genetically predisposed individuals. CD affects approximately 1% of the populations of Europe and the United States, but the rate varies between countries (Singh et al. 2018). In CD, the ingestion of gluten present in wheat, rye, and barley induces an abnormal immune response, which eventually may lead to the development of crypt hyperplasia and villous atrophy in the small intestine, as well as chronic inflammation in the intestinal epithelium and lamina propria (LP; Stamnaes and Sollid 2015). The small-intestinal damage is paralleled by gastrointestinal symptoms, such as malabsorbtion, abdominal distention, and chronic diarrhea (Stamnaes and Sollid 2015). CD has also been associated with a variety of extra-intestinal manifestations, including skin disease dermatitis herpetiformis, hepatitis, osteoporosis, and infertility (Bonciani et al. 2012; Castillo et al. 2015; Khashan et al. 2010).

The genetic susceptibility to CD is conferred by distinct major histocompatibility complex (MHC) class II alleles coding for human leukocyte antigen (HLA) molecules. Approximately 90% of all CD-patients carry a HLA-DQ2 haplotype and almost all the reminder HLA-DQ8 haplotype (Megiorni and Pizzuti 2012). At the population level, approximately 40% possess these alleles, but only a minority of these individuals acquires CD during their lifetime (Lindfors et al. 2019). CD is common in certain risk groups, including first-degree relatives and patients suffering from autoimmune diseases, such as type 1 diabetes mellitus (Lindfors et al. 2019). The prevalence of CD has increased over the last century, which has been suggested to be contributed by the hygiene-hypothesis (Lohi et al. 2007). The increased percentage of CD may also be explained by improved diagnostic methods, including serological tests and duodenal biopsies. Dietary and environmental factors, such as intestinal microbiota, have also been suggested to contribute to the CD progression (De Re et al. 2017).

At present, the only effective treatment for CD is a life-long gluten-free diet (GFD) in which wheat, rye, and barley are excluded from diet. Strict GFD usually results in recovery of the gastrointestinal mucosa and alleviation of symptoms (Laurikka et al. 2016). Untreated CD is associated with decreased quality-of-life (Ukkola et al. 2011). Dietary treatment increases the quality-of-life, although it may not reach the level of the general population, possibly due to major dietary restrictions (Ukkola et al. 2011). Furthermore, despite GFD, symptoms may sometimes persist and the mucosal recovery may take years (Laurikka et al. 2016).

2.2 Immunology of coeliac disease

Food antigens do not generally induce a systemic immune response, a phenomenon known as oral tolerance which is not well understood in humans (Stamnaes and Sollid 2015). However, it is known that there is a breakage of oral tolerance towards dietary gluten in CD (Stamnaes and Sollid 2015). The gluten induces innate and adaptive immune responses, and it has been suggested that both innate and adaptive immune responses are required for the development of full-blown CD (Stamnaes and Sollid 2015). Next, I will discuss the role of the intestinal immune system, duodenal bulb, antigens, and the induced immune responses in the pathogenesis of CD.

2.2.1 Intestinal immune system

Intestinal epithelium and mucosal layer provide mechanical and chemical protection by forming tight connections between intestinal epithelial cells, producing mucus, and secreting antimicrobial peptides (Konig et al. 2016; Spencer and Sollid 2016). $CD3^+$ small-intestinal intraepithelial lymphocytes (IELs) are located in the intestinal epithelial cell layer and act as a protective mechanism by killing infected or damaged epithelial cells (Lindfors et al. 2019; Stamnaes and Sollid 2015). Approximately 75% of the IELs are composed of $CD8^+$ T cells expressing T cell receptor (TCR) chains $\alpha\beta^+$, 10% $CD4^+$ T cells expressing $TCR\alpha\beta^+$, and 15% either $CD4^+$ or $CD8^+$ T cells expressing $TCR\gamma\delta^+$ (Cukrowska et al. 2017). $CD8^+$ T cells recognize HLA-I-peptide complex, which activates a cell-mediated cytotoxic response, whereas $CD4^+$ T cells recognize HLA-II-peptide complex, which in turns induces a humoral response (Megiorni and Pizzuti 2012). IELs also express both inhibitory and activating natural killer (NK) receptors (Lindfors et al. 2019).

Primary intestinal immune response induction occurs in gut-associated lymphoid tissue (GALT), which is considered as its own immunological compartment, as it differs from the peripheral lymphoid system in several aspects, including the immediate juxtaposition of the mucosal epithelium and lymphoid tissue, and the specialized antigen uptake mechanisms (Mesin et al. 2012). GALT also maintains activated lymphocytes even in the absence of infection. GALT consists of aggregated lymphoid follicles, termed Peyer's patches (PPs), isolated lymphoid follicles (ILFs), and diffuse lymphoid tissue (Mesin et al. 2012). All lymphoid tissue types consist of a three-dimensional framework of reticular fibers, reticular cells, and lymphocytes. PPs are organized structures composed of clustered lymphoid follicles and interfollicular $CD4^+$ T cell zones (Knoop and Newberry 2012). Lymphoid follicles consist of developing B cells, including immature, CD19 B cell lineage differentiation marker

positive B cells that express IgD and IgM with antigen-specific B cell receptors (BCRs; Knoop and Newberry 2012). PPs are covered with microfold cells located in the follicle-associated epithelium (FAE) of GALT and antigen-presenting cells (APCs) in the subepithelial layer, including $CD11c^+$ dendritic cells (DCs; Knoop and Newberry 2012). In addition to PPs, more compactly structured ILFs have been recently discovered in humans (Moghaddami et al. 1998) and in mice (Hamada et al. 2002). ILFs contain a B cell composition similar to the lymphoid follicles of PPs, and they are covered with $CD4^+$ T cells and APCs (Hamada et al. 2002; Knoop and Newberry 2012). However, unlike PPs which remain in mature state throughout life, ILFs range from immature cryptopatches to fully matured structures that contain compensatory mechanisms for humoral responses (Knoop and Newberry 2012). In contrast to the firmly structured PPs and ILFs, diffuse lymphoid tissue is a widely dispersed constituent of lymphatic organs and loosely aggregated cells in LP, particularly in the connective tissue underlining the small intestine (Cesta 2006). The structure and the cell composition of each lymphoid tissue type are illustrated in Figure 1.

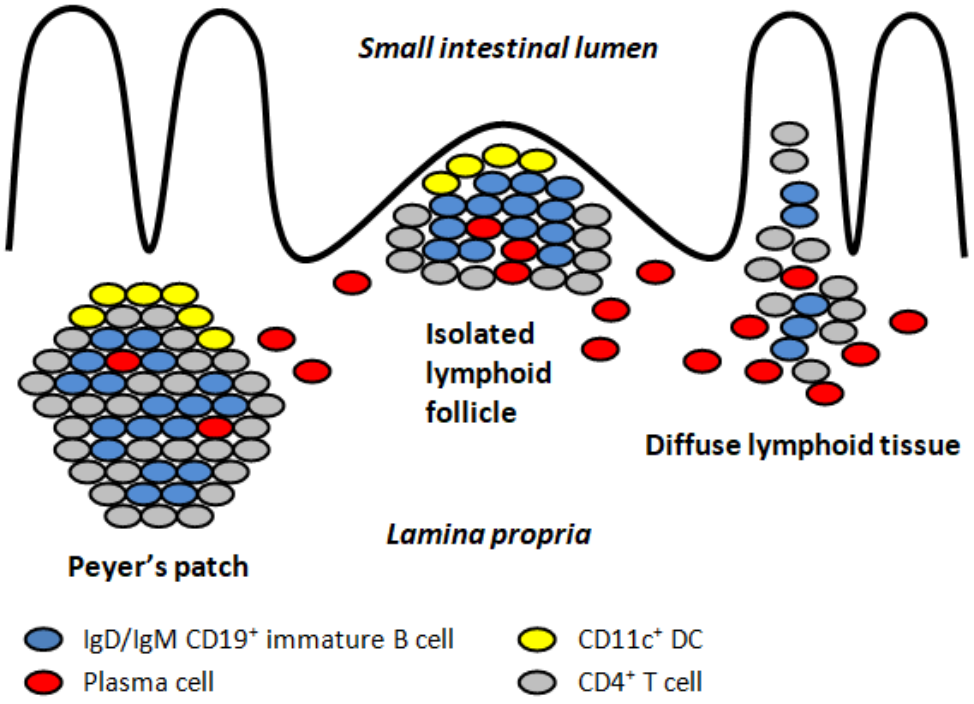

Figure 1. Cell composition of the lymphoid tissues in the small intestine. Peyer's patches, isolated lymphoid follicles, and diffuse lymphoid tissue contain $CD4^+$ T cells and immature $CD19^+$ B cells expressing IgD and/or IgM on their surface. Peyer's patches and isolated lymphoid follicles also contain $CD11c^+$ dendritic cells (DCs). Immature B cells differentiate into plasma cells, which migrate to the intestinal lamina propria. Figure drawn according to Knoop and Newberry 2012.

2.2.2 Duodenal bulb

Duodenal bulb is an approximately five centimeters long area between stomach and duodenum, as seen in Figure 2. It starts at the pylorus and ends at the gall bladder neck. It has a round shape and a smoother surface compared to the duodenum that has longer villi and other folded structures (http://duodenal.org/duodenal-bulb/; 21.9.2018). Duodenal bulb participates in the food digestion process along with the rest of the intestinal tract.

CD-related lesions, such as crypt hyperplasia and villous atrophy, are generally investigated in the distal duodenum and proximal jejunum to confirm the diagnosis for CD (Bonamico et al. 2008; Vogelsang et al. 2001). However, according to Bonamico and co-workers, these characteristic lesions can also be found in duodenal bulb biopsies (Bonamico et al. 2008). They also observed that sometimes the duodenal bulb mucosa was affected, while the rest of the intestinal region showed no lesions. According to McCarty and co-workers, biopsy and histological examination of duodenal bulb in addition to the distal duodenum increases the diagnostic yield of CD (McCarty et al. 2018). This could be explained by the high amount of lymphoid tissues in the duodenal bulb and that the bulb is the first one to be reached by gluten (Bonamico et al. 2008; McCarty et al. 2018; Stoven et al. 2016; Vogelsang et al. 2001). Despite these results, the reliability of duodenal bulb biopsies in diagnosis of CD is uncertain due to common abnormal histological findings in the duodenal bulb area (Stoven et al. 2016; Taavela et al. 2016). Furthermore, the quality of the biopsy specimens from duodenal bulb may vary considerably (Lindfors et al. 2019; Taavela et al. 2016).

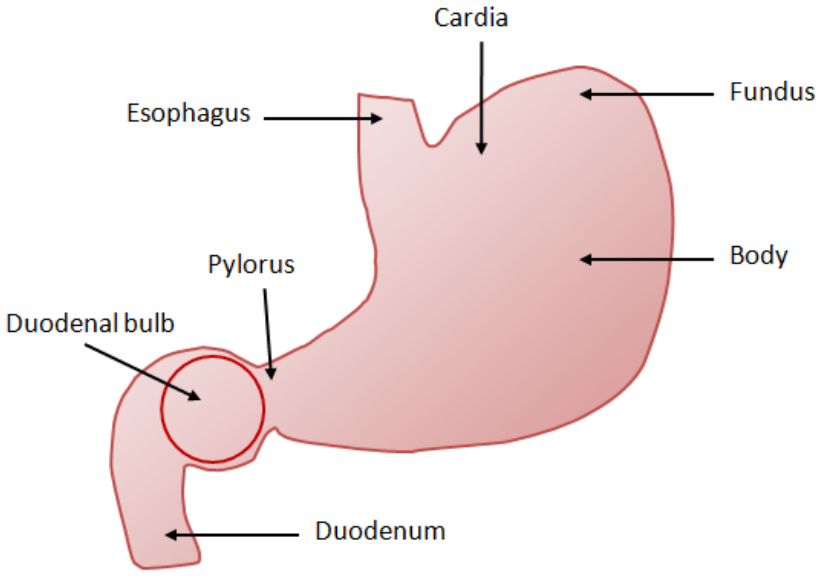

Figure 2. Schematic structure of the gastrointestinal tract from stomach to duodenum. The location of the duodenal bulb is marked with an arrow and a circle.

2.2.3 Gluten and transglutaminase 2 as antigens in coeliac disease

Gluten is a storage protein found in wheat, rye, and barley. It is composed of insoluble prolamine polypeptides, namely gliadins and glutenins in wheat, secalin in rye, and hordein in barley (Castillo et al. 2015). Gluten is highly resistant to proteolytic degradation in the gastrointestinal system due to high proline residue content, resulting in generation of relatively long, immunogenic gluten-derived peptides, which can be exposed to intestinal immune cells (Lindfors et al. 2019). Gliadin fractions are classified into α-, β-, δ-, and ω-types based on their primary structure (Stoven et al. 2012). The α-type is considered most harmful for CD-patients, and it can elicit both innate and adaptive immune responses (Stamnaes and Sollid 2015; Stoven et al. 2012). Gluten-derived peptides can pass through the intestinal epithelium into LP using either transcellular or paracellular transport (Lindfors et al. 2019). The exact transportation mechanism is not known, although several suggested models exist, such as a tight junction disassembly induced by gluten, which increases paracellular permability of the epithelium (Clemente et al. 2003), and transportation using different intracellular trafficking routes (Barone et al. 2010).

Transglutaminase 2 (TG2) is a multifunctional Ca^{2+}-dependent enzyme member of the transglutaminase family. TG2 catalyzes post-translational modification of proteins by transamidation or deamidation, and in addition acts as a GTPase/ATPase, protein disulfide isomerase, and protein kinase (Nurminskaya and Belkin 2012). It is ubiquitously expressed in both the intracellular and the extracellular spaces of various types of tissues and organs, although it is only enzymatically active in the extracellular space for reasons that are not understood (De Re et al. 2017). It is believed that TG2 is associated in the small intestine with fibronectin in the basement membrane supporting the epithelial cell layer (Stamnaes and Sollid 2015). TG2 is involved in many cellular processes, including adhesion, migration, growth, apoptosis, and differentiation (De Re et al. 2017; Meresse et al. 2012; Nurminskaya and Belkin 2012). It contributes to various physiological responses and pathological states, including wound healing, inflammation, autoimmunity, and tumor growth (De Re et al. 2017; Nurminskaya and Belkin 2012).

TG2 has an undisputed role in CD due to its capability to modify gluten post-translationally by deamidation, which changes glutamine residues in specific glutamine-proline rich motifs into negatively charged glutamic acid residues (Molberg et al. 1998). These modified peptides are known as deamidated gliadin peptides (DGPs). DGPs contain epitopes with an increased binding affinity to either HLA-DQ2 or HLA-DQ8, but not to other MHC class II molecules

(Stamnaes and Sollid 2015). These HLA-DQ molecules on APCs present DGPs to $CD4^+$ T cells, which is crucial for the pathogenesis of CD (Bodd et al. 2012; Iversen et al. 2015). TG2 can also cross-link proteins by forming an isopeptide bond between glutamine and lysine. The cross-linking of TG2 and DGPs forms TG2-DGP complexes that would allow DGP-specific $CD4^+$ T cells to present TG2 to TG2-specific B cells (Iversen et al. 2015). However, the mechanism by which TG2 becomes autoantigen in CD is not yet completely understood (De Re et al. 2017; Stamnaes and Sollid 2015).

2.2.4 Innate immune response in coeliac disease

Innate immune response in CD is suggested to be mediated by cytokine interleukin (IL)-15, which is secreted by intestinal epithelial cells and DCs after stimulation by gliadin peptides and other antigens present in wheat (Iacomino et al. 2016; Lindfors et al. 2019). In CD, the number of cells secreting IL-15 is increased in both the LP and the intestinal epithelium (Iacomino et al. 2016). IL-15 participates in the activation and selective expansion of IELs, particularly cells expressing $TCR\gamma\delta^+$ (Meresse et al. 2012). The density of IELs is elevated in CD, and they participate in the intestinal epithelial damage by killing epithelial cells, possibly due to the loss of inhibitory natural killer cell group (NKG) 2A receptors and up-regulation of activating NKG2C receptors (Lindfors et al. 2019; Meresse et al. 2012; Stamnaes and Sollid 2015). IL-15 also increases the expression of NKG2D receptor and its epithelial ligand, major histocompatibility class I polypeptide-related sequence A (MICA), which is located on intestinal epithelial cells (Stamnaes and Sollid 2015). The interaction between NKG2D and MICA induces cytotoxic response in $CD8^+$ $TCR\alpha\beta^+$ IELs at an increased level, which leads to barrier dysfunction and leaking of the epithelium (Cukrowska et al. 2017; Meresse et al. 2012). Other secreted cytokines associated with innate immune response include type I interferons and IL-18 (Lindfors et al. 2019).

The immunoregulatory system has an important role in downregulating the inflammation in the early phase of CD. The number of $CD4^+CD25^+$ regulatory T (T_{reg}) cells is increased already in the early phase of CD, and IL-15 is not able to impair the production of anti-inflammatory interferon (IFN)-γ and transforming growth factor β of these cells (Borrelli et al. 2013; De Re et al. 2017). However, in active CD, IL-15 appears to increase the resistance of T cells against the suppression of T_{reg} cells (Hmida et al. 2012). This may be explained by the higher amount of IL-15 receptor expression on T_{reg} cells in active CD (Borrelli et al. 2013). The basic mechanism of the innate immune response in CD is illustrated in Figure 3.

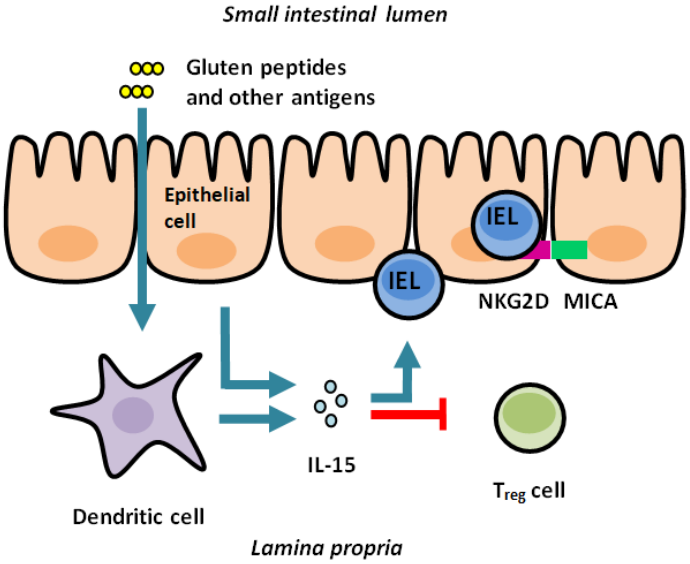

Figure 3. Innate immune response in coeliac disease. The response is initiated by gliadins and other antigens present in wheat. Dendritic cells recognize these molecules and secrete interleukin (IL)-15 along with the intestinal epithelial cells. IL-15 increases the expression of NKG2D receptors on intraepithelial lymphocytes (IELs), which recognize MICA on adjacent intestinal epithelial cells. NKG2D-MICA interactions are increased, leading to enhanced cytotoxic responses of IELs. In addition, IL-15 can inhibit the regulatory effects of regulatory T (T_{reg}) cells. Figure drawn according to Lindfors et al. 2019 and Meresse et al. 2012.

2.2.5 Adaptive immune response in coeliac disease

Adaptive immune response in CD is hallmarked by $CD4^+$ T cell and B cell responses and antibodies produced by CD-specific plasma cells. CD-specific adaptive immune response is induced by DGPs, which are thought to be recognized by HLA-DQ2/DQ8 molecules on the surface of different APCs, including microfold cells and $CD11c^+$ DCs (Mesin et al. 2012; Stamnaes and Sollid 2015). Thereafter, DGP-presenting DCs and other APCs are assumed to migrate to GALT, where they induce the activation of immature $CD4^+$ T cells (Meresse et al. 2012). These DGP-reactive $CD4^+$ T cells acquire a pro-inflammatory phenotype and are thought to provide help to TG2-targeting B cells to differentiate into plasma cells that produce autoantibodies against TG2. $CD4^+$ T cells also recruit NK cells to further stimulate the destruction of the intestinal epithelial cells (Stamnaes and Sollid 2015). DGP-specific $CD4^+$ T cells can form a pool of memory cells that may explain the fast response against gluten upon its reintroduction after GFD (Stamnaes and Sollid 2015). The innate immune cytokine, IL-15, also activates $CD4^+$ T cells, which secrete pro-inflammatory cytokines, such as IFN-γ and IL-21 (Stamnaes and Sollid 2015). IL-15 may also synergize with cytokines produced by $CD4^+$ T cells that stimulates the expansion of cytotoxic $CD8^+$ T cells (Meresse et al. 2012). IL-21 stimulates $CD4^+$ T cells to produce more IFN-γ and IL-15, and it has been suggested that IL-21 connects innate and adaptive immune responses (Sarra et al. 2013).

There are many suggested models that depict the connection between B cells, T cells, and TG2-DGP complexes. These models are based on the ability of TG2-specific B cells to internalize and process TG2-DGP complexes and to release covalently linked DGP T cell epitopes for MHC class II presentation to T cells (Iversen et al. 2015; Stamnaes and Sollid 2015). Therefore, B cells may contribute equally well to the activation of T cells and may act as APCs (Stamnaes and Sollid 2015). TG2-DGP complex can be formed via two ways: either by coupling to the active site cysteine via a thioester-bond or by coupling to surface exposed lysine residues via isopeptide bonds (Di Niro et al. 2012; Stamnaes and Sollid 2015). These covalently linked complexes are unusually stable but short-lived enzyme-substrate intermediates (Stamnaes and Sollid 2015). TG2 can also form TG2-multimers, which could also contain DGP T cell epitopes while retaining recognition by anti-TG2 autoantibodies (Stamnaes and Sollid 2015). The basic mechanism of the adaptive immune response in CD is illustrated in Figure 4.

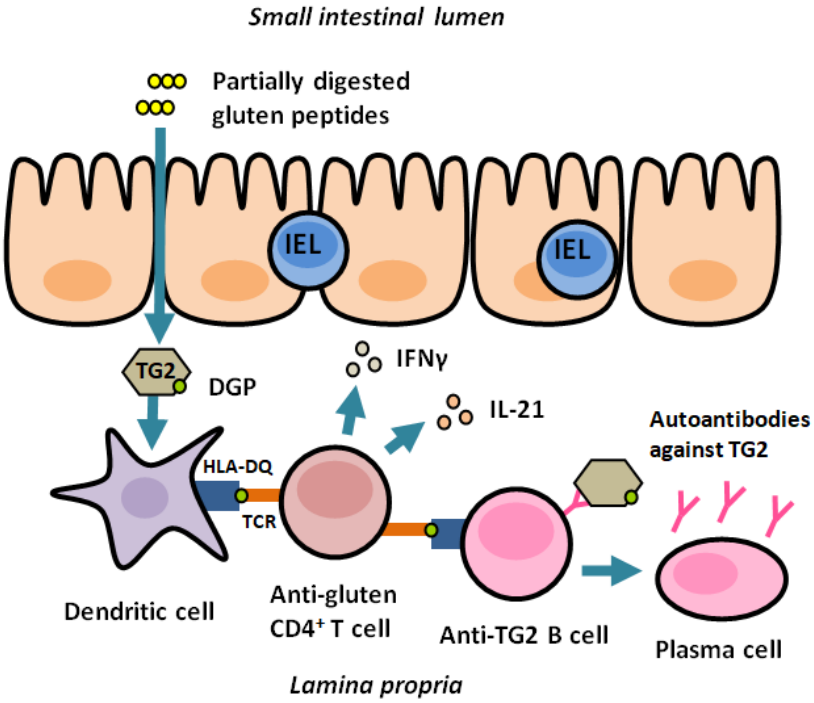

Figure 4. Adaptive immune response in coeliac disease. Gluten-derived peptides are transferred through the intestinal epithelial cell layer to the lamina propria, where their deamidation into deamidated gliadin peptides (DGPs) by transglutaminase 2 (TG2) is thought to occur. Dendritic cells and other antigen-presenting cells recognize DGP and present HLA-DQ-bound DGP to $CD4^+$ T cells, which recognize the molecule via their T cell receptors (TCRs). These DGP-specific $CD4^+$ T cells possibly activate TG2-specific B cells with the help of TG2-DGP complexes. Activated B cells differentiate into TG2-specific plasma cells that produce autoantibodies against TG2. Activated $CD4^+$ T cells also secrete pro-inflammatory cytokines, including interferon (IFN)-γ and interleukin (IL)-21. Figure drawn according to Lindfors et al. 2019, Meresse et al. 2012, and Stamnaes and Sollid 2015.

2.3 Antibodies in coeliac disease

Antibodies against TG2 and DGPs are a characteristic feature of CD, and they appear in very early phases of the disease, possibly months or years before any noticeable morphological changes in the small-intestinal mucosa (Di Niro et al. 2012; Kurppa et al. 2009; Lindfors et al. 2019). CD-associated antibodies were first characterized in the 1970s as anti-reticulin antibodies, which bind to reticulin fibres of the endomysium in rodent connective tissue (Seah et al. 1971). In the 1980s, these antibodies were characterized as native gliadin antibodies and IgA endomysial antibodies (EmA) binding endomysium in the smooth muscle of the monkey esophagus (Chorzelski et al. 1983). The target of EmA was later identified as TG2 (Dieterich et al. 1997). Since then, CD-specific TG2-targeting serum autoantibodies have been used in the diagnostics of CD along with histological evaluation (Mesin et al. 2012). Anti-TG2 autoantibodies are commonly measured by quantitative enzyme-linked immunosorbent assay (Collin et al. 2005; Mesin et al. 2012). Human umbilical cord is used in testing for EmAs from CD-patients' sera (Ladinser et al. 1994). Other diagnostic methods have also been developed, such as a rapid point-of care test for TG2-autoantibodies (Korponay-Szabo et al. 2005; Lindfors et al. 2019). CD-specific anti-TG2 autoantibodies can be observed in cryosected small intestine biopsies using either direct or indirect immunofluorescence. Next, I will discuss the different antibodies associated with CD and their potential role in the disease.

2.3.1 Antibodies associated with coeliac disease

Anti-DGP antibodies are predominantly IgA and IgG, while anti-TG2 autoantibodies are predominantly IgA (Di Niro et al. 2012; Giersiepen et al. 2012; Iversen et al. 2015). Patients who have humoral IgA deficiency produce IgG or IgM autoantibodies against TG2 (Meresse et al. 2012). Approximately 4–24% of IgA-producing intestinal plasma cells are specific for TG2 and 1% for DGP (Di Niro et al. 2016; Iversen et al. 2015; Steinsbo et al. 2014).

Anti-TG2 autoantibodies have been found to be a more specific hallmark of CD compared to anti-DGP antibodies (Iversen et al. 2015). Both anti-DGP antibodies and anti-TG2 autoantibodies are present in the circulation, and anti-TG2 autoantibodies are also found as deposits in the basement membrane of the small-intestinal mucosa along the villous and crypt epithelium and around blood vessels (Korponay-Szabo et al. 2004; Mesin et al. 2012; Stamnaes and Sollid 2015). These deposits are highly specific for CD, and they seem to precede and predict the histological changes (Salmi et al. 2006; Tosco et al. 2008). Anti-TG2 autoantibodies have also extra-intestinal target sites in other organs, such as liver, muscles, and kidney (Korponay-Szabo et al. 2004).

IgA is locally secreted in the LP of the small intestine, where it binds to polymeric Ig receptor and is actively transported into the luminal region (Spencer and Sollid 2016). IgA is mostly dimeric in the intestinal area and consists of IgA1 and IgA2 subclasses, whereas in serum IgA is mostly in monomeric form (Spencer and Sollid 2016).

In normal small-intestinal mucosa, IgA antibodies can be detected inside the plasma and epithelial cells, but they do not target TG2 (Hietikko et al. 2018). In CD, there exists TG2-specific plasma cells in the small-intestinal mucosa that secrete IgA autoantibodies against TG2, and data by Di Niro and co-workers indicates that TG2-specific autoantibodies located in serum are secreted by plasma cells that are clonally related to intestinal TG2-specific plasma cells but reside outside the small intestine (Di Niro et al. 2016).

Several other autoantibodies have been found in CD-patients, such as autoantibodies against actin (Clemente et al. 2000), calreticulin (Tuckova et al. 1997), desmin (Teesalu et al. 2001), and transglutaminase 3 (Salmi et al. 2016). The role of these autoantibodies in the pathogenesis and diagnosis of CD is still unknown. However, autoantibodies targeting transglutaminase 3 have been associated with dermatitis herpetiformis (Salmi et al. 2016).

2.3.2 Role of antibodies in coeliac disease

The exact role of anti-TG2 and anti-DGP antibodies in the pathogenesis of CD is still unclear (Stamnaes and Sollid 2015). Secretory CD-specific IgA is suggested to mediate the transcytosis of gluten-derived peptides through the intestinal epithelium by binding to a transferrin receptor CD71, which is overexpressed in CD-patients (Lebreton et al. 2012; Matysiak-Budnik et al. 2008; Rauhavirta et al. 2011). It has been shown that TG2-targeting IgD, and to a minor extent IgM, expressed on B cell surface can become cross-linked by TG2 and form a multimerized receptor that may promote the activation of TG2-specific B cells (Di Niro et al. 2012; Giersiepen et al. 2012; Iversen et al. 2015). It has also been suggested that TG2-specific autoantibodies may affect the function and activity of TG2, but thus far, the results have been contradictory (Byrne et al. 2010; Myrsky et al. 2009). On the other hand, the role of TG2-specific B cells as potential APCs for gluten-reactive T cells may suggest that the B cells participate in the pathogenesis of CD by amplifying the anti-gluten T cell response rather than the antibodies themselves (Stamnaes and Sollid 2015).

2.4 Development of B cells in coeliac disease

The generation of TG2-targeting B cells and plasma cells in GALT has a major role in CD, as TG2 is considered the main autoantigen of CD (Mesin et al. 2012). However, a high number of studies on the development of B cells in the GALT are based on mice, and therefore most of the current knowledge may not be applicable when comparing to human B cell development (Knoop and Newberry 2012).

In mice, PPs have been considered as the primary induction site of mucosal IgA-producing B cells (Knoop and Newberry 2012). However, recent studies have demonstrated that murine ILFs can also induce B cell maturation and IgA response (Knoop and Newberry 2012). Next, I will discuss the basic development of human B cells, the development and role of ILFs, and the development of TG2-specific B cells in CD.

2.4.1 Development of B cells

The development of B cells is based on the formation of antigen-recognizing BCRs. These receptors contain an Ig superfamily structure, which forms the recognition site against an antigen (Rothenberg 2014). The Ig structure is made of two identical Ig heavy chains and two identical Ig light chains that are assembled into disulfide-bonded heterodimers (Rothenberg 2014). A single gene encodes the Ig heavy chain, whereas two different genes, Igκ and Igλ, encode the Ig light chain (Rothenberg 2014). Each of the three gene loci contains a variable and a constant region. The variable region consists of variable (V), diversity (D), and joining (J) segments. Ig heavy chain contains V_H, D_H, and L_H segments, whereas Ig light chain contains V_L and J_L segments.

The development of B cells initiates in the bone marrow, which contains pluripotent hematopoietic stem cells (HSCs). These cells differentiate first into multipotent progenitors, which then give rise to common lymphoid progenitors (CLPs). The activation of B cell developmental pathway is induced by transcription factors E box binding protein 2A (E2A) and early B cell factor 1 (EBF1; Ramirez et al. 2010; Rothenberg 2014). After the initiation of differentiation, E2A and EBF1 activate transcription factors Ikaros and Pax5 in pro-B cells that induce the beginning of genetic recombination and expression of proteins required for the display of pre- and mature BCRs (Ramirez et al. 2010; Rothenberg 2014). In addition, pro-B cells activate the expression of CD19 (Rothenberg 2014). Pax5 is considered as the commitment factor that maintains the B cell identity by activating B cell-specific genes and repressing genes associated with other cell lineages (Medvedovic et al. 2011).

Genes encoding BCR are assembled in a somatic gene rearrangement, known as the V(D)J recombination, in which VDJ-segments are joined through successive rearrangements (He et al. 2007). The V(D)J recombination is mediated by proteins RAG1 and RAG2 (Rothenberg 2014). According to the current knowledge, the expression of both RAG1 and RAG2 is limited to B cell and T cell lineages (Rothenberg 2014). The rearrangement initiates on genes that encode the Ig heavy chain (Rothenberg 2014). The progression of the development depends the success of the rearrangement, which is verified in the primary development checkpoint during the transition from pro-B cell stage to pre-B cell stage (Rothenberg 2014). After successful verification, the pre-B cells rearrange the gene segments coding for the Igκ or Igλ light chain genes (Rothenberg 2014). The V(D)J rearrangement produces surface IgM positive mature naïve B cells with a large antibody repertoire (Chen et al. 2009). After the BCRs are maturated, mature naïve B cells migrate from the bone marrow to colonize secondary lymphoid organs, including the GALT (Ramirez et al. 2010). In the GALT, B cells acquire surface IgD of the same specificity as IgM by going through alternative splicing of the Ig heavy chain constant region (Chen et al. 2009).

Mature naïve B cells localize to the different lymphoid tissue structures that contain lymphoid follicles. After encountering an antigen, mature naïve B cells encounter $CD4^+$ T cells at the border of the lymphoid follicle and the interfollicular $CD4^+$ T cell zone for cognate interaction and undergo a secondary diversification, known as somatic hypermutation (SHM), in which amino acid sequence in the variable regions of the Ig is altered to enhance the binding affinity and specificity of the BCR and antibodies (Zan and Casali 2013). SHM forms an expansion of affinity-matured mutant B cell clones with high-affinity antibodies.

Constant segments that are proximal to the variable region can be deleted through a mechanism known as class-switch recombination (CSR), which generates IgA, IgG, and IgE subtypes with the same antigen binding specificity as IgM (Chen et al. 2009; Zan and Casali 2013). CSR occurs through either a T cell-dependent follicular pathway or T cell-independent extra-follicular pathway. T cell-dependent pathway is mediated by antigen-activated $CD4^+$ T cells located in the $CD4^+$ T cell zone. These cells express tumor necrosis factor (TNF) superfamily member CD40L, which promotes CSR by binding to the CD40 receptor on B cells (Chen et al. 2009; Knoop and Newberry 2012). This also increases the expression of enzyme activation-induced cytidine deaminase (AID), which promotes both CSR and SHM of the Ig genes (Knoop and Newberry 2012; Mesin et al. 2012). T cell-independent pathway is mediated by antigen-activated DCs, macrophages, and intestinal epithelial cells. These cells

secrete TNF superfamily members B cell activating factor and a proliferation-inducing ligand, which bind to their corresponding receptors on B cells (Chen et al. 2009; Spencer and Sollid 2016). B cells that undergo T cell-dependent pathway are associated with extensive SHM, whereas B cells expanding in T cell-independent pathway acquire fewer mutations (Mesin et al. 2012).

B cells undergo SHM and CSR in germinal centers that are associated with the T cell-dependent pathway (Mesin et al. 2012). Germinal centers are located in the lymphoid follicles of PPs and ILFs (Mesin et al. 2012). B cells in germinal centers are surrounded by other cells that support the clonal expansion and selection of the B cells, including follicle DCs, follicular T cells, and macrophages (Spencer and Sollid 2016). Germinal centers are surrounded by a mantle zone consisting of $CD27^{-}IgM^{+}IgD^{+}$ mature naïve B cells (Spencer and Sollid 2016). The mantle zone is surrounded by a marginal zone consisting of B cells which do not express IgD (Spencer and Sollid 2016). Each germinal center appears to represent a clone of cells derived from one antigen-stimulated lymphocyte (Mesin et al. 2012).

GALT germinal center initiation requires CD40-mediated signal from T cells, although germinal center -associated B cell and IgA responses may not always require cognate T cell and B cell interactions (Mesin et al. 2012). In fact, it has been observed that germinal centers can form in PPs based on BCR specificity without requiring interaction between T cells and B cells (Mesin et al. 2012). Furthermore, it has been suggested that germinal centers are not essential for antibody formation and may be involved in long-term antibody responses (Spencer and Sollid 2016).

After CSR, mature naïve B cells differentiate into plasmablasts (Spencer and Sollid 2016). During the differentiation, the expression of EBF1 and Pax5 is silenced (Rothenberg 2014). Plasmablasts produce less antibodies than plasma cells and are characterized by the expression of proliferation marker Ki-67, which is devoid in the plasma cells (Mesin et al. 2012). Plasmablasts can enter the blood through the lymphatic system and home back to the intestine, where they finally maturate into plasma cells and are guided to the intestinal LP by chemokines from intestinal epithelial cells (He et al. 2007; Spencer and Sollid 2016). The main phases of the human intestinal B cell development are illustrated in Figure 5.

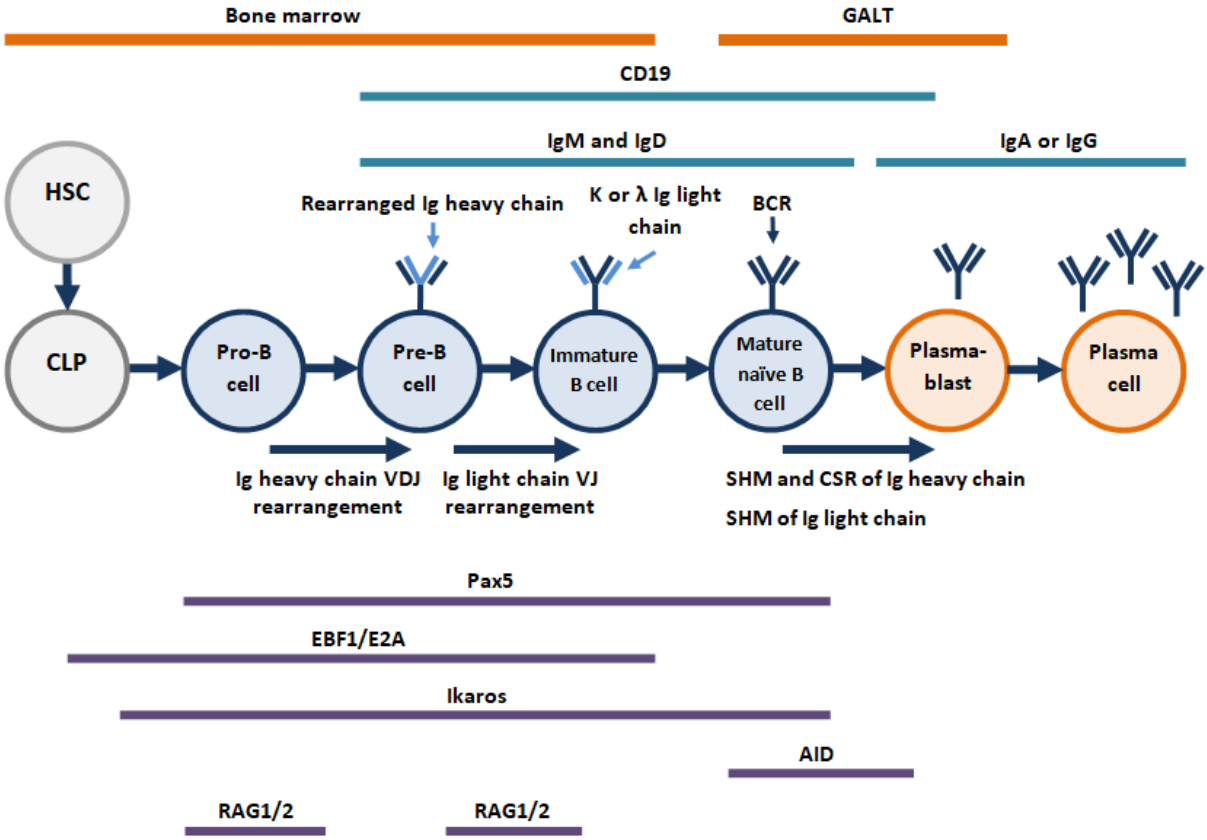

Figure 5. Developmental pathway of human intestinal B cells. The main phases of the B cell development, the expression of stage-specific factors, and the location of the development stages are illustrated in this figure. Hematopoietic stem cells (HSCs) in the bone marrow first differentiate into common lymphoid progenitors (CLPs), which give rise to B cell lineage due to expression of early B cell factor 1 (EBF1), E box binding protein 2A (E2A), Pax5, and Ikaros. $CD19^+$ progenitor B cells undergo VDJ-recombination mediated by RAG1 and RAG2, in which Ig heavy and Ig light chains are rearranged and the B cell receptor (BCR) is assembled as IgM and IgD. Mature naïve B cells migrate to GALT and undergo somatic hypermutation (SHM) and class-switch recombination (CSR) that are initiated by activation-induced cytidine deaminase (AID). B cells differentiate into IgA^+ or IgG^+ plasmablasts which migrate to blood. After homing back to GALT, plasmablasts mature into plasma cells that produce antibodies of the same Ig class and specificity of the BCR. Figure drawn according to Ramirez et al. 2010, Rothenberg 2014, and Spencer and Sollid 2016.

Plasma cells are non-proliferating cells that serve as antibody-secreting cells in the bone marrow or mucosal tissue, including the small intestine (Spencer and Sollid 2016). LP of the small intestine contains terminally differentiated plasma cells, which are maintained by the local supportive microenvironment (Mesin et al. 2012). Plasma cells are localized throughout the LP, although most of the cells occupy the diffuse subepithelial connective tissue (Spencer and Sollid 2016). The intestinal LP also contains macrophages, DCs, and neutrophils, which may participate in the plasma cell production (Mesin et al. 2012). In mice, DCs produce TNF superfamily members that promote IgA production in B cells expressing complementary receptors (Knoop and Newberry 2012).

Plasma cells are characterized by the cell markers CD138 and CD27, whereas the expression of CD19 and CD45 is more heterogeneous (Mesin et al. 2012). Intestinal plasma cell repertoire consists of both unmutated IgA antibodies and somatically mutated IgA antibodies (Mesin et al. 2012). Mature plasma cells cannot switch antibody classes (Spencer and Sollid 2016). According to Spencer and Sollid, plasma cells in the small intestine and serum express lower levels of surface IgA than corresponding memory B cells (Spencer and Sollid 2016).

2.4.2 Development and function of isolated lymphoid follicles

The function and development of ILFs in humans is not well understood. However, there have been many studies on murine ILFs. Therefore, most of the current knowledge about ILFs is based on mice, which may differ significantly from human ILFs.

ILFs are compact structures filled with closely packed lymphocytes that appear postnatally throughout the small intestine, ranging from early phase cryptopatches to fully matured ILFs (Buettner and Lochner 2016). In mice, ILFs are located at the base of a crypt or in villi that are thicker and shorter in a longitudinal section than the surrounding normal villi (Hamada et al. 2002; Lorenz and Newberry 2004). Similarly to PPs, ILFs contain a large B cell area with a germinal center, covered with FAE and $CD4^+$ T cells (Mesin et al. 2012). However, unlike PPs, ILFs lack distinct interfollicular T cell zones (Wang et al. 2006). Murine ILFs contain mostly immature $CD19^+$ B cells that have not yet gone through CSR (Knoop and Newberry 2012). In mice, most of the B cells display $B220^+CD19^+CD23^+IgM^{low}IgD^{high}CD5^-Mac\text{-}1^-$ phenotype that express stem cell factor c-kit, and a smaller subset express IgA^+ (Hamada et al. 2002; Pabst et al. 2005).

Because the structure of ILFs shares similarities with PPs, ILFs are also assumed as inductive sites for intestinal immune reactions (Pabst et al. 2005). According to Tsuji and co-workers, ILFs contribute significantly to IgA synthesis in the small intestine of mice (Tsuji et al. 2008). Unlike to T cell-dependent IgA response in the lymphoid follicles of PPs, the activation of B cells and induction of AID for IgA CSR in the murine ILFs may not require T cell interactions (Tsuji et al. 2008). Instead, the activation of B cells appears to be mediated only by ILF DCs, and many AID-expressing B cells are located close to these DCs (Tsuji et al. 2008).

Cryptopatches are aggregates of lymphoid tissue inducer (LTi) cells and lymphoid tissue organizer (LTo) cells that act as precursors to ILFs (Knoop and Newberry 2012; Nochi et al. 2013). In mice, intestinal epithelial cells produce IL-7 that binds to the IL-7 receptor on LTi

cells. This induces the expression of lymphotoxin $\alpha_1\beta_2$ that binds to the lymphotoxin β receptor on LTo cells and up-regulates the expression of various chemokines and adhesion molecules that participate in the recruitment and retention of lymphocytes into cryptopatches (Hamada et al. 2002; Nochi et al. 2013). Cryptopatches can develop into ILFs in response to signal from the commensal microbiota (Knoop and Newberry 2012). In mice, the amount of cryptopatches can outnumber ILFs (Knoop and Newberry 2012). The genesis and function of cryptopatches in human GALT is not well understood (Nochi et al. 2013). However, in a study performed by Nochi and co-workers, they found out that murine cryptopatches can initiate human GALT genesis (Nochi et al. 2013).

According to the current knowledge, *de novo* development of murine cryptopatches does not occur throughout adulthood (Knoop and Newberry 2012). Instead, cryptopatches may develop into ILFs throughout adulthood as an immune response (Knoop and Newberry 2012). In fact, the development and function of ILFs is considered to be more dynamic than of PPs because the amount, size, and cellular composition of ILFs can highly vary, depending on the antigen load in the intestine (Knoop and Newberry 2012; Tsuji et al. 2008). Pabst and co-workers found out that in mice, the size, location, and cellular composition of lymphoid aggregations are heterogeneous and that most lymphoid structures cannot be classified neither as ILF or cryptopatch (Pabst et al. 2005). According to their study, the size and formation of aggregated lymphoid structures depends on the strain and age of studied animals (Pabst et al. 2005). Therefore, comparing data obtained from different species at different ages can be difficult, as the developmental speed varies between species (Pabst et al. 2005).

2.4.3 Current concept of the development of transglutaminase 2-specific B cells

TG2-specific plasma cells represent 5–25% of all plasma cells in the coeliac lesion with a limited number of clones, which suggests a huge clonal expansion in the small intestine (Stamnaes and Sollid 2015). The reason why intestinal TG2-specific plasma cells have fewer mutations than other plasma cells in the intestinal LP is not known. The low mutation rate could be related to T cells that provide help to TG2-specific B cells via TG2-DGP complexes (Spencer and Sollid 2016). According to Mesin and co-workers, intestinal anti-TG2 autoantibody repertoire has a very limited number of somatic mutations in the Ig heavy chain encoded by VH5 gene, which could be related to the structural properties of the VH5 region (Mesin et al. 2012). In addition, all VH5 antibodies do not bind to TG2, which shows that the anti-TG2 reactivity does not depend on unspecific binding of the VH5 region (Di Niro et al. 2012).

Besides the uniquely limited number of mutations, it is not well understood where the development of TG2-specific B cells takes place, and whether it is T cell-dependent or T cell-independent (Mesin et al. 2012). It is assumed that in mice, the IgA responses generated in lymphoid follicles require the T cell-dependent pathway, whereas extra-follicular IgA responses are T cell-independent (Tsuji et al. 2008). T cell-dependent pathway in germinal centers commonly results in long-lived plasma cells that sustain antigen-specific response for decades (Iversen et al. 2015). However, intestinal TG2-specific plasma cells have been observed to disappear within a month after patients commence GFD, which indicates that intestinal TG2-specific plasma cells are not long-lived (Di Niro et al. 2016). Furthermore, SHM and AID-mediated CSR have been observed to occur outside of germinal centers in both mice and humans, suggesting that intestinal immature B cells can develop directly in the LP (Di Niro et al. 2016; He et al. 2007; Mesin et al. 2012). Therefore, it has been suggested that TG2-specific B cells may develop at extra-follicular sites without germinal center reactions (Iversen et al. 2015; Mesin et al. 2012). This theory is further supported by the scarce SHM, which suggests that the development either occurs via T cell-independent pathway without germinal center formation, or the cells undergo limited rounds of selection in a germinal center (Mesin et al. 2012). In a study performed by Di Niro and co-workers, they could not detect AID in TG2 positive plasmablasts in the small intestine, which suggests that TG2-specific B cells may undergo CSR outside the small-intestinal mucosa (Di Niro et al. 2012).

Even if the TG2-specific B cells would develop at extra-follicular sites, T cells could still be involved in the response, as the clinical observation of anti-TG2 autoantibodies is strictly gluten- and HLA-dependent (Di Niro et al. 2012). This is further supported by Di Niro and co-workers who observed that B cells engineered to express an anti-TG2 BCR could process and present TG2-DGP complexes, resulting in activation of gluten-specific $CD4^+$ T cells which in turn activate the B cells (Di Niro et al. 2012).

Most of the studies on the development of human TG2-specific B cells have been based on isolated TG2-specific plasma cells derived from CD-patients' small intestine, which is insufficient for studying the earlier phases of B cell development as majority of the development occurs in GALT (Spencer and Sollid 2016). Access to GALT structures of CD-patients would help to dissect this, but so far this has been restricted due to ethical aspects and methodological limitations (Spencer and Sollid 2016).

3 Objectives

The aim of this thesis was to investigate the presence of early phase TG2-specific B cells in the duodenal bulb and to determine whether they are located in the lymphoid follicles or at extra-follicular sites. The first phase was to screen cryosected duodenal bulb biopsy specimens from untreated CD-patients and non-CD controls to identify specimens containing ILFs or other lymphoid tissues. The second phase was to detect early phase TG2-specific B cells using both direct and indirect immunofluorescence staining and co-localization with TG2 and antibodies against immature B cell markers.

4 Materials and Methods

4.1 Screening of duodenal bulb biopsies

The first phase of the study was to screen 211 duodenal bulb biopsy specimens from Finnish children and 268 specimens from Romanian children and adults to identify specimens with potential ILFs, PPs, or diffuse lymphoid tissue. The specimens had been collected from both untreated CD-patients and healthy individuals. Finnish and Romanian specimens were used due to accessibility. The specimens consisted of cryosected, 5-µm-thick sections stained using immunohistochemistry to evaluate CD latency. Each specimen consisted of three slides, and each slide contained three identically stained sections. The sections had been stained using monoclonal antibodies against TCR$\alpha\beta^+$, TCR$\gamma\delta^+$, or HLA-DR. The screening was performed blinded to prevent possible bias. Specimens were observed using Olympus BX60 microscope (Olympus, Tokyo, Japan) and images were acquired using Olympus Colorview camera and edited with CellD image software (Olympus Soft Imaging Solutions GmbH, Muenster, Germany).

A total of 16 specimens with most potential lymphoid tissue structures were selected for the immunofluorescence staining optimization. New 5-µm-thick sections were cut from the original biopsy specimens, and three sections were layered on each slide. The slides were stored at –20°C until they were stained.

4.2 Immunofluorescence staining of duodenal bulb biopsies

Both direct and indirect immunofluorescence staining methods were based mainly on protocols by Di Niro and co-workers, and Hietikko and co-workers (Di Niro et al. 2012; Hietikko et al. 2018). First, the protocol was optimized for the blocking, washing, and incubation phases of the recombinant TG2 (rTG2) and direct IgA immunofluorescence stainings by using the selected duodenal bulb biopsies and additional small intestine biopsies. The entire protocol was performed at room temperature. In the optimized protocol, the slides were first air-dried for 20 min and then fixed in 96% alcohol – acetone for 3 min, as it is commonly used for cryosected tissue sections (Dominguez-Sola and Cattoretti 2017). After the fixation, the slides were air-dried for 2 min and washed with phosphate-buffered saline (PBS) for 3 × 2 min. The slides were blocked to prevent unspecific binding by using 5% milk powder – 5% bovine serum albumin (BSA) – 5% normal goat serum 30 µl/section for 30 min in a moisturized chamber. For rTG2 staining, the sections were incubated with biotinylated human rTG2 (1:100; T002, Zedira, Darmstadt, Germany) 30 µl/section for 60 min in a

moisturized chamber. To detect the rTG2, the sections were incubated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated streptavidin (1:100; 43-4314, Invitrogen, Paisley, UK) 30 µl/section for 30 min in a moisturized chamber while protected from light. For direct IgA stainings, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human anti-IgA antibody (1:40; F0204, Dako A/S, Glostrup, Denmark) or TRITC-conjugated goat anti-human anti-IgA antibody (1:40; A18786, Thermo Fisher Scientific, Waltham, USA) 30 µl/section for 60 min in a moisturized chamber. TRITC-conjugated streptavidin was used as a secondary control for rTG2 and IgA-TRITC. For IgA-FITC, no secondary control was available. All dilutions were made in 5% milk powder – 5% BSA. The slides were washed with 0.05% TWEEN – PBS for 3 × 5 min between and after the incubations. Finally, the slides were mounted with 4',6-diamidino-2-phenylindole (DAPI)-containing VectaShield Mounting Medium (H-1200, Vector Laboratories, Burlingame, USA) and a cover glass. DAPI was used to counterstain nuclei. Stained slides were stored at +4°C for shorter periods and at –20°C for longer periods while protected from light.

Indirect immunofluorescence staining was based on the optimized protocol. After the blocking phase, the sections were incubated with primary antibody dilutions 30 µl/section for 60 min in a moisturized chamber. In secondary controls, 30 µl 5% milk powder – 5% BSA was used for each section in the primary incubation. After the primary incubation, the sections were incubated with appropriate secondary antibody dilutions 30 µl/section for 30 min in a moisturized chamber while protected from light. All used antibodies were diluted in 5% milk powder – 5% BSA. A description of the primary and secondary antibodies used in this study is provided in Table 1 and Table 2. The slides were washed with 0.05% TWEEN – PBS for 3 × 5 min between and after the incubations. The rest of the protocol was the same as in the optimized rTG2 and IgA immunofluorescence stainings.

Table 1. Primary anti-human antibody dilutions used in the indirect immunofluorescence staining.

Antibody	Host species	Dilution	Catalogue	Manufacturer
Anti-IgD	Rabbit	1:25	ab195581	Abcam, Cambridge, UK
Anti-IgM	Mouse	1:25	9020-01	Southern Biotech, Birmingham, USA
Anti-AID	Rat	1:100	#4959	Cell Signaling Technology, Danvers, USA
Anti-Pax5	Mouse	1:250	SC-13146	Santa Cruz Biotechnology, Dallas, USA

AID: activation-induced cytidine deaminase

Table 2. Secondary antibody dilutions used in the indirect immunofluorescence staining.

Antibody	Host species	Dilution	Catalogue	Manufacturer
Alexa Fluor 488 anti-rabbit	Goat	1:500	A11034	Life Technologies, Carlsbad, USA
Alexa Fluor 568 anti-rabbit	Goat	1:500	A11036	Life Technologies
Alexa Fluor 488 anti-mouse	Goat	1:500	A11001	Invitrogen
Alexa Fluor 488 anti-rat	Donkey	1:500	A21208	Invitrogen
Alexa Fluor 568 anti-rat	Donkey	1:500	ab175475	Abcam

After the optimization phase, four specimens from untreated CD-patients and three specimens from non-CD controls were selected for the final immunofluorescence staining. Fixation was not included in the final staining because it may affect the three-dimensional structure of the epitopes in the specimens, and therefore decrease the quality of the staining. The final staining material consisted of rTG2 to detect TG2-specific B cells and plasma cells, antibodies against IgA to detect IgA-specific B cells and plasma cells, antibodies against IgD and IgM to detect early phase B cells, and antibodies against AID and Pax5 to detect developing B cells. All used staining combinations for the co-localizations are described in Table 3.

Table 3. Immunofluorescence double staining combinations and the target of the co-localization. The emission wavelength (488 nm = green, 568 nm = red) of TRITC, FITC, and the secondary antibodies is described for each primary antibody.

Combination	Target
rTG2 (568) + IgA-FITC (488)	Mature naïve TG2-specific B cells, plasmablasts, or plasma cells
rTG2 (568) + IgD (488)	Early phase TG2-specific B cells before class-switch
rTG2 (568) + IgM (488)	Early phase TG2-specific B cells before class-switch
rTG2 (568) + AID (488)	TG2-specific B cells with ongoing IgA class-switch
rTG2 (568) + Pax5 (488)	TG2-specific B cells before transition into plasma cells
IgA-TRITC (568) + AID (488)	B cells with ongoing IgA class-switch
IgA-TRITC (568) + Pax5 (488)	IgA-specific B cells before transition into plasma cells
IgD (488) + AID (568)	B cells with ongoing class-switch
IgD (568) + Pax5 (488)	Early phase B cells

AID: activation-induced cytidine deaminase; FITC: fluorescein isothiocyanate; rTG2: recombinant transglutaminase 2; TRITC: tetramethylrhodamine isothiocyanate

4.3 Fluorescence microscopy of duodenal bulb biopsies

During the immunofluorescence staining optimization phase, the specimens were observed with widefield fluorescence using Olympus BX60 microscope or Olympus IX51 Fluorescence Microscope. Images were acquired using Olympus BX60 microscope with Olympus Colorview camera and CellD image software, or Olympus IX51 microscope with Olympus DP30BW camera and DP manager software (Olympus). For the final staining, only Olympus IX51 microscope was used. The same exposure time was used for all specimens of each staining combination. All images were organized using ImageJ software (Rasband WS, ImageJ, National Institute of Mental Health, Bethesda, USA; Schneider et al. 2012).

4.4 Ethical considerations

The material of this study consisted of duodenal bulb and small intestine biopsy cryosections from prospectively collected cohorts. The specimens were collected from healthy and CD-affected Finnish and Romanian individuals. The collection and use of Finnish specimens was approved by Ethics Committee of the Pirkanmaa Hospital District (ethical approval R11187), and the collection and use of Romanian specimens was approved by University of Medicine and Pharmacy Carol Davila and National Institute for Mother and Child Health (ethical approvals 20534.2011 and 8971.2017). The specimens were kindly provided by acting professor and pediatric gastroenterologist Kalle Kurppa, and pediatric gastroenterologist Alina Popp.

5 Results

5.1 Identification of lymphoid structures in duodenal bulb biopsies

The study comprised 479 screened cryosected duodenal bulb biopsy specimens. Out of all the screened specimens, 67 (14.0%) contained structures resembling ILFs, 13 (2.70%) contained structures resembling PPs, 62 (12.9%) contained structures resembling diffuse lymphoid tissue, and the reminder 337 (70.4%) did not contain any recognizable lymphoid structures. The specimens that contained lymphoid tissue structures were evaluated, and the 16 most high-quality specimens were selected for the immunofluorescence staining optimization. For the final staining, seven pediatric specimens were selected, which included three specimens from Finnish untreated CD-patients, two specimens from non-CD Finnish individuals, one specimen from a Romanian untreated CD-patient, and one specimen from a non-CD Romanian individual. More detailed information is provided in Table 4 and Table 5. Every specimen included a structure resembling ILF, PP, or diffuse lymphoid tissue, which are shown in Figure 6 and Figure 7.

Table 4. Selected untreated coeliac disease patient duodenal bulb biopsy specimens for the immunofluorescence staining. Nationality and the found lymphoid tissue structures are described for each coeliac disease (CD)-patient. In addition, the diagnostic information has been provided, including endomysial antibody (EmA) dilutions and anti-transglutaminase 2 (TG2) autoantibody values from serum samples, and the number of CD-associated cells and IgA deposits in duodenal bulb biopsies. EmA dilution 1:≥5 is considered positive. The cut-off value for TG2 is >5 AU/ml. The reference values are 37 cells/mm for $CD3^+$ intraepithelial lymphocytes (IELs) and 4.3 cells/mm for T cell receptor (TCR)-$\gamma\delta^+$ IELs. IgA deposits were graded from 0 (negative) to 3 (high). All reference values were from Hietikko and co-workers (Hietikko et al. 2018).

Untreated CD-patient	Nationality	EmA dilution	TG2 (AU/ml)	Cells/mm	IgA deposits	Found structures
1	Finnish	1:100	22	N/A	N/A	Isolated lymphoid follicle or Peyer's patch
2	Finnish	1:500	112	$CD3^+$ 65 TCR$\gamma\delta^+$ 18	>3	Isolated lymphoid follicle or diffuse lymphoid tissue
3	Finnish	1:50	15	$CD3^+$ 106 TCR$\gamma\delta^+$ 42	>3	Diffuse lymphoid tissue
4	Romanian	N/A	N/A	N/A	N/A	Isolated lymphoid follicle or diffuse lymphoid tissue

N/A: data not available

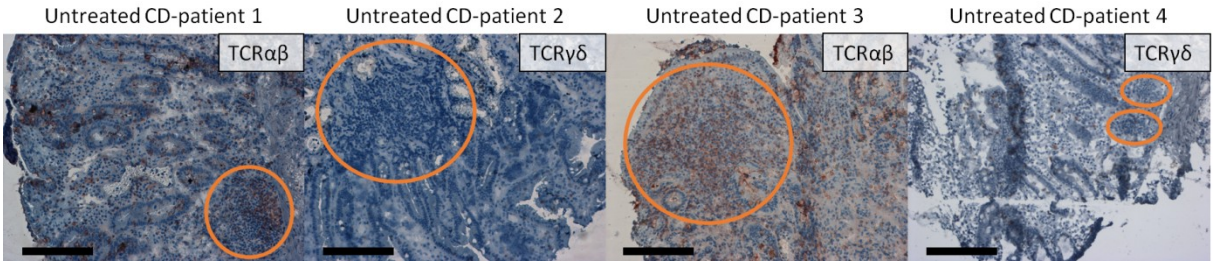

Figure 6. Brightfield images of the selected untreated coeliac disease patient duodenal bulb biopsy specimens. The specimens had been stained using immunohistochemistry to detect T cell receptor (TCR)-$\alpha\beta^+$ or TCR$\gamma\delta^+$ T cells. Structures that resemble lymphoid tissue are marked with a circle. Images were acquired using Olympus BX60 microscope with magnification ×10. Scale bar = 200 µm. CD: coeliac disease

Table 5. Selected non-coeliac control duodenal bulb biopsy specimens for the immunofluorescence staining. Nationality and the found lymphoid tissue structures are described for each control. In addition, the diagnostic information has been provided, including endomysial antibody (EmA) dilutions and anti-transglutaminase 2 (TG2) autoantibody values from serum samples, and the number of CD-associated cells and IgA deposits in duodenal bulb biopsies. EmA dilution 1:≥5 is considered positive. The cut-off value for TG2 is >5 AU/ml. The reference values are 37 cells/mm for $CD3^+$ intraepithelial lymphocytes (IELs) and 4.3 cells/mm for T cell receptor (TCR)-$\gamma\delta^+$ IELs. IgA deposits were graded from 0 (negative) to 3 (high). All reference values were from Hietikko and co-workers (Hietikko et al. 2018).

Control	Nationality	EmA dilution	TG2 (AU/ml)	Cells/mm	IgA deposits	Found structures
1	Finnish	1:5	Negative	$CD3^+$ 16 TCR$\gamma\delta^+$ 5.1	Negative	Isolated lymphoid follicle
2	Finnish	Negative	Negative	$CD3^+$ 20 TCR$\gamma\delta^+$ 2	Negative	Isolated lymphoid follicle or Peyer's patch
3	Romanian	N/A	N/A	N/A	N/A	Diffuse lymphoid tissue

N/A: data not available

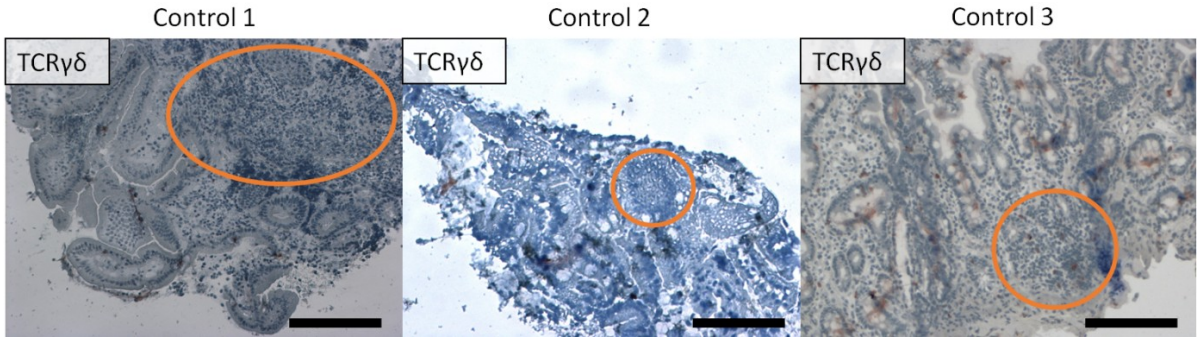

Figure 7. Brightfield images of the selected non-coeliac control duodenal bulb biopsy specimens. The specimens had been stained using immunohistochemistry to detect T cell receptor (TCR)-$\gamma\delta^+$ T cells. Structures that resemble lymphoid tissue are marked with a circle. Images were acquired using Olympus BX60 microscope with magnification ×10. Scale bar = 200 µm.

5.2 Optimization of immunofluorescence staining

In the optimization of the immunofluorescence staining protocol, the primary aim was to find optimal concentrations for the staining incubations. The initial concentrations for antibodies against IgD and IgM were 1:100, 1:250, and 1:500, which are represented in Figure 8. The dilutions were chosen based on an article by Chen and co-workers (Chen et al. 2009). Because the dilutions did not appear to reduce the background staining, a higher concentration of 1:25 was decided to use for both antibodies, which is shown in Figure 9. By adding 0.05% TWEEN to the PBS washing buffer that was used between and after incubations, unspecific binding decreased as seen when comparing Figure 8 and Figure 9. The specimens in Figure 9 were washed with 0.05% TWEEN – PBS, whereas the specimens in Figure 8 were washed with PBS only.

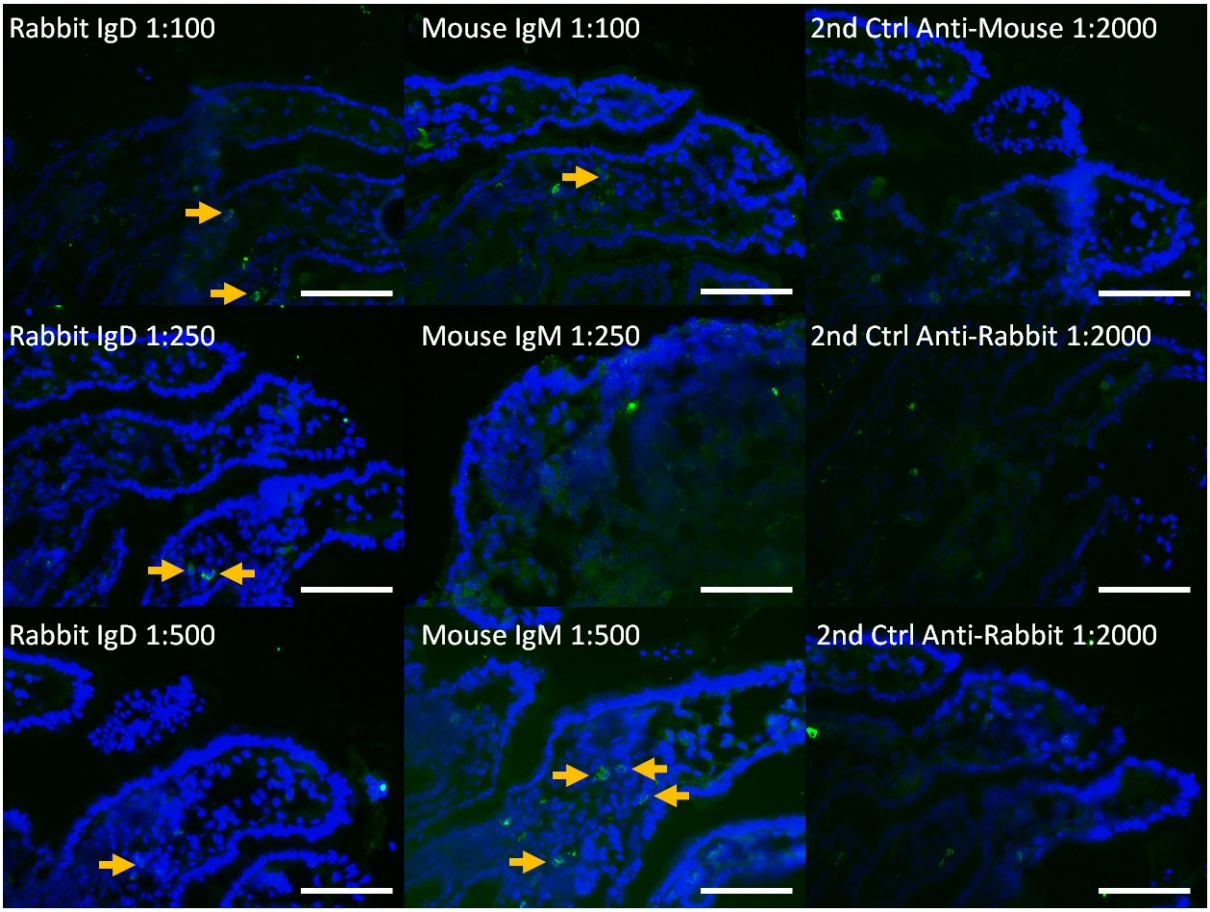

Figure 8. Concentration optimization for the antibodies against IgD and IgM using dilutions 1:100, 1:250, and 1:500. Images of IgD and IgM were taken from the same duodenal bulb biopsy specimen. All specimens were washed with phosphate-buffered saline only. IgD and IgM are shown in green and DAPI in blue. Positive cells are marked with an arrow. Fluorescence images were acquired using Olympus BX60 microscope with magnification ×20. Scale bar = 100 µm. 2nd Ctrl: secondary control

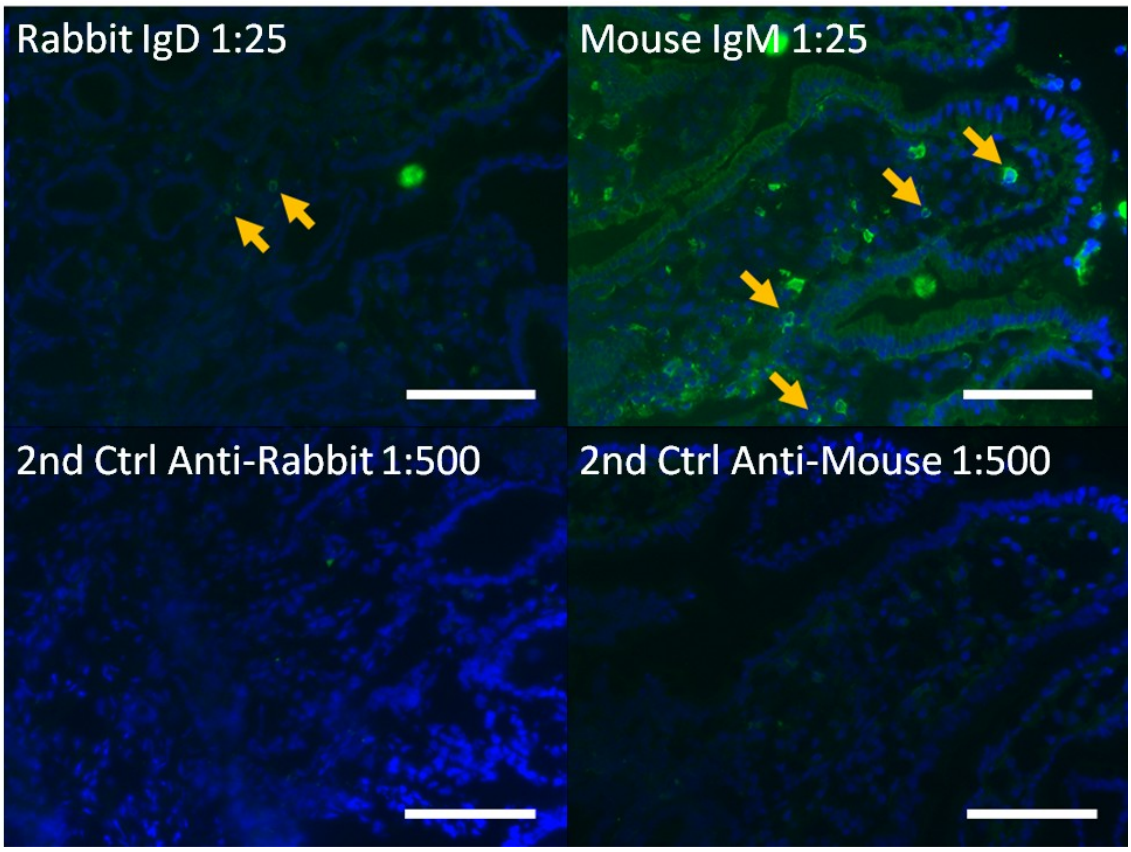

Figure 9. Concentration optimization for the antibodies against IgD and IgM using dilution 1:25. All duodenal bulb biopsy specimens were washed with 0.05% TWEEN – phosphate-buffered saline. IgD and IgM are shown in green and DAPI in blue. Positive cells are marked with an arrow. Fluorescence images were acquired using Olympus BX60 microscope with magnification ×20. Scale bar = 100 µm. 2nd Ctrl: secondary control

The concentrations were also optimized for rTG2 and antibodies against AID and Pax5. For rTG2, a dilution of 1:100 was used, which is shown in Figure 10. The dilution was chosen based on an article by Hietikko and co-workers (Hietikko et al. 2018). In addition, a positive control test was performed with rTG2 using small intestine biopsy sections from CD-affected individuals. The initiate dilutions for AID and Pax5 were 1:50, 1:100, and 1:250, which are represented in Figure 11. Dilutions that achieved optimal results were 1:100 for AID and 1:250 for Pax5. For the final staining, IgA-FITC and IgA-TRITC were used at a dilution of 1:40, which was based on the protocol by Hietikko and co-workers (Hietikko et al. 2018).

Because fixation may alter the structure of the desired epitopes, a specimen was tested without using fixation. According to the results, there was no significant difference in the quality of the staining. Therefore, fixation was decided to not be included in the final staining.

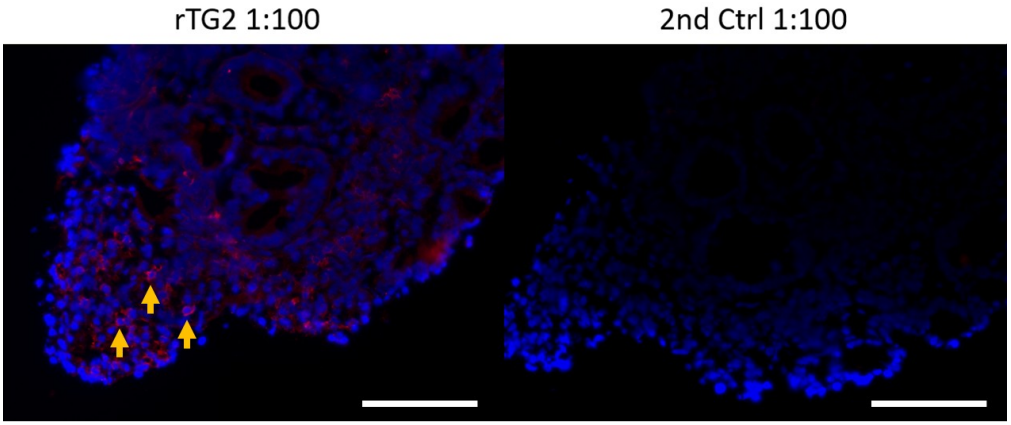

Figure 10. Concentration optimization for recombinant transglutaminase 2 using dilution 1:100 and a duodenal bulb biopsy specimen from an untreated CD-patient. Recombinant transglutaminase (rTG2) is shown in red and DAPI in blue. Positive cells are marked with an arrow. Fluorescence images were acquired using Olympus BX60 microscope with magnification ×20. Scale bar = 100 µm. 2nd Ctrl: secondary control

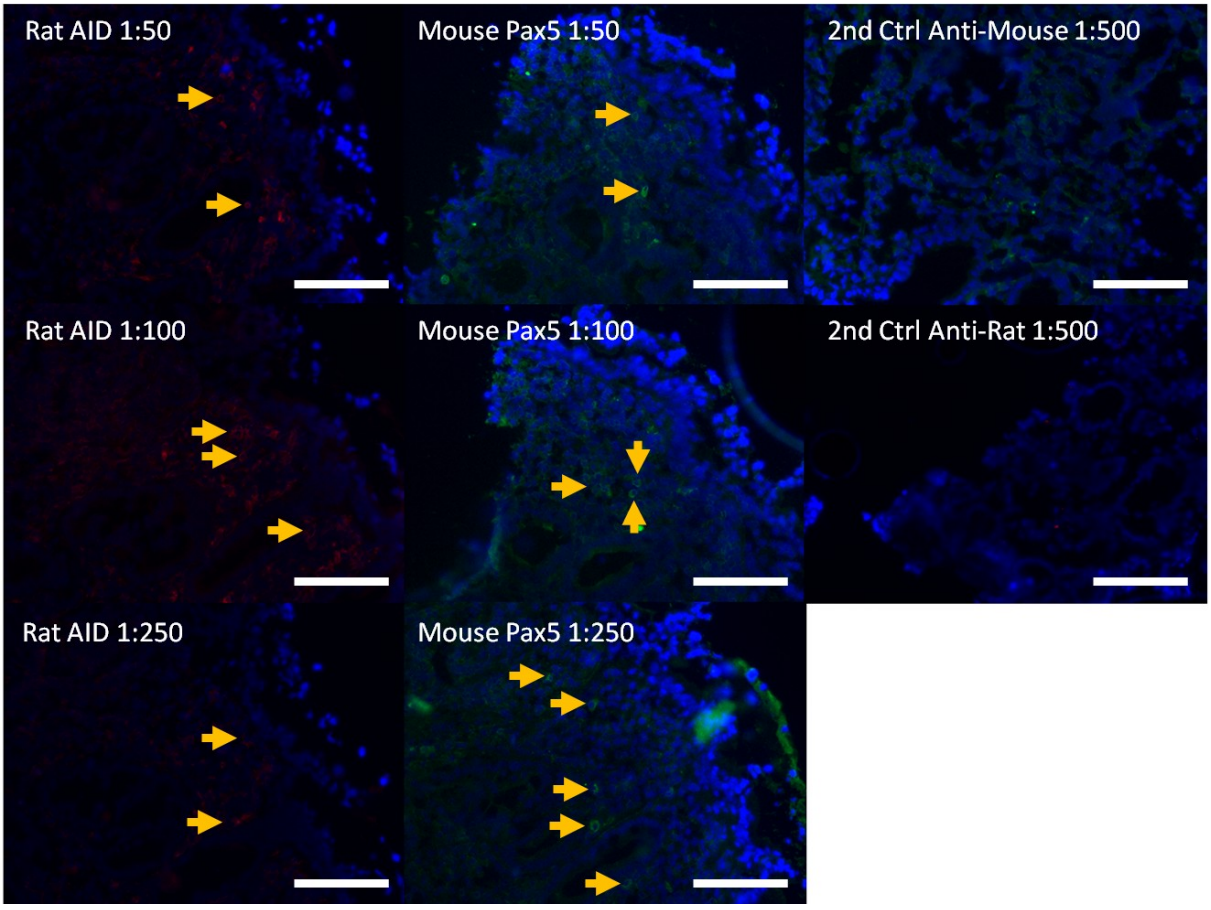

Figure 11. Concentration optimization for the antibodies against activation-induced cytidine deaminase (AID) and Pax5 using dilutions 1:50, 1:100, and 1:250. Images of AID and Pax5 were taken from the same duodenal bulb biopsy specimen. AID is shown in red, Pax5 in green, and DAPI in blue. Positive cells are marked with an arrow. Fluorescence images were acquired using Olympus BX60 microscope with magnification ×20. Scale bar = 100 µm. 2nd Ctrl: secondary control

5.3 Identification of B cells in duodenal bulb biopsies

The selected four untreated CD-patient and three non-CD control duodenal bulb biopsy specimens were stained using different rTG2 and antibody combinations to identify B cells and lymphoid structures. Combination rTG2-IgA was used to identify mature naïve TG2-specific B cells that had gone through CSR, TG2-specific plasmablasts, or TG2-specific plasma cells. Combinations rTG2-IgD and rTG2-IgM were used to detect early phase TG2-specific B cells that had yet to undergo CSR. Combination rTG2-AID was used to detect TG2-specific B cells with ongoing CSR. Combination rTG2-Pax5 was used to detect TG2-specific B cells that had not yet matured into plasma cells. In addition, IgA and IgD combined with AID and Pax5 were used to detect all early phase B cells regardless of their antigen specificity. Next, I will present the results of each staining combination.

First, the presence of IgA positive and TG2-specific B cells, plasmablasts, and plasma cells was investigated using rTG2-IgA combination. A single co-localization was detected in three untreated CD-patient sections, whereas no co-localizations were detected in non-CD controls. All three double positive cells were located in the neck of a villus. The number of IgA single positive cells was high in all sections. Results are presented in Figure 12.

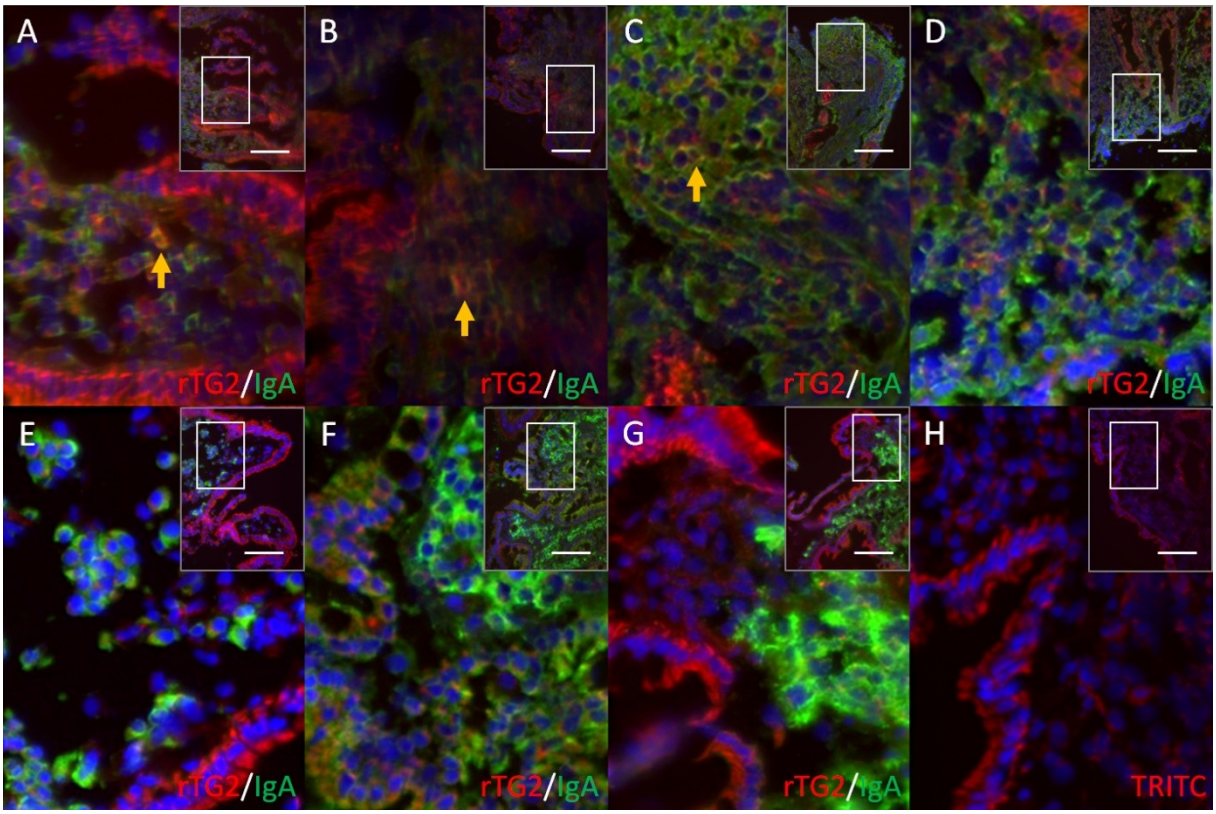

Figure 12. Representative images of immunofluorescence double staining with recombinant transglutaminase 2 and IgA using duodenal bulb biopsy sections from untreated coeliac disease (CD)-patients 1–4 (A–D), non-CD controls 1–3 (E–G), and a secondary control (H). A single double positive cell was detected in three untreated CD-patient sections (A–C). Recombinant transglutaminase 2 (rTG2) is shown in red, IgA in green, DAPI in blue, and co-localization in yellow. Double positive cells are marked with an arrow in the enlarged images. Images were acquired using Olympus IX51 microscope with magnification ×20. The original image is shown in the top-right corner and the white rectangle represents the area of the enlarged image. Scale bar = 100 µm.

The presence of early phase TG2-specific B cells that had yet to undergo CSR was investigated using combinations rTG2-IgD and rTG2-IgM. Four cells with rTG2-IgD co-localization were detected in two untreated CD-patient sections, two double positive cells in each. One rTG2-IgD double positive cell was detected in a non-CD control. All of the rTG2-IgD double positive cells were located in the villus layer. Few IgD single positive cells were detected in all specimens. A small IgD single positive cell cluster was detected in one untreated CD-patient section and in one non-CD control section. No rTG2-IgM double positive cells were detected in untreated CD-patient sections. One double positive cell located in the neck of a villus was detected in one non-CD control. Few dispersedly localized IgM single positive cells were detected in all sections, although the number was higher in untreated CD-patients. Results of rTG2-IgD and rTG2-IgM combinations are presented in Figure 13 and Figure 14.

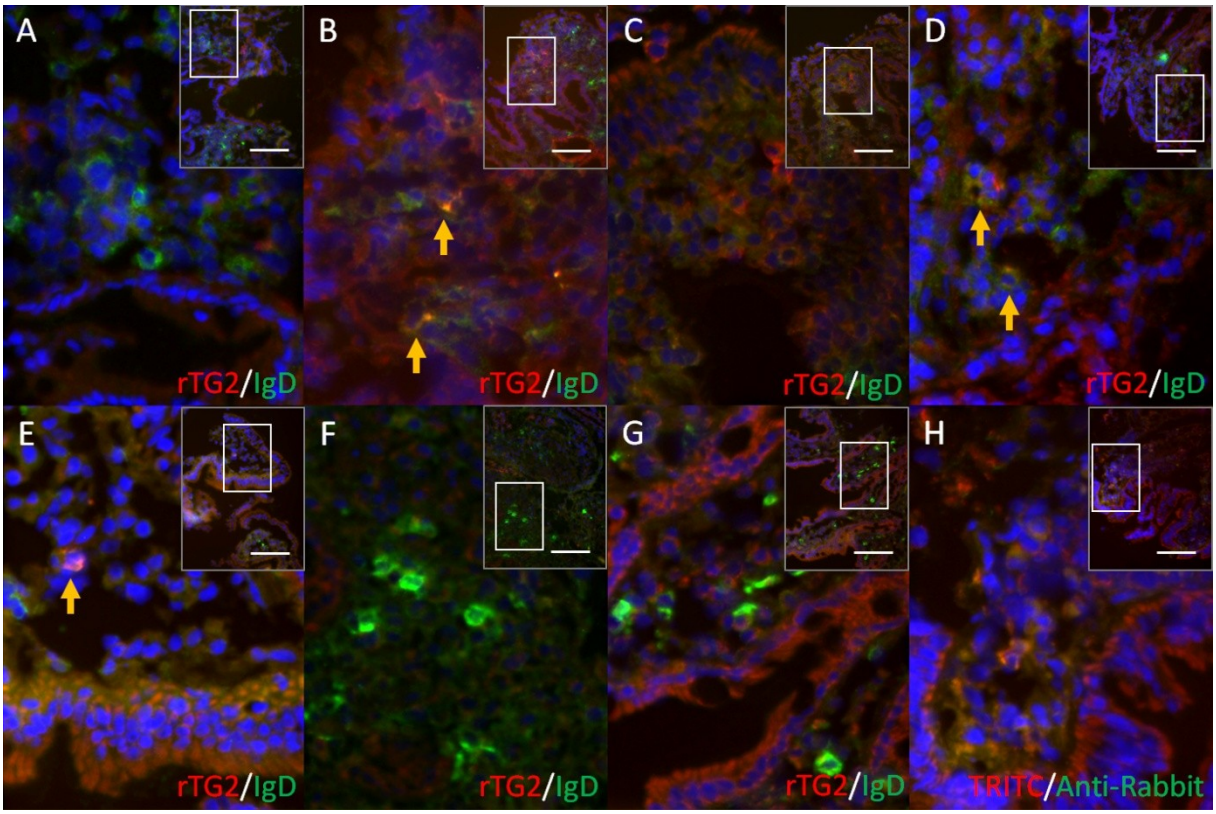

Figure 13. Representative images of immunofluorescence double staining with recombinant transglutaminase 2 and IgD using duodenal bulb biopsy sections from untreated coeliac disease (CD)-patients 1–4 (A–D), non-CD controls 1–3 (E–G), and a secondary control (H). A total of four double positive cells were detected in two untreated CD-patient sections (B, D) and one double positive cell in one untreated CD-patient section (E). A small cluster of IgD single positive cells was detected in one untreated CD-patient section (A) and in one non-CD control section (F). Recombinant transglutaminase 2 (rTG2) is shown in red, IgD in green, DAPI in blue, and co-localization in yellow. Double positive cells are marked with an arrow in the enlarged images. Images were acquired using Olympus IX51 microscope with magnification ×20. The original image is shown in the top-right corner and the white rectangle represents the area of the enlarged image. Scale bar = 100 µm.

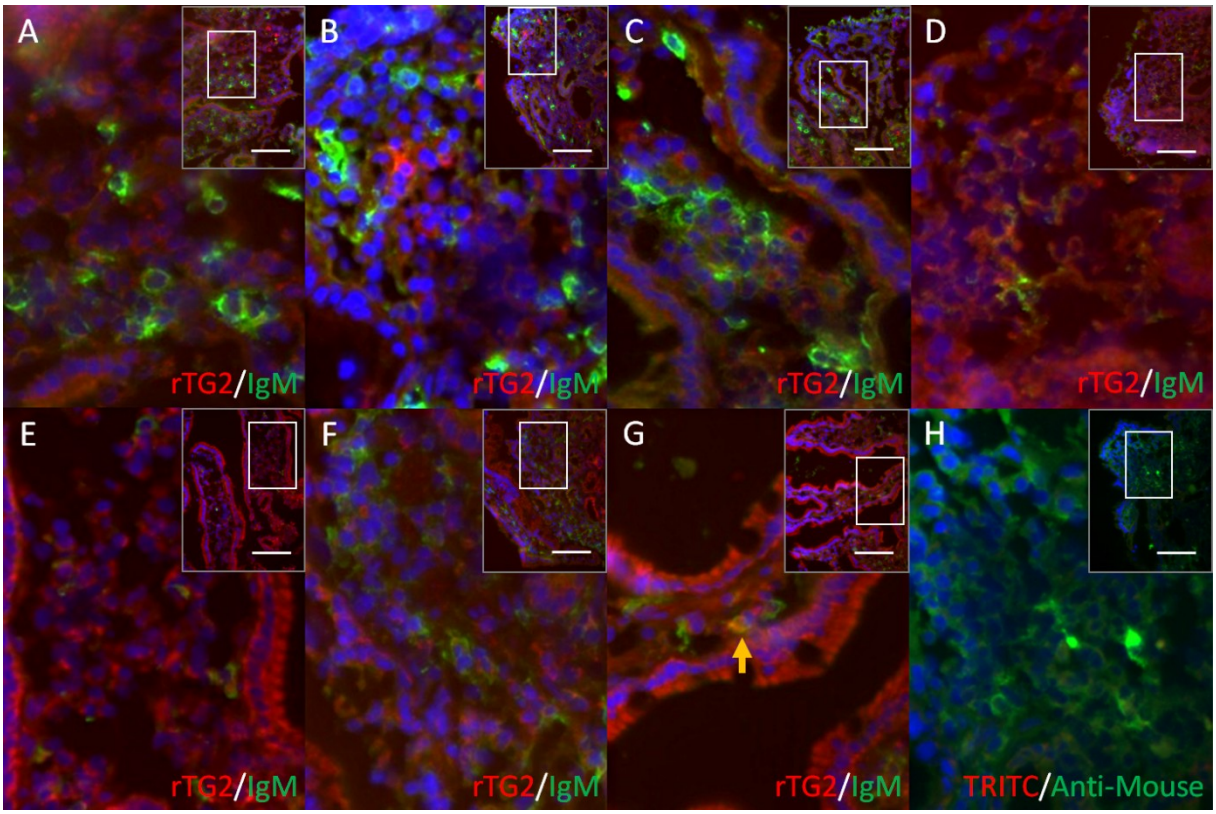

Figure 14. Representative images of immunofluorescence double staining with recombinant transglutaminase 2 and IgM using duodenal bulb biopsy sections from untreated coeliac disease (CD)-patients 1–4 (A–D), non-CD controls 1–3 (E–G), and a secondary control (H). One double positive cell was detected in a non-CD control section (G). Recombinant transglutaminase 2 (rTG2) is shown in red, IgM in green, DAPI in blue, and co-localization in yellow. The double positive cell is marked with an arrow in the enlarged image. Images were acquired using Olympus IX51 microscope with magnification ×20. The original image is shown in the top-right corner and the white rectangle represents the area of the enlarged image. Scale bar = 100 µm.

The presence of TG2-specific B cells with ongoing CSR was investigated using rTG2-AID co-localization. Altogether four cells with rTG2-AID co-localization were detected in three untreated CD-patient sections. One section contained two double positive cells and two sections contained a single double positive cell. One of such cells was located in a villus and the three other cells were located in the neck of a villus or close to the intestinal epithelium. No co-localizations were detected in non-CD controls. Few AID single positive cells were detected in all sections. Results are presented in Figure 15.

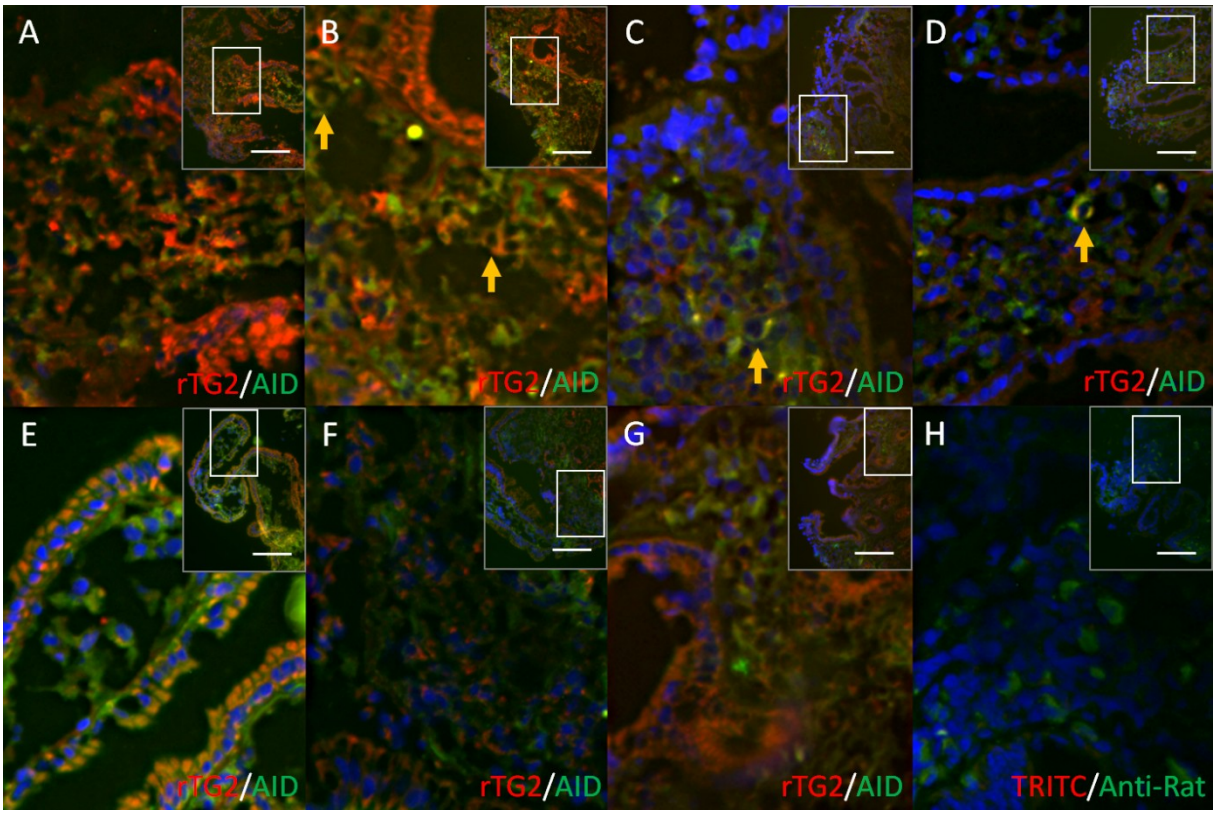

Figure 15. Representative images of immunofluorescence double staining with recombinant transglutaminase 2 and activation-induced cytidine deaminase (AID) using duodenal bulb biopsy sections from untreated coeliac disease (CD)-patients 1–4 (A–D), non-CD controls 1–3 (E–G), and a secondary control (H). A total of four double positive cells were detected in three untreated CD-patient sections (B–D). Recombinant transglutaminase 2 (rTG2) is shown in red, AID in green, DAPI in blue, and co-localization in yellow. Double positive cells are marked with an arrow in the enlarged images. Images were acquired using Olympus IX51 microscope with magnification ×20. The original image is shown in the top-right corner and the white rectangle represents the area of the enlarged image. Scale bar = 100 µm.

The presence of TG2-specific B cells that had not yet matured into plasma cells was investigated using rTG2-Pax5 combination. No cells with rTG2-Pax5 co-localization were detected in untreated CD-patients. One double positive cell located in the neck of a villus was detected in two non-CD control sections. A low number of dispersedly localized Pax5 single positive cells were detected in all sections. Results are presented in Figure 16.

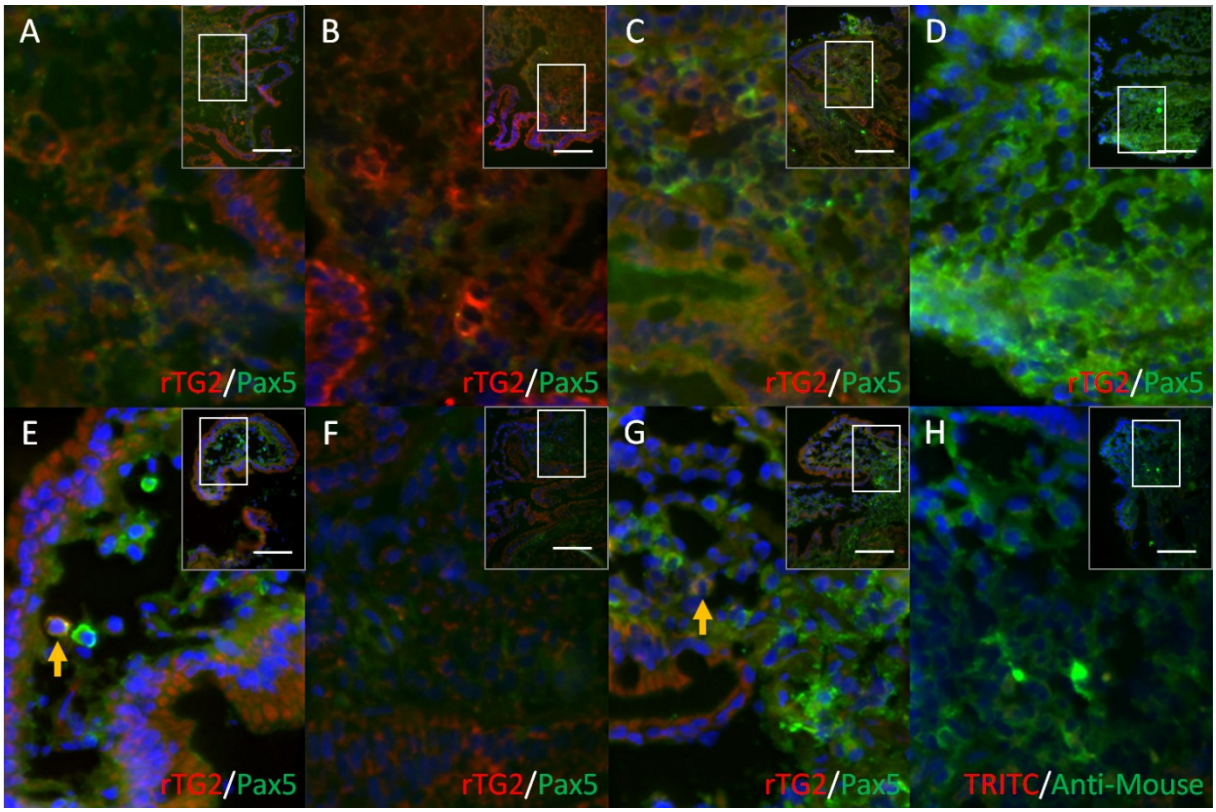

Figure 16. Representative images of immunofluorescence double staining with recombinant transglutaminase 2 and Pax5 using duodenal bulb biopsy sections from untreated coeliac disease (CD)-patients 1–4 (A–D), non-CD controls 1–3 (E–G), and a secondary control (H). A single double positive cell was detected in two non-CD control sections (E, G). Recombinant transglutaminase 2 (rTG2) is shown in red, Pax5 in green, DAPI in blue, and co-localization in yellow. Double positive cells are marked with an arrow in the enlarged images. Images were acquired using Olympus IX51 microscope with magnification ×20. The original image is shown in the top-right corner and the white rectangle represents the area of the enlarged image. Scale bar = 100 µm.

After the TG2-specific co-localizations, IgA-AID combination was used to detect all IgA positive B cells that were undergoing CSR to identify potential lymphoid follicle structures. A single IgA-AID double positive cell was detected in two untreated CD-patient sections and in one non-CD control section. All three double positive cells were located in villi. A high amount of dispersedly localized IgA single positive cells and a low number of dispersedly localized AID single positive cells were detected in all sections. Results are presented in Figure 17.

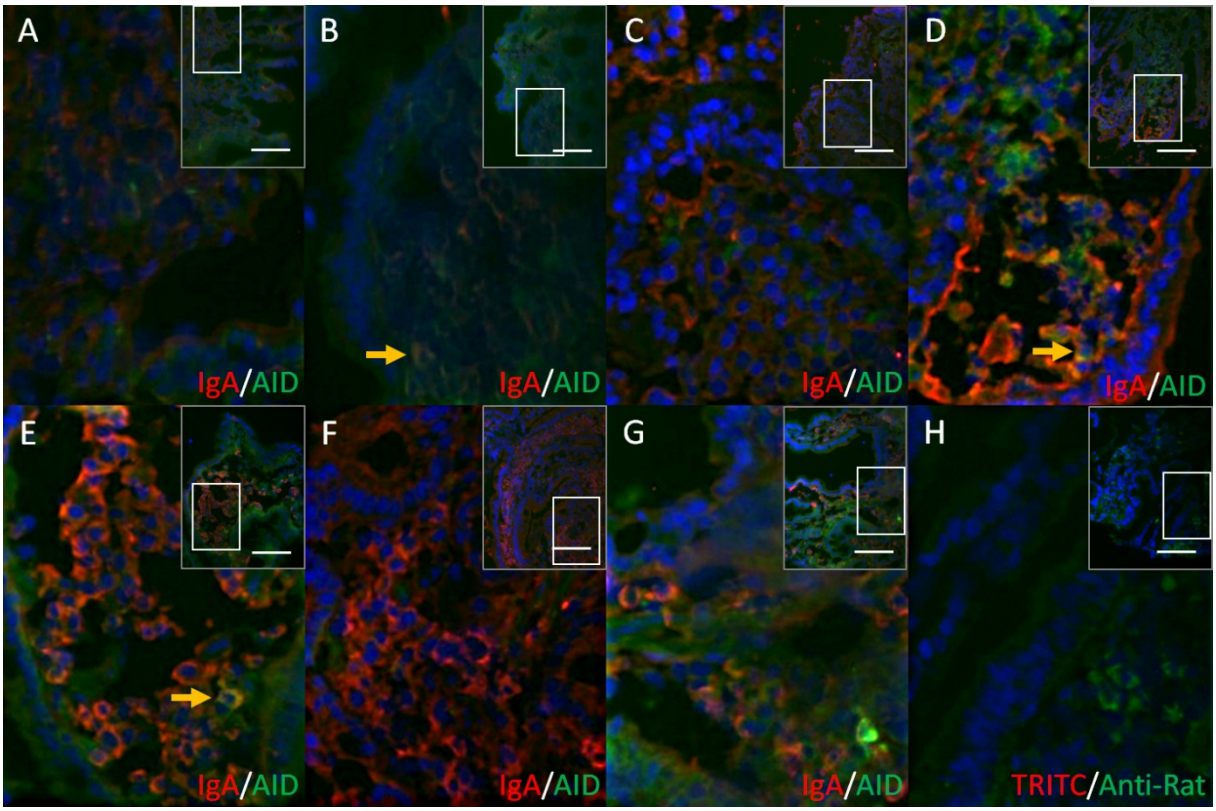

Figure 17. Representative images of immunofluorescence double staining with IgA and activation-induced cytidine deaminase (AID) using duodenal bulb biopsy sections from untreated coeliac disease (CD)-patients 1–4 (A–D), non-CD controls 1–3 (E–G), and a secondary control (H). One double positive cell was detected in two untreated CD-patient sections (B, D) and in one non-CD control section (E). IgA is shown in red, AID in green, DAPI in blue, and co-localization in yellow. Double positive cells are marked with an arrow in the enlarged images. Images were acquired using Olympus IX51 microscope with magnification ×20. The original image is shown in the top-right corner and the white rectangle represents the area of the enlarged image. Scale bar = 100 µm.

The presence of all IgA positive B cells before maturation into plasma cells was detected using IgA-Pax5 combination. Multiple double positive cells were detected in all untreated CD-patient sections and in two non-CD control sections. Most of the double positive cells were in small clusters located in villi or close to the intestinal epithelium. The number of dispersedly localized IgA single positive cells was high in all sections, and a low amount of dispersedly localized Pax5 positive cells was detected as well. Results are presented in Figure 18.

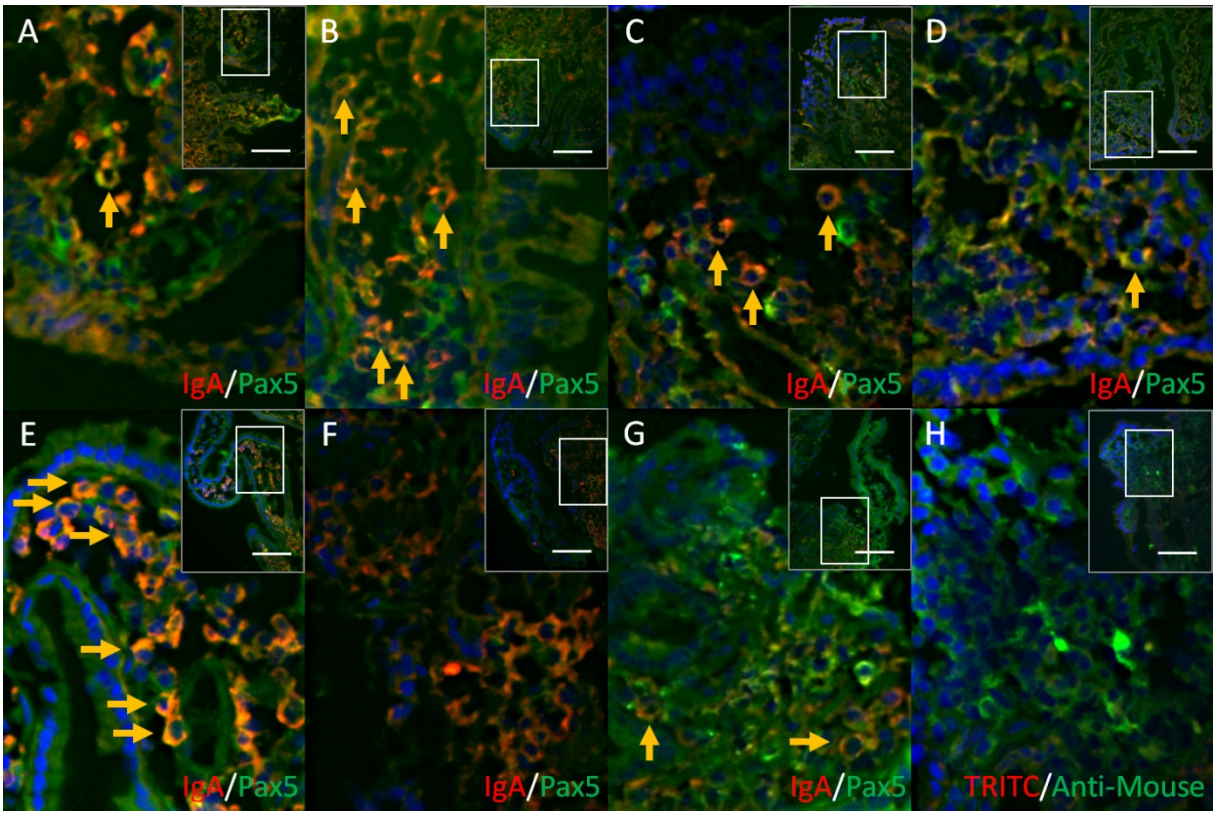

Figure 18. Representative images of immunofluorescence double staining with IgA and Pax5 using duodenal bulb biopsy sections from untreated coeliac disease (CD)-patients 1–4 (A–D), non-CD controls 1–3 (E–G), and a secondary control (H). Double positive cells were detected in all untreated CD-patient sections (A–D) and in two non-CD control sections (E, G). IgA is shown in red, Pax5 in green, DAPI in blue, and co-localization in yellow. Double positive cells are marked with an arrow in the enlarged images. Images were acquired using Olympus IX51 microscope with magnification ×20. The original image is shown in the top-right corner and the white rectangle represents the area of the enlarged image. Scale bar = 100 µm.

The presence of all B cells that were undergoing CSR in potential lymphoid follicle structures was investigated using IgD-AID combination. A total of six double positive cells were detected in three untreated CD-patient sections and a total of three double positive cells in two non-CD control sections. All double positive cells were located close to the intestinal epithelium. Few dispersedly localized IgD and AID single positive cells were detected in all sections. Results are presented in Figure 19.

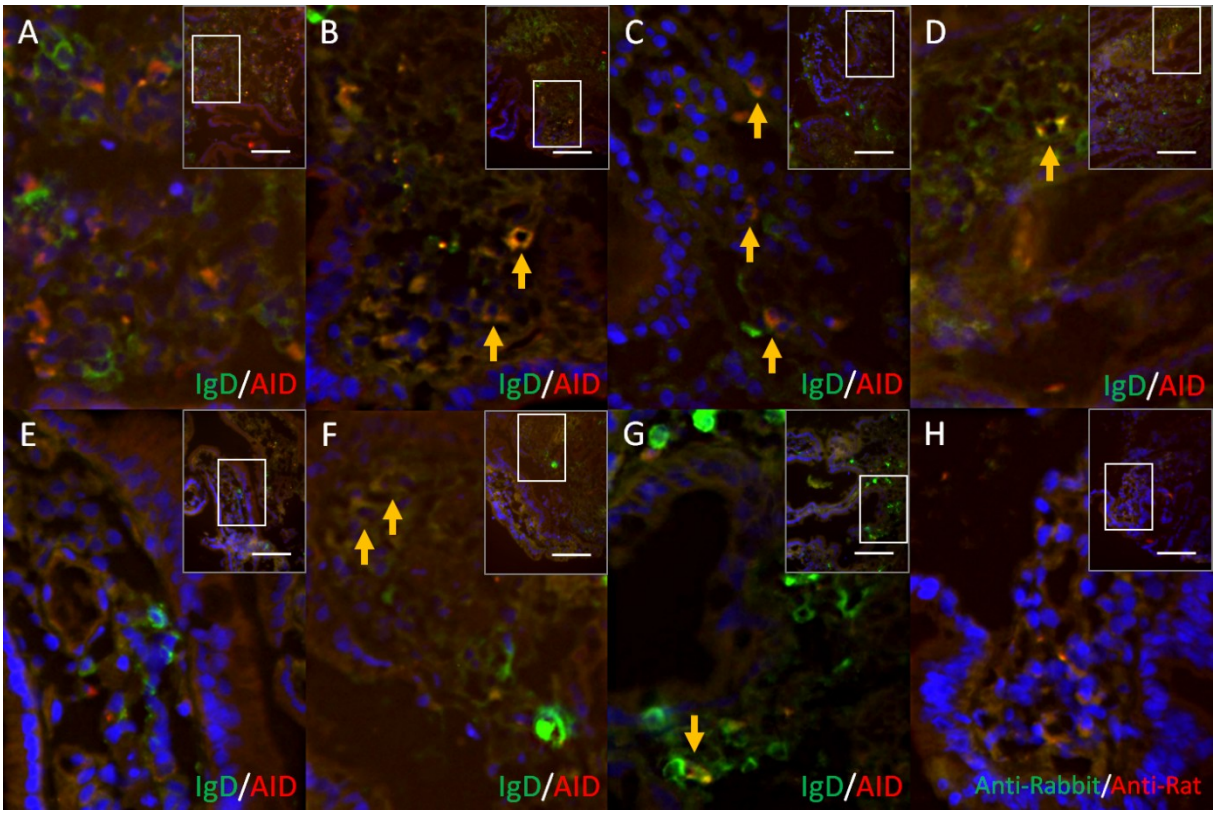

Figure 19. Representative images of immunofluorescence double staining with IgD and activation-induced cytidine deaminase (AID) using duodenal bulb biopsy sections from untreated coeliac disease (CD)-patients 1–4 (A–D), non-CD controls 1–3 (E–G), and a secondary control (H). Double positive cells were detected in three untreated CD-patient sections (B–D) and in two non-CD control sections (F–G). IgD is shown in green, AID in red, DAPI in blue, and co-localization in yellow. Double positive cells are marked with an arrow in the enlarged images. Images were acquired using Olympus IX51 microscope with magnification ×20. The original image is shown in the top-right corner and the white rectangle represents the area of the enlarged image. Scale bar = 100 µm.

Finally, the presence of all early phase B cells was investigated using IgD-Pax5 combination. A total of six double positive cells were detected in three untreated CD-patient sections and a total of five double positive cells in all non-CD control sections. One potential double positive cell cluster was detected in one untreated CD-patient section and in one non-CD control section. All double positive cells were located close to the intestinal epithelium. A low number of dispersedly localized IgA and Pax5 single positive cells were detected in all sections. Results are presented in Figure 20.

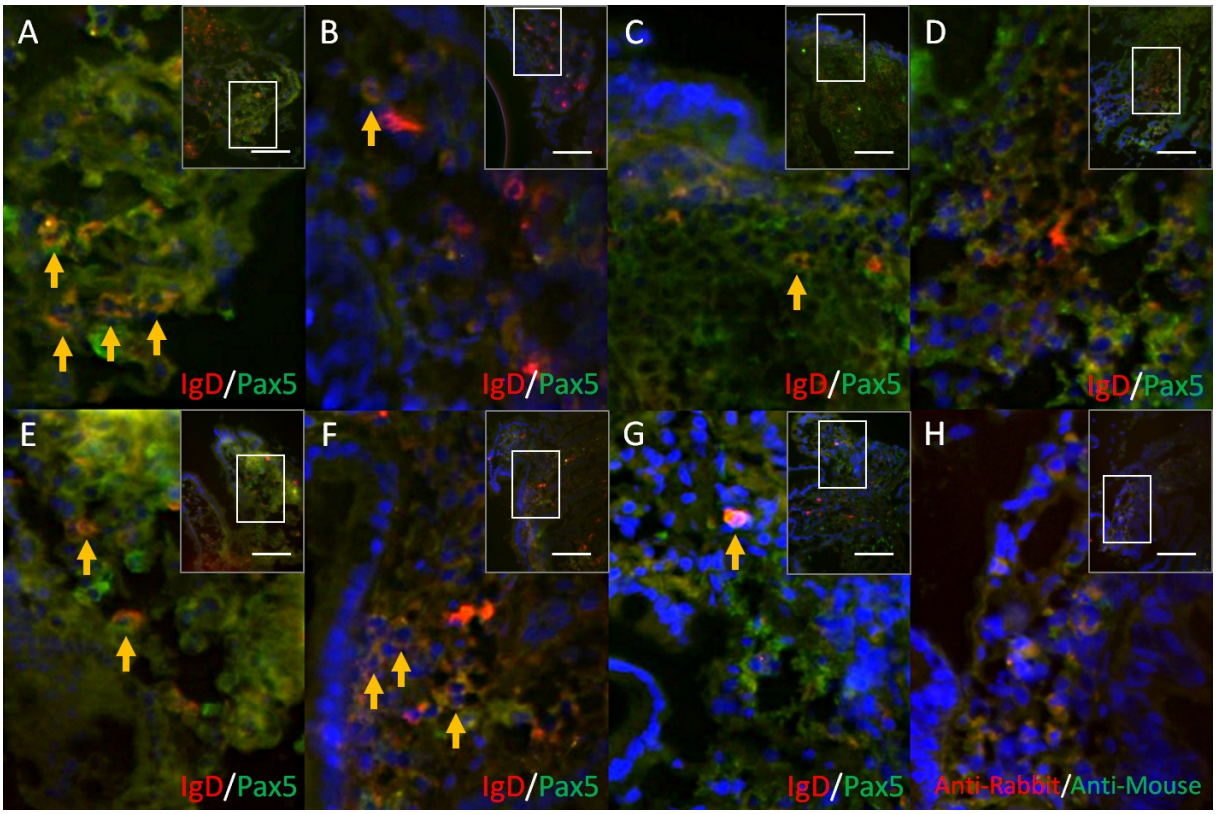

Figure 20. Representative images of immunofluorescence double staining with IgD and Pax5 using duodenal bulb biopsy sections from untreated coeliac disease (CD)-patients 1–4 (A–D), non-CD controls 1–3 (E–G), and a secondary control (H). Double positive cells were detected in three untreated CD-patient sections (A–C) and in all non-CD control sections (E–G). One potential double positive cell cluster was detected in an untreated CD-patient section (A) and in a non-CD control section (F). IgD is shown in red, Pax5 in green, DAPI in blue, and co-localization in yellow. Double positive cells are marked with an arrow in the enlarged images. Images were acquired using Olympus IX51 microscope with magnification ×20. The original image is shown in the top-right corner and the white rectangle represents the area of the enlarged image. Scale bar = 100 µm.

When comparing all results, rTG2 appeared to be most co-localized with AID. The expression of IgA and IgD single positive cells was more abundant compared to Pax5 and AID single positive cells, and the number of IgA, IgM, and IgD single positive B cells was higher than the number of TG2-specific B cells of the specific Ig class. The number of positive untreated CD-patients and non-CD controls for each combination is summarized in Table 6.

Table 6. Results of the immunofluorescence double staining combinations. Target of the co-localization and the amount of positive untreated coeliac disease (CD)-patients and non-CD controls are described for each combination.

Combination	Target	CD-patients	Controls
rTG2-IgA	Mature naïve TG2-specific B cells, plasmablasts or plasma cells	3/4	Negative
rTG2-IgD	Early phase TG2-specific B cells before class-switch	2/4	1/3
rTG2-IgM	Early phase TG2-specific B cells before class-switch	Negative	1/3
rTG2-AID	TG2-specific B cells with ongoing IgA class-switch	3/4	Negative
rTG2-Pax5	TG2-specific B cells before transition into plasma cells	Negative	2/3
IgA-AID	B cells with ongoing IgA class-switch	2/4	1/3
IgA-Pax5	IgA-specific B cells before transition into plasma cells	4/4	2/3
IgD-AID	B cells with ongoing class-switch	3/4	2/3
IgD-Pax5	Early phase B cells	3/4	3/3

AID: activation-induced cytidine deaminase; rTG2: recombinant transglutaminase 2

Despite the few positive cell clusters detected in the stained sections, only one structure was detected in one untreated CD-patient that resembled the potential lymphoid tissue in the screened section stained for TCRγδ$^+$ T cells using immunohistochemistry, which is shown in Figure 21. The structure contained a circular zone of IgD positive B cells. No co-localization with Pax5 or rTG2 was detected in the structure.

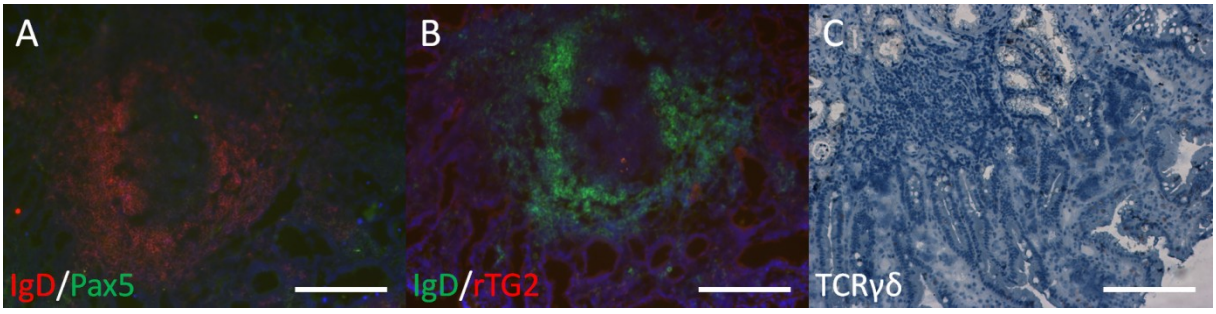

Figure 21. Potential lymphoid structure in an untreated CD-patient duodenal bulb biopsy section. All three images are from the same location of untreated CD-patient 2. (A) IgD-Pax5 combination using immunofluorescence staining. IgD is shown in red and Pax5 in green. (B) IgD-recombinant transglutaminase 2 (rTG2) combination using immunofluorescence staining. IgD is shown in green and rTG2 in red. (C) T cell receptor (TCR)-γδ positive T cells stained using immunohistochemistry. DAPI is shown in blue in all images. No Pax5 or TG2 positive cells were detected in the double stained sections (A–B). Immunofluorescence images (A–B) were acquired using Olympus IX51 microscope and the brightfield image (C) using Olympus BX60 with magnification ×10. Scale bar = 200 µm.

6 Discussion

This study aimed to investigate the location of early phase TG2-specific B cells in the duodenal bulb of CD-patients using immunofluorescence staining and co-localization with rTG2 against IgD, IgM, IgA, AID, and Pax5. The amount of studies on the early development of human TG2-specific B cells has been minimal, as most of the research material consists of either isolated cells from the small intestine (Spencer and Sollid 2016) or diagnostic biopsies from the duodenum and jejunum of CD-patients, which are devoid of lymphoid tissues that contain sites for B cell development. Furthermore, several studies on lymphoid tissues, particularly ILFs, are based on mice or other species (Hamada et al. 2002; Nochi et al. 2013; Pabst et al. 2005; Tsuji et al. 2008; Wang et al. 2006). There are fundamental differences that exist between species, which prevents extrapolation from mice to humans (Spencer and Sollid 2016). In addition, the TG2-specific B cell response has not been studied in mice. Therefore, comparing the results of this study with previous studies was difficult. Nonetheless, the gained results raised speculations and hypotheses, which I will discuss next.

6.1 Identification of lymphoid tissues in duodenal bulb biopsies

This study comprised 479 cryosected duodenal bulb biopsies from untreated CD-patients and non-CD individuals. The high amount of material was to ensure that there were enough high-quality specimens with potential lymphoid structures. Cryosections were used due to better preservation of the epitopes compared to histological methods that include fixation with formalin (Son et al. 2013). However, cryosections also have a weaker morphology compared to formalin-fixed specimens (Son et al. 2013).

Considering the high amount of starting material, the number of screened specimens that contained potential lymphoid structures was low. This is explained by the lack of recognizable structures and the varying morphology of the duodenal bulb biopsies (Lindfors et al. 2019; Taavela et al. 2016). The potential abnormal histological findings in the duodenal bulb biopsies may explain the difficulty in recognizing potential lymphoid structures (Stoven et al. 2016; Taavela et al. 2016). In addition, many of the Romanian duodenal bulb biopsies had been collected from individuals with various infections, which increased the risk of the specimens containing non-CD-specific lymphoid structures. Because of this, only one Romanian untreated CD-patient and one non-CD control without any apparent infection were included in the final staining.

As for the number of the potential lymphoid structures, the amount of detected ILFs and diffuse lymphoid tissue was the highest, whereas the number of PPs was the lowest. This is explained by the distinct structure of the PPs with high specificity, whereas ILFs and diffuse lymphoid tissue lack a distinct shape, and therefore the potential ILF and lymphoid tissue structures may include non-specific cell aggregates.

6.2 Identification of transglutaminase 2-specific B cells in duodenal bulb

High-quality duodenal bulb biopsy specimens from four untreated CD-patients and three non-CD controls were selected for the immunofluorescence double staining. Using rTG2, it was possibly to visualize TG2-specific B cells and plasma cells in the sections (Di Niro et al. 2016). In addition, early phase TG2-specific B cells were detected using co-localization with antibodies specifically targeting IgA, IgD, IgM, AID, and Pax5. To confirm the potential lymphoid structures that were detected during the screening, a double staining with IgA and IgD combined with AID and Pax5 was performed.

A low amount of IgA positive TG2-specific cells was detected in three out of four untreated CD-patient sections. These cells could be either late phase TG2-specific B cells, plasmablasts, or plasma cells. All double positive cells were found in the apical area of the mucosa in proximity of the lumen, where TG2-specific plasma cells are generally localized (Di Niro et al. 2016). Therefore, it is a high possibility that the detected cells were plasma cells, which suggests that TG2-specific plasma cells may localize to the duodenal bulb mucosa. This could be confirmed by using a co-localization with rTG2 and an anti-CD138 antibody, which is used to detect plasma cells (Di Niro et al. 2016). No double positive cells were observed in untreated CD-patient 4. A possible explanation could be that the double positive cells were located in other sections of the biopsy specimen. On the other hand, no diagnosed data was provided for the patient, and therefore the patient could possibly be negative for CD-specific IgA antibodies. In parallel to the studies by Di Niro and co-workers, and Hietikko and co-workers using small-intestinal biopsies, no TG2-IgA double positive cells were found in the non-CD control duodenal bulb biopsy specimens (Di Niro et al. 2016; Hietikko et al. 2018).

Few rTG2-IgD co-localizations were detected in two untreated CD-patients, which suggests that TG2-specific B cells that have yet to undergo CSR may exist in the duodenal bulb. All double positive cells were found in the villus layer in proximity of the lumen. As majority of the lymphoid tissues have been found to be located further in the LP, including PPs and ILFs in mice (Knoop and Newberry 2012; Lorenz and Newberry 2004), this could suggest that the

cells were not part of any lymphoid structure. This is further supported by the fact that the double positive cells were not surrounded by other IgD single positive B cells as in lymphoid follicles (Buettner and Lochner 2016).

Despite the few detected IgD positive TG2-specific cells, no rTG2-Pax5 co-localizations were detected in the untreated CD-patient sections. This is puzzling, as the expression of Pax5 is associated with developing B cell lineages (Medvedovic et al. 2011; Rothenberg 2014). Considering the three-dimensional structure of the GALT and the two-dimensional biopsy sections, it is possible that the identified IgD positive and TG2-specific B cells were detected in a different layer than in the sections that were stained using anti-Pax5 antibody. This is supported by the fact that a low amount of Pax5 single positive cells was detected in the sections parallel to IgD single positive cells.

No rTG2-IgM co-localizations were detected in the untreated CD-patient sections. This could be explained by the fact that in mice, most GALT B cells express phenotype $IgD^{high}IgM^{low}$ (Hamada et al. 2002), and therefore the number of IgM positive cells could be low and possibly located in other layers that were not stained. Overall, the amount of IgD and IgM positive B cells was low in all sections, which suggests that the number of early phase B cells in the duodenal bulb may be low.

Few rTG2-AID co-localizations were detected in three out of four untreated CD-patient sections. As AID is associated with CSR that mostly occurs in germinal centers of lymphoid follicles, the location of the double positive cells may give a hint whether the TG2-specific B cells develop in lymphoid follicles or at extra-follicular sites (Dominguez-Sola and Cattoretti 2017; He et al. 2007). All double positive cells were detected in the villus layer without any recognizable forms of cell aggregations, which was in parallel to the rTG2-IgD co-localizations. Therefore, the results would again suggest that the development of TG2-specific B cells may occur at extra-follicular sites of the duodenal bulb mucosa. Of note, the results seem to conflict with the study by Di Niro and co-workers, in which they did not detect AID in TG2 positive plasmablasts of the small intestine and suggested that TG2-specific B cells may undergo CSR outside the small-intestinal mucosa, although their study may not be comparable with this study as they used a different method (Di Niro et al. 2012).

Overall, the number of TG2-specific B cells was significantly higher in untreated CD-patients than in non-CD controls. Interestingly, few rTG2-IgD, rTG2-IgM, and rTG2-Pax5 co-localizations were detected in non-CD controls 1 and 3. The possibility of an error during the

staining is unlikely, as the specimens were positive in multiple staining combinations. The EmA dilution of 1:5 from the serum sample of the non-CD control 1 could indicate a potential early phase CD. Non-CD control 3 was a Romanian child without a diagnosed CD, but no further data was provided. Therefore, the individual could have a potential early phase CD as well or an infection that could affect the results. On the other hand, the fact that no rTG2-IgA double positive cells were detected in the non-CD controls could suggest that early phase TG2-specific cells expressing IgD and IgM could develop in non-CD individuals, but the immune system induces the apoptosis of these cells before they undergo CSR. Nonetheless, using control specimens from fully healthy individuals with all diagnosed data available in further studies would possibly prevent similar results.

Additional co-localizations using IgA and IgD combined with AID and Pax were used to detect all B cells to identify possible lymphoid structures in the sections. As expected, the amount of double positive cells was higher in all combinations compared to the rTG2 co-localizations. Particularly, the number of IgA single positive cells was high in all sections, suggesting that a high number of IgA positive B cells, plasmablasts, and plasma cells targeting other antigens than TG2 could exist in the duodenal bulb.

Few IgD single positive cell aggregates were identified close to the intestinal epithelium or at the base of crypts, which could indicate potential ILF structures. However, no recognizable structures were found at the sites of the rTG2 co-localizations. When comparing to the potential lymphoid structures that were identified during the screening, only one similar structure was found in one untreated CD-patient. The structure consisted of a circular zone of IgD positive cells and an inner zone that contained no IgD positive cells. According to Iversen and co-workers, germinal centers are surrounded by a zone of IgD positive cells, but there are no IgD positive cells within the germinal center (Iversen et al. 2015). Therefore, the structure could be identified as a germinal center of a potential ILF. No TG2-specific cells were detected in the structure, which suggests that the germinal center has formed in response to a different antigen. Interestingly, no Pax5 positive cells were detected in the structure, which seems to parallel the results of rTG2-Pax5 co-localization.

All in all, the results suggest that a low number of developing TG2-specific B cells may exist in the duodenal bulb and at extra-follicular sites, which supports the hypothesis. However, the limitations of this study must be taken into consideration before accepting with all the results. First, the use of antibodies as detection tools in GALT has been considered challenging due to

the presence of similar molecules and antibody receptors expressed in cell populations within GALT (Dominguez-Sola and Cattoretti 2017). Second, interpolating a three-dimensional structure from two-dimensional data is difficult, particularly when the precise structural data is crucial to fully understand the function of the lymphatic networks in the GALT. Finally, the cryosected sections provide a temporally limited view on the analysis of cell dynamics during the development in GALT.

6.3 Future aspects

The high amount of starting material provided a sufficient number of high-quality specimens for this study. However, considering the limitations of the two-dimensional data provided by the cut sections, more specimens could be included in further investigations, including specimens from individuals with different ages. Furthermore, additional staining combinations could be used to confirm the different developmental stages of the B cells, including Ki-67 to detect proliferating plasmablasts and CD138 to detect plasma cells. Triple staining combinations, such as rTG2-IgD-CD19, could be used to identify B cells at different stages with high accuracy. A triple staining using rTG2-AID with IgA or IgD would make it possible to identify the Ig class of the AID positive cells. Considering the lack of recognizable lymphoid structures in this study, there was no need to identify different lymphoid tissue types. However, if a need would arise in further studies, antibodies against CD11c expressed on FAE DCs could be used to identify ILFs and PPs from diffuse lymphoid tissue. Lastly, confocal microscopy could be included to confirm the co-localization of the epitopes, particularly combinations with rTG2.

7 Conclusions

A recent hypothesis suggests that the development of CD-patient TG2-specific B cells may occur outside lymphoid follicles. Therefore, this study aimed to identify the location of early phase TG2-specific B cells in the duodenal bulb, which is known to contain a high number of lymphoid tissues with lymphoid follicles. This was achieved by studying prospectively collected cohorts, which included cryosected duodenal bulb biopsy specimens from Finnish and Romanian CD-patients and healthy individuals. The cohorts are unique, as they represent the largest collection of frozen human duodenal bulb biopsies to study the development of CD-specific B cells in this specific location. The biopsy specimens were screened, and seven high-quality specimens were selected for immunofluorescence double staining to detect TG2-specific B cells and lymphoid structures.

A low amount of TG2-specific B cells expressing IgD or AID was detected in the CD-patient biopsy sections. However, the double positive cells did not appear to form recognizable follicle-like cell clusters or be located in areas which would typically contain lymphoid tissues with lymphoid follicles. Both IgD and AID are associated with early phase B cells. In addition, AID has been associated with CSR that commonly occurs in lymphoid follicles which contain aggregates of B cells. Therefore, the results suggest that developing TG2-specific B cells may exist in the duodenal bulb and that the development may take place in the mucosa outside lymphoid follicles, as the hypothesis suggests. Interestingly, TG2-specific B cells expressing Pax5 were only detected in two non-CD controls but not in the CD-patients, even though Pax5 is assumed to be expressed in all developing B cells. This could suggest that the amount of developing TG2-specific B cells in the duodenal bulb is very low.

Further studies on the development of TG2-specific B cells can be based on this knowledge that a low amount of early phase TG2-targeting B cells may exist in the duodenal bulb mucosa. More research on the early development of TG2-targeting B cells can help to understand the exact mechanism how CD is activated, and therefore help in discovering new treatment options.

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