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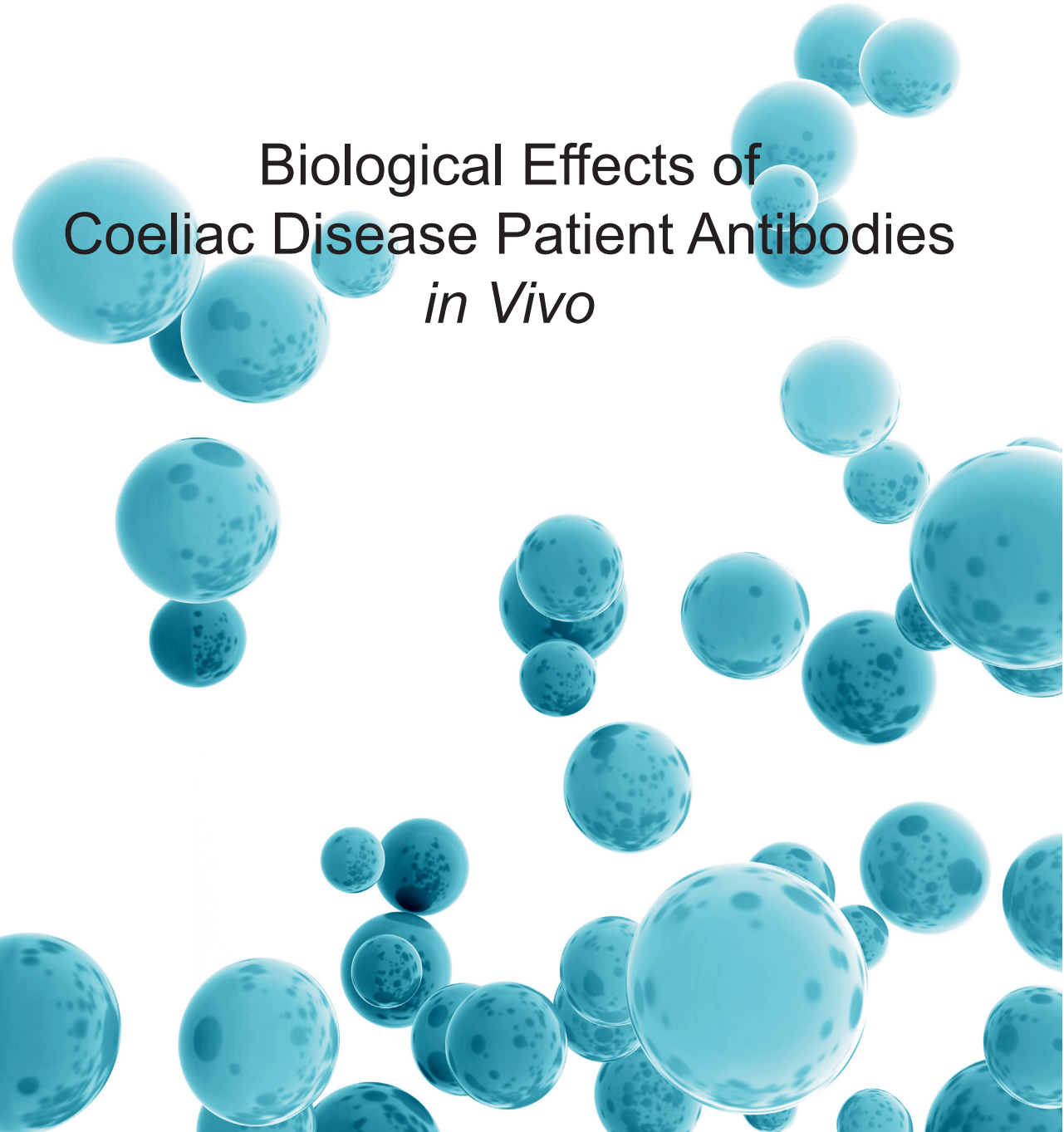

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Biological Effects of Coeliac Disease Patient Antibodies *in Vivo*

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ACADEMIC DISSERTATION
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“Life is not about waiting for the storms to pass.
It's about learning how to dance in the rain.”

Vivian Greene

Abstract

In coeliac disease, dietary gluten from wheat, barley and rye induces an autoimmune reaction in genetically susceptible individuals. This involves the formation of coeliac disease-specific antibodies targeting mainly transglutaminase 2 (TG2). TG2 autoantibodies are present both in the circulation and as deposits in the small-intestinal mucosa already in very early phases of the disease. Usually these autoantibodies belong to immunoglobulin (Ig) class A, but IgA deficient coeliac patients have IgG class antibodies in their circulation, and IgM antibodies have been found in the small-intestinal mucosa. Typically patients also evince small-bowel mucosal damage which develops gradually from normal villous morphology to inflammation and finally to crypt hyperplasia and villous atrophy. Furthermore, it has been shown that coeliac patients evince abnormalities in small-intestinal vasculature, which supplies an essential mechanical support for the villous structure. The clinical presentation of the disease is variable, ranging from asymptomatic to classical intestinal manifestations such as diarrhoea and abdominal pain, and even to extraintestinal symptoms in different organs involving for instance liver, skin, muscles and brain. Interestingly, many of these have been reported to occur while the small-intestinal morphology is still normal.

Many studies have shown that TG2 autoantibodies have biological effects *in vitro*, but there is controversy as to their contribution to the disease pathogenesis. The present work aimed to demonstrate *in vivo* effects of coeliac disease patient antibodies and especially TG2-targeted autoantibodies relevant to the pathogenesis of the disease. Studies **I** and **II** were conducted using a passive transfer method, where either sera or serum total IgG fraction from IgA deficient coeliac disease patients (**I**) or patient-derived recombinantly produced TG2 autoantibodes (**II**) were injected into mice lacking T cells. In study **III**, *in vitro*, *ex vivo* and *in vivo* matrigel assays were utilized to investigate the effects of TG2 autoantibodies on vascular formation and functionality.

In studies **I** and **II**, mice receiving coeliac patient-derived sera, total IgG or monoclonal TG2 autoantibodies evinced a slight, albeit significant, deterioration of the mucosal morphology in the small intestine. In addition, an increased

density of infiltrative cells in the lamina propria was observed. Autoantibody deposits targeted to TG2 were also found in the small-intestinal mucosa of the mice. None of these features was observed in control mice. Interestingly, an increased occurrence of mild diarrhoea and delayed weight gain was observed in a subset of the mice injected with coeliac patient sera or total IgG in study **I**. In contrast, in study **II**, injections of TG2 autoantibodies led to no such difference in the occurrence of mild diarrhoea between groups and the weights of the mice were fairly stable throughout the study period.

The results from study **III** clearly showed that coeliac patient-derived antibodies inhibited angiogenesis *in vitro*, *ex vivo* and *in vivo*. In *in vitro* studies the cells were less mobile in the presence of coeliac antibodies compared to controls and *ex vivo* results further revealed that, in the presence of coeliac patient TG2-targeted autoantibodies, cells outgrowing from mouse aortas were round and did not exhibit cellular processes characteristic for the leading edge during migration as in controls. Thus it might be assumed that inhibited angiogenesis is accounted for defective cell migration. In addition, the *in vivo* study revealed impaired functionality of vessels in the presence of coeliac antibodies.

For the first time, TG2 targeted autoantibody deposits were shown in the small-intestinal mucosa of mice. Importantly, autoantibody deposits occur in conjunction with mild enteropathy in mice. The condition of mice receiving coeliac patient-derived sera, total IgG or TG2 autoantibodies resembled early-phase disease in coeliac patients.

Based on the data from the present study, it seems conceivable that an increased density of inflammatory cells in the lamina propria, together with the slightly increased levels of tumor necrosis factor (TNF)-α and interleukin (IL)-27 seen in study **II**, may contribute to small-intestinal mucosal deterioration and thus play a role in the pathogenesis of coeliac disease. In addition, study **III** revealed the anti-angiogenic effects of coeliac TG2 autoantibodies and thus it may be assumed that small-intestinal deposits may contribute to the development of villous atrophy by impairing the intestinal vascularity and leaving the villi without proper mechanical support. The results from the present study would also imply that the development of clinical features requires, in addition to the TG2 autoantibodies used in the present study, also TG2 autoantibodies targeting other epitopes in TG2, entirely other antibody populations and/or longer exposure to the antibodies. Altogether, this study provided new evidence on the biological effects of coeliac disease-specific autoantibodies *in vivo*.

Tiivistelmä

Keliakialle on ominaista, että ravinnosta saatava vehnän, ohran ja rukiin gluteeni aiheuttaa autoimmuunivasteen geneettisesti alttiissa yksilössä. Osana tätä prosessia on keliakia-spesifisten vasta-aineiden muodostuminen. Nämä vasta-aineet kohdistuvat pääosin transglutaminaasi 2 (TG2)-entsyymiä vastaan ja niitä löytyy sekä potilaiden verenkierrosta että kerääntyminä ohutsuolen limakalvolta jo taudin varhaisessa vaiheessa. Yleensä kyseiset autovasta-aineet kuuluvat immunoglobuliini (Ig) A-luokkaan, mutta mikäli kyseessä on IgA-puutoksinen keliakiapotilas, verenkierrossa olevat TG2-autovasta-aineet ovat IgG-luokkaa ja suolen vasta-ainekerääntymät IgM-luokkaa. Keliakialle yleistä on myös immuunireaktion aiheuttama ohutsuolen limakalvovaurio, joka kehittyy asteittain lievän tulehduksen kautta lopulta suolikuopakkeiden liikakasvuun ja nukkalisäkkeiden tuhoutumiseen. Mielenkiintoista kyllä, keliakiapotilailla on havaittu muutoksia myös ohutsuolen verisuonistossa, joka toimii tärkeänä mekaanisena tukena suolinukan rakenteelle. Taudin kliininen kuva on hyvin vaihteleva. Potilas saattaa olla täysin oireeton, hänellä voi olla gastrointestinaalisia oireita kuten ripulia ja vatsakipuja tai täysin suolen ulkopuolisia ongelmia esimerkiksi maksassa, lihaksissa tai aivoissa. Potilaalla saattaa olla yllämainittuja oireita jo ohutsuolen limakalvon rakenteen ollessa vielä normaali.

Monissa solutason tutkimuksissa on osoitettu, että TG2-autovasta-aineilla on biologisia vaikutuksia, mutta tästä huolimatta niiden osallisuudesta taudin syntyyn ei ole ollut lopullista selvyyttä. Tämän väitöskirjatyön tarkoituksena on näyttää keliakiavasta-aineiden ja erityisesti TG2-autovasta-aineiden mahdollisia vaikutuksia taudin syntyyn elävän eliön tasolla. Työt **I** ja **II** toteutettiin menetelmällä, jossa IgA-puutoksisilta keliakiapotilailta peräisin olevaa seerumia, seerumista puhdistettua IgG-fraktiota (**I**) tai soluviljelmissä tuotettuja keliakia-potilailta peräisin olevia TG2-autovasta-aineita (**II**) injektoitiin hiiriin, joilla ei ole T-soluja. Työssä **III** tutkittiin TG2-autovasta-aineitten vaikutusta verisuonten muodostumiseen ja toimivuuteen soluilla sekä elävässä kudoksessa ja eliössä matrigeelin käyttöön perustuvalla menetelmällä.

Töissä **I** ja **II** havaittiin, että keliakiapotilailta peräisin olevalla seerumilla, IgG-fraktiolla tai TG2-autovasta-aineilla injektoiduilla hiirillä oli lieviä mutta kuitenkin tilastollisesti merkittäviä muutoksia ohutsuolen limakalvorakenteessa. Kyseisillä hiirillä oli myös lisääntynyt määrä soluja ohutsuolen lamina propriassa eli tukikalvolla, joka sijaitsee limakalvon epiteelikerroksen alla. Lisäksi ohutsuolesta löytyi TG2-autovasta-ainekertymiä. Mitään yllämainituista piirteistä ei havaittu kontrollieläimillä. Mielenkiintoista oli, että työssä **I** hiirillä, joihin injektoitiin keliaakikkojen seerumia tai IgG-fraktiota, esiintyi lievää ripulia enemmän kuin kontrolleilla ja painon kehitys oli hidastunut. Työssä **II** vastaavia eroja ryhmien välillä ei havaittu.

Työssä **III** osoitettiin, että keliaakikoilta peräisin olevat vasta-aineet estävät verisuonten syntymistä elävässä kudoksessa. Solutason kokeissa solujen havaittiin liikkuvan vähemmän keliakiavasta-aineiden läsnäollessa verrattuina kontrolleihin. Lisäksi keliakia-vasta-aineilla käsitellyt hiiren kudoksesta lähtöisin olevat solut eivät kyenneet muodostamaan vaeltamiseen tarvittavia ulkonemia. Saattaakin olla, että solujen puutteellinen kyky vaeltaa myötävaikutti kyseisissä hiirissä havaittuun vähentyneeseen verisuonten muodostumiseen. Keliakia-vasta-aineet myös heikensivät verisuonten toimivuutta.

Tässä väitöskirjatyössä siis löydettiin ensimmäistä kertaa TG2-autovasta-aineita kerääntyminä hiiren ohutsuolen limakalvolta. Lisäksi tärkeä löydös oli, että vasta-ainekertymät olivat hiiren ohutsuolessa yhtäaikaisesti suolivaurion kanssa. Näiden hiirten tila, joihin injektoitiin keliaakikoiden seerumia, IgG-fraktiota tai TG2-autovasta-aineita, muistutti alkavaa keliakiaa ihmisillä.

Tämän työn tulosten perusteella on mahdollista, että lisääntynyt solumäärä lamina propriassa samoin kuin lievästi nousseet sytokiinitasot (tuumorinekroositekijä-α, TNF-α, ja interleukiini-27, IL-27) työssä **II** saattavat vaikuttaa ohutsuolen limakalvon vaurion kehittymiseen. Työn **III** tulokset näyttivät selvästi vasta-aineiden häiritsevän uusien verisuonten syntymistä ja kehittymistä ja näin ollen voitaisiin ajatella, että myös vasta-ainekertymät myötävaikuttavat nukkalisäkkeiden tuhoutumiseen heikentämällä verisuonistoa ja sen myötä verisuoniston nukkalisäkkeille tuomaa mekaanista tukea. Lisäksi tämän työn tulokset viittaavat siihen, että työssä **II** käytettyjen TG2-autovasta-aineiden lisäksi keliakian kliinisten oireiden syntyminen vaatii myös TG2-autovasta-aineita, jotka kohdistuvat muita TG2-epitooppeja kohtaan, kokonaan muita vasta-ainepopulaatioita tai/ja pidempää altistumisaikaa vasta-aineille. Kaiken kaikkiaan, tämä väitöskirjatyö paljastaa uusia keliakia-spesifisten TG2-autovasta-aineiden biologisia vaikutuksia.

Contents

List of original publications

This thesis is based on the following original publications, referred to in the text by Roman numerals **I-III**:

I Kalliokoski S, Caja S, Frias R, Laurila K, Koskinen O, Niemelä O, Markku M, Kaukinen K, Korponay-Szabó IR, Lindfors K. Injection of celiac disease patient sera or immunoglobulins to mice reproduces a condition mimicking early developing coeliac disease. J Mol Med (Berl). 2015 Jan;93(1):51-62. doi: 10.1007/s00109-014-1204-8.

II Kalliokoski S, Ortín Piqueras V, Frías R, Sulic AM, Määttä J. A. E., Kähkönen N, Viiri K, Huhtala H, Pasternack A, Laurila K, Sblattero D, Korponay-Szabó IR, Mäki M, Caja S, Kaukinen K, Lindfors K. Transglutaminase 2-specific coeliac disease autoantibodies induce morphological changes and signs of inflammation in the small bowel mucosa of mice. Amino Acids. 2016 Aug 9. doi: 10.1007/s00726-016-2306-0.

III Kalliokoski S, Sulic AM, Korponay-Szabó IR, Szondy Z, Frias R, Perez MA, Martucciello S, Roivainen A, Pelliniemi LJ, Esposito C, Griffin M, Sblattero D, Mäki M, Kaukinen K, Lindfors K, Caja S. Coeliac Disease-Specific TG2-Targeted Autoantibodies Inhibit Angiogenesis Ex Vivo and In Vivo in Mice by Interfering with Endothelial Cell Dynamics. PLoS One. 2013 Jun 18;8(6):e65887.

Abbreviations

ABC	avidin-biotin complex
AEC	3-amino-9-ethylcarbazole
AGA	anti-gliadin antibody
APC	antigen-presenting cells
ARA	anti-reticulin antibody
BCR	B cell receptor
BSA	bovine serum albumin
CD IgA	total immunoglobulin A fraction purified from coeliac disease patient-derived serum
CD Mab	recombinantly produced monoclonal coeliac disease-specific transglutaminase 2-targeted miniantibody
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DGP	deamidated gliadin peptides
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
EmA	endomysial antibody
ENA-78	epithelial neutrophil-activating peptide
EPO	erythropoietin
[^{18}F]FDG	2-deoxy-2[^{18}F]-fluoro-*D*-glucose
Foxp3^{+}	forkhead box P3 transcription factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
HLA	human leukocyte antigen
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cell
IFN-γ	interferon-γ
IEL	intraepithelial lymphocyte
Ig	immunoglobulin
IL	interleukin
KSCN	potassium thiocyanate

MHC	major histocompatibility complex
MICA	major histocompatibility complex class I molecule A
MIP-2	macrophage inflammatory protein-2
mRNA	messenger ribonucleic acid (RNA)
NK	natural killer
NKG2D	natural killer cell group 2
non-CD IgA	total immunoglobulin A fraction purified from healthy control serum
non-CD Mab	recombinantly produced monoclonal control miniantibody
PBS	phosphate-buffered saline
PET	positron emission tomography
qPCR	quantative real-time PCR
SEM	standard error of mean
TCR	T cell receptor
TG2	transglutaminase 2
TG3	transglutaminase 3
TG6	transglutaminase 6
TLR	Toll-like receptor
TNF-α	tumour necrosis factor-α
Treg	$CD4^+CD25^+$ regulatory T cells
Vh/CrD	villous height/crypt depth ratio
vWF	von Willebrandt factor

1 Introduction

Coeliac disease is one of the numerous autoimmune conditions, and hallmarked by the presence of disease-specific antibodies targeted mainly towards transglutaminase 2 (TG2) (Dieterich et al. 1997). Gluten triggers the production of TG2 autoantibodies in the small-intestinal mucosa of genetically susceptible individuals (Marzari et al. 2001). These TG2 autoantibodies are present in the circulation of untreated coeliac disease patients, but have also been found in the small-bowel mucosa as deposits co-localizing with extracellular TG2 below the epithelial layer and around blood vessels (Korponay-Szabó et al. 2004, Koskinen et al. 2008).

Small-intestinal mucosal damage, namely atrophy and crypt hyperplasia, is still the golden standard in the diagnosis of coeliac disease (Husby et al. 2012). Disease-specific autoantibodies can be present in patients' mucosa and sera already prior to actual damage, and such a condition is called early developing coeliac disease (Kaukinen et al. 2001, Kurppa et al. 2009, Salmi et al. 2006a). It has in fact been shown that small-intestinal TG2-specific deposits precede villous atrophy (Salmi et al. 2006a, Tosco et al. 2008). Mucosal damage develops gradually from mucosal inflammation to crypt hyperplasia and finally to villous atrophy, a process which may take years or even decades (Lähdeaho et al. 2005, Mäki et al. 1990, Marsh 1992). Coeliac disease patients may present with a wide variety of gastrointestinal symptoms, but also extraintestinal manifestations, including dermatitis herpetiformis, osteoporosis, liver and neurological problems, and infertility. It is nowadays commonly observed that patients have no or only mild gastrointestinal symptoms such as delayed weight gain in childhood, diarrhoea and abdominal pain, or only extraintestinal signs are present. Some may be completely asymptomatic (Kelly et al. 2015). The degree of mucosal injury does not necessarily correlate with the level of symptoms (Kaukinen et al. 2001, Kurppa et al. 2009).

Interestingly, untreated coeliac disease patients have also been reported to evince abnormalities in their small-intestinal mucosal vasculature, which supplies an essential mechanical support to the intestinal villi. Such an observation was first made on 1980s (Cooke and Holmes 1984), but the gluten-dependency of the

vascular damage in coeliac patients was confirmed only more recently (Myrsky et al. 2009b). It is of note that TG2-targeted autoantibodies disturb angiogenesis, the formation of new vessels, and increase vascular permeability *in vitro* (Myrsky et al. 2008, Myrsky et al. 2009a).

The role of the TG2-targeted autoantibodies in coeliac disease is not fully understood despite the convincing *in vitro* results. It has been shown that TG2 autoantibodies modulate both epithelial and endothelial cellular biology (Barone et al. 2007, Nadalutti et al. 2014, Rauhavirta et al. 2011, Zanoni et al. 2006). The autoantibodies have nevertheless usually been given only a minor role in the disease pathogenesis despite their usefulness in diagnostics.

The purpose of the present study was to investigate the biological effects of coeliac disease patient antibodies, and more specifically TG2 autoantibodies, *in vivo* in mice. The passive transfer method was utilized to demonstrate the pathogenic role of antibodies in coeliac disease. In addition, *in vitro*, *ex vivo* and *in vivo* matrigel assays were designed to address the question what kind of effects TG2 autoantibodies have on vascular formation and functionality, and to discover the mechanism underlying this.

Review of the literature

2 Coeliac disease

Coeliac disease is a systemic autoimmune disorder triggered by dietary gluten from wheat, rye and barley in genetically susceptible individuals. The first modern clinical description of the condition was given by Samuel Gee (1888). However, the disorder remained poorly understood until the harmful effect of wheat gluten was noted in the 1950s (Dicke et al. 1953) and a gluten-free diet was proposed as treatment (van de Kamer et al. 1953). Only a year later the basis for the current diagnostic criteria was set up when villous atrophy and crypt hyperplasia were demonstrated as histological features of coeliac disease (Paulley 1954). Soon thereafter, the availability of devices to take biopsy increased (Royer et al. 1955, Shiner 1956) and also the possibility of antibodies being involved in the disease was described for the first times (Berger 1958, Heiner et al. 1962, Taylor et al. 1961).

The presence of enteropathy, defined by villous atrophy, crypt hyperplasia and increased intraepithelial lymphocytes found in duodenal biopsy, has long remained as cornerstone of the coeliac disease diagnosis (Marsh 1992). Such small-bowel mucosal damage evolves gradually and may take years or even decades to develop, and histologic characteristics of the coeliac small-bowel mucosa may vary from normal morphology to flat lesion (Lähdeaho et al. 2005, Mäki et al. 1990, Marsh 1992). In the early phase of the disease, patients might already have disease-specific autoantibodies present in the circulation and small-intestinal mucosa despite normal mucosal morphology (Kurppa et al. 2009, Salmi et al. 2006a), similarly to what has been observed among the first-degree relatives of patients (Dogan et al. 2012, Uenishi et al. 2014). Serological tests showing these coeliac disease-specific autoantibodies in patients' sera greatly support the diagnostics and thus biopsy is no longer always needed for diagnosis in paediatric patients (Husby et al. 2012). Strict avoidance of gluten from wheat, barley and rye is still the only available treatment for coeliac disease (Mäki 2014). New treatment options are being developed, but so far they have not replaced the gluten-free diet (Lindfors et al. 2012).

The connection with autoimmunity was suggested on the 1990s (Mäki et al. 1991a, Mäki 1994), and since then the autoimmune aspects of coeliac disease

have become commonly accepted. It has been noted that coeliac patients have an increased risk of developing other autoimmune diseases such as type 1 diabetes mellitus, thyroid autoimmune disorders and Sjögren's syndrome (Collin et al. 1994, Sategna Guidetti et al. 2001, Ventura et al. 1999). Correspondingly, the prevalence of coeliac disease is higher in several autoimmune disorders. In addition, patients with selective IgA deficiency have a ten-fold increased risk of coeliac disease (Collin et al. 1992).

Formerly coeliac disease was regarded as a rare disease of childhood (Davidson and Fountain 1950), but in the 1980s the diagnosis became more common also in older children and adults, and the clinical presentation shaded into milder forms (Logan et al. 1983, Mäki et al. 1988). At that time the prevalence of coeliac disease was still reported to be even as low as 0.1 % (Stevens et al. 1987), but the disease has since been seen to be much more common in many regions. Based on a report from 2010, the overall prevalence of coeliac disease is approximately 1 % in the general population in Europe (Mustalahti et al. 2010), similarly to the United States (Fasano et al. 2003). In Finland and Sweden the figure is as high as 2-3 %, while in Germany it is only 0.3 % (Mustalahti et al. 2010). Interestingly, coeliac disease seropositivity doubled in the two decades between 1980 and 2000 in the Finnish population, indicating the actual increase in the prevalence of the disease (Lohi et al. 2007).

In the past, coeliac disease was thought to affect mostly individuals of European origin. However, recent publications show that the disorder is also common in the Middle East, India, Pakistan and North Africa, where the highest seroprevalence, 5.6 %, is described among Saharawi children (Aziz et al. 2007, Catassi et al. 1999, Kochhar et al. 2012, Sood et al. 2006). Coeliac disease has also been described among people of Amerindian or African American ancestry (Brar et al. 2006, Mendez-Sanchez et al. 2006, Parada et al. 2011) and in China (Wu et al. 2010).

Coeliac disease develops in genetically predisposed individuals. Major histocompatility complex (MHC) II is the most important genetic factor (Trynka et al. 2011). The majority of coeliac disease patients, 90 %, express human leukocyte antigen (HLA)-DQ2.5 (DQA1*05, DQB1*02) (Sollid et al. 1989) and the remainder either HLA-DQ2.2 (DQA1*02:01, DQB1*02:02) or HLA-DQ8 (DQA1*03, DQB1*03:02) (Sollid and Lie 2005). It has been established that HLA-DQ2 gene has a strong dose effect, since HLA-DQ2 homozygous individuals have an at least 5-fold increased risk of disease development compared with HLA-DQ2 heterozygous individuals (Mearin et al. 1983). The

HLA-DQ molecules are important in the pathogenesis of coeliac disease in that they predispose to it by presenting gluten to $CD4^+$ T cells (Bodd et al. 2012, Lundin et al. 1994, Lundin et al. 1993). However, the presence of these HLA-DQ molecules is not sufficient for disease development, as 30 % of the general population have this genotype (Karell et al. 2003). Also non-HLA susceptibility genes have been localized and identified to clarify the complex genetics of coeliac disease. In 2010, a large genome-wide association study revealed 26 non-HLA associated loci related to coeliac disease (Dubois et al. 2010), and soon the number was raised to 39 when the issue was studied by the Immunochip platform (Trynka et al. 2011). Based on recent results it seems that a long noncoding RNA is also associated with susceptibility to coeliac disease, as it for instance represses expression of certain inflammatory genes under homeostatic conditions (Castellanos-Rubio et al. 2016).

2.1 Clinical features

2.1.1 Gastrointestinal manifestations

In the 1970s, coeliac disease was regarded as a severe malabsorption syndrome, the most typical symptoms being diarrhoea, malnutrition, weight loss and failure to thrive in childhood (Young and Pringle 1971). Deficiencies of nutrients such as various vitamins, iron, calcium and folic acid were found in patients as a consequence of malabsorption (Visakorpi and Mäki 1994). However, since the 1980s, changes in the clinical presentation have been observed (Mäki et al. 1988, Visakorpi and Mäki 1994). A recent study showed that for instance poor growth was common in Finland before the 1980s, but has become rarer up to the present decade (Kivelä et al. 2015). Also the proportion of children with gastrointestinal symptoms has decreased, and interestingly, at the turn of the 21st century, the most typical gastrointestinal symptoms diarrhoea and vomiting were replaced by constipation and abdominal pain (Kivelä et al. 2015). Thus the clinical picture has altered over time and the classical symptoms caused mostly by malabsorption are no longer the norm. Coeliac patients can present with minimal or no gastrointestinal symptoms and with diverse extraintestinal manifestations (Collin et al. 1997, Kivelä et al. 2015, Mäki et al. 1988, Murray et al. 2003).

Of note, the severity of clinical symptoms and histological findings do not necessarily correlate (Kaukinen et al. 2001, Murray et al. 2008, Thomas et al.

2009). It has been shown that early-stage coeliac disease patients positive for coeliac antibodies may suffer from various gastrointestinal symptoms even before the development of villous atrophy (Kaukinen et al. 2001, Kurppa et al. 2009). On the other hand, clinically silent coeliac disease has been described as a condition where patients have positive coeliac antibodies and small-intestinal mucosal lesion while remaining asymptomic (Mäki et al. 1991b, Vilppula et al. 2008). Clinically silent coeliac patients are frequently found in at-risk groups and family members (Mäki et al. 1991b).

2.1.2 Extraintestinal manifestations

In addition to gastrointestinal manifestations, coeliac disease patients may have extraintestinal symptoms. In many cases, these remain the only presenting signs of coeliac disease for a long period even in the absence of gastrointestinal symptoms with or without small-intestinal damage. This is why the disease might often be difficult to recognize (Korponay-Szabó et al. 2015).

Dermatitis herpetiformis, a skin form of coeliac disease, is one of the first well-established extraintestinal manifestations of coeliac disease. This most common gluten-dependent extraintestinal symptom typically affects the extensor surfaces of the skin in the knees and elbows (Collin and Reunala 2003). The majority of dermatitis herpetiformis patients show morphological changes in their small bowel (Reunala et al. 1984) and coeliac-specific autoantibodies are present in most untreated dermatitis herpetiformis patients (Dieterich et al. 1999, Salmi et al. 2014).

Coeliac disease has been linked to a variety of neurological conditions such as peripheral neuropathy, ataxia and epilepsy (Hadjivassiliou et al. 2014, Hadjivassiliou et al. 2002, Luostarinen et al. 1999). Coeliac patients have also been described with liver problems such as hypertransaminasaemia (Bardella et al. 1999, Volta et al. 1998) and even severe liver failure (Kaukinen et al. 2002). Furthermore, osteoporosis as well as osteopenia are fairly common manifestations of coeliac disease due to low bone-mineral density (Meyer et al. 2001, Mustalahti et al. 1999). Extraintestinal manifestations related to coeliac disease also include conditions such as muscular weakness (Korponay-Szabó et al. 2004), dental enamel defects (Aine et al. 1990), infertility and risk of pregnancy failure (Alstead and Nelson-Piercy 2003, Khashan et al. 2010, Rostami et al. 2001).

2.2 Damage in the small-intestinal mucosa of coeliac patients

2.2.1 Small-intestinal mucosal morphology

Small-bowel biopsy specimens, where villous atrophy and crypt hyperplasia is usually seen in coeliac patients consuming a gluten-containing diet, has long been the cornerstone of the coeliac disease diagnosis (Walker-Smith et al. 1990). Noteworthily, diagnosis should always be made from well-oriented and good-quality specimens, which allow correct evaluation of morphological changes (Collin et al. 2005, Taavela et al. 2013). The damage is usually most severe in the proximal parts of the small intestine, but it may be patchy and variable along the whole small intestine (Hopper et al. 2008, Ravelli et al. 2010).

The small-bowel mucosal damage in coeliac disease evolves gradually (Figure 1) as a result of an immunological response to gluten (more details in section 2.3) and may take a long time to develop (Lähdeaho et al. 2005, Mäki et al. 1990, Marsh 1992). According to the Marsh classification a spectrum of histological signs can be present and they include increased infiltration of lymphocytes (Marsh I), elongation of crypts (Marsh II) and finally villous atrophy with crypt hyperplasia (Marsh III) (Marsh 1992). Subsequently, Marsh III was divided into three subgroups depending on the degree of villous atrophy (Oberhuber et al. 1999). Villous height and crypt depth can also be measured and the villous height crypt depth ratio (Vh/CrD) calculated in order to evaluate the degree of mucosal damage. A ratio above 2.0 has usually been the cut-off for normal values (Kuitunen et al. 1982).

2.2.2 Immunohistochemical markers of small-intestinal damage

In addition to Vh/CrD, several immunohistochemical markers can be determined from small-intestinal biopsies. In the epithelial cell layer, the number of intraepithelial lymphocytes (IEL) carrying a certain type of surface molecule, CD3, is usually increased in coeliac disease patients (Järvinen et al. 2003, Kuitunen et al. 1982). IELs express different T cell receptors (TCR), of which a combination of α and β chains ($\alpha\beta^+$) is most commonly seen, but $\gamma\delta^+$ IELs are nevertheless more specific for coeliac disease (Järvinen et al. 2003, Savilahti et al. 1990, Selby et al. 1983). In the lamina propria, the changes in T lymphocyte densities are fairly small and amounts of specific cell types are similar to those in

controls (Selby et al. 1983, Verkasalo et al. 1990). However, the number of Ig containing cells is increased in the lamina propria in coeliac disease, most of them being plasma cells (Lancaster-Smith et al. 1977, Savilahti 1972). These plasma cells secrete both TG2 and DGP antibodies in the coeliac patient's small-intestinal mucosa (Di Niro et al. 2012, Steinsbø et al. 2014).

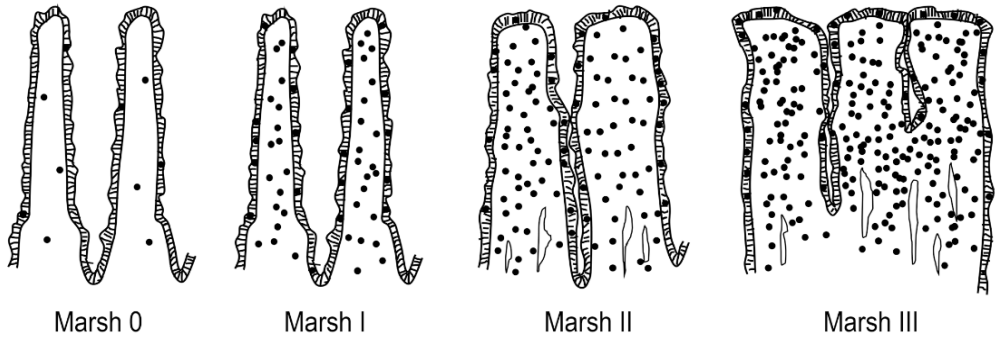

Figure 1. The gradual development of small-bowel mucosal lesion in coeliac disease. Marsh classifications include normal mucosa (Marsh 0), increased infiltration of lymphocytes (Marsh I), elongation of crypts (Marsh II) and villous atrophy with crypt hyperplasia (Marsh III). The figure is modified from Marsh 1992.

2.2.3 Small-intestinal vasculature

The vasculature supplies an important mechanical support to the small-intestinal villi. In each villous, a single arteriole traverses to the tip and creates a capillary tuft, which consists of capillaries branched from the arteriole (Matheson et al. 2000). However, several intestine-related diseases, for example inflammatory bowel disease and peritoneal adhesions, are characterized or contributed to by dysregulated growth or formation of blood vessels during the process called angiogenesis (Carmeliet 2003). It has been demonstrated that patients with inflammatory bowel disease have increased vascularization in the inflamed colonic mucosa and increased levels of several angiogenic factors (Alkim et al. 2012, Danese et al. 2006).

In coeliac disease, the possible role of angiogenesis in the pathogenesis is not completely understood. In the 1970s, endothelial swelling was observed in the small-bowel mucosal capillaries of coeliac disease patients after ingestion of gluten (Shiner and Ballard 1972, Shiner 1973). Abnormalities in the coeliac

small-intestinal mucosal vasculature were first described by Cooke and Holmes in the 1980s (Cooke and Holmes 1984). The investigators visualized the vasculature in post-mortem small-intestinal tissue by ink injections and observed remarkable differences between a coeliac and a non-coeliac disease patient. Most importantly, the overall organization of the vasculature was altered and capillary tufts were absent in the coeliac patient (Cooke and Holmes 1984). It was shown more recently that ingestion of gluten leads to an altered appearance of the small-bowel mucosal microvasculature in coeliac disease patients (Myrsky et al. 2009b). A clear difference in the overall organization of the mucosal vasculature between coeliacs and controls was already noted under a stereomicroscope when examining whole biopsies, and the observation was further confirmed by immunofluorescence stainings. In line with the study by Cooke and Holmes, also here a lack of capillary tufts in coeliac mucosa was described and instead the capillaries seemed to form a continuous subepithelial layer. In quantitative analysis of stainings, the number and maturity of the vessels in the coeliac small-intestinal mucosa was decreased when compared to controls. After one year on a gluten-free diet, the mucosal vasculature of the coeliac patients was normalized, resembling that of controls (Myrsky et al. 2009b). It was also verified by immunofluorescent staining that coeliac disease-specific IgA class autoantibodies form deposits around blood vessels and recognize TG2 on small-intestinal mucosal walls, as was previously shown (Hadjivassiliou et al. 2006, Kaukinen et al. 2005, Korponay-Szabó et al. 2004, Salmi et al. 2006a).

2.3 Pathogenesis of coeliac disease

The ingestion of gluten induces coeliac disease in HLA-DQ2- or -DQ8 positive individuals and such loss of oral tolerance to gluten may occur at any time in a person's life (Catassi et al. 2010). The exact mechanism underlying the development of small-intestinal mucosal lesion in coeliac disease is not fully understood. However, only a small proportion of genetically predisposed individuals will develop active disease, which suggests an important role for both immunologic and environmental factors in the complex coeliac jigsaw (Kupfer and Jabri 2012).

The environmental factors influencing coeliac disease development are still partly unknown, but they might involve for instance certain viral infections such as adeno- and rotaviruses, as these have been suggested to increase the risk of

coeliac disease (Kagnoff et al. 1987). Another factor might be that nowadays hygiene is so much improved in modern countries that lack of microbial exposure may increase the risk of autoimmune disease (Kondrashova et al. 2008, Lohi et al. 2007). In addition, changes in the intestinal microbiota (Verdu et al. 2015) are one possible contributor. Of note, the literature has not revealed a typical 'coeliac microbiota signature', but several studies have demonstrated that coeliac disease patients have altered faecal and duodenal microbiota compositions compared with healthy individuals (Verdu et al. 2015). Differing microbial colonization might be partially explained by factors such as birth delivery mode (Decker et al. 2010, Mårild et al. 2012), early antibiotic use (Canova et al. 2014) and feeding practices in childhood (Ivarsson et al. 2000, Ou et al. 2009).

Prolamins are the main storage proteins of wheat, barley and rye and they can evoke coeliac disease in genetically susceptible individuals. Oats appears to be mostly safe for coeliac patients (Janatuinen et al. 1995), although *in vitro* studies have shown that the immunogenicity of oats varies depending on the cultivar used (Comino et al. 2015). Prolamins are called as gliadins and glutenins in wheat, hordeins in barley and secalin in rye (Anand et al. 1978). Wheat storage proteins are also known as gluten, however, in the context of coeliac disease, gluten refers to the harmful proteins of wheat, barley and rye. Gluten is rich in proline and glutamine residues (Vader et al. 2003). Such a high proline content makes gluten particularly resistant to proteolytic degradation in the gastrointestinal tract (Shan et al. 2002). Wheat gluten-derived gliadins can be divided into α-, β-, γ- and ω-gliadin based on their structural differences, and it has been shown that α-gliadin is the most harmful for coeliac patients (Stoven et al. 2012). As a result of insufficient degradation of gluten, toxic and immunogenic gliadin peptides are generated (Shan et al. 2002). These so-called toxic peptides, for example α-gliadin peptide p31-43, can cause an innate immune response (Maiuri 2003). In contrast, adaptive immunity is activated by immunogenic peptides such as gliadin peptide p57-68 and the longer form p56-88, the 33mer (Arentz-Hansen et al. 2000, Qiao et al. 2004, Shan et al. 2002).

Gluten has been shown to exert several *in vitro* effects. For instance, in organ cultures carried out with coeliac patient-derived small-intestinal biopsies, gluten induces secretion of autoantibodies (Picarelli et al. 1996, Stenman et al. 2008), increases proliferation of crypt epithelial cells (Barone et al. 2007, 2000) and induces secretion and expression of interleukin (IL)-15 (Maiuri et al. 2000). In addition, *in vitro* studies made with Caco-2 cells have shown that gluten induces increased intestinal epithelial permeability (Drago et al. 2006, Rauhavirta et al.

2011, Sander et al. 2005). Increased permeability is also seen in untreated coeliac disease patients (Fasano et al. 2000, Madara and Trier 1980). However, the exact mechanism by which gluten-derived peptides cross the epithelium is not known. Ménard and co-workers (2012) have established that both toxic and immunogenic peptides can enter epithelial cells by an intracellular route in intestinal biopsies from coeliac patients. More specifically, these peptides enter the epithelial cells by endocytosis (Caputo et al. 2010, Zimmermann et al. 2014), but possibly use different trafficking routes in the cells (Barone et al. 2010, Schumann et al. 2008, Zimmer et al. 2010). In addition, it has been suggested that gliadin peptides can be transcytosed through the enterocytes by entering a recycling pathway together with secretory IgA, which is bound to transferrin receptor CD71, and thus are able to avoid lysosomal degradation (Lebreton et al. 2012). Another route to translocate gliadin peptides is the paracellular pathway. It has been observed that gluten induces a release of zonulin, a regulator of the epithelial tight junctions (Fasano et al. 2000, Tripathi et al. 2009), which might induce paracellular permeability of the epithelium (Clemente et al. 2003).

2.3.1 Innate immunity

The so-called toxic gliadin peptides, for example α-gliadin peptide p31-43, can elicit an IL-15-mediated innate immune response in the small intestine of coeliac disease patients, leading to intestinal epithelial damage (Hüe et al. 2004, Maiuri et al. 2003) (Figure 2). Intestinal epithelia, dendritic cells and macrophages are the major source of IL-15 (Di Sabatino et al. 2006, Maiuri et al. 2001), whose expression is actually increased both in the lamina propria and the epithelium of coeliac disease patients' small-intestinal mucosa (Di Sabatino et al. 2006, Iacomino et al. 2016, Maiuri et al. 2000, Mention et al. 2003).

Small-intestinal IELs, which play an important role in epithelial damage, are composed primarily of $CD8^+$ T cells expressing $\alpha\beta^+$, but also $\gamma\delta^+$, whose density is elevated in active coeliac disease (Han et al. 2013, Järvinen et al. 2003). Also a few natural killer (NK)-like cells are typically found among IELs (Jabri and Ebert 2007). IL-15 up-regulates both the natural killer cell group 2D (NKG2D) receptor on IELs and its epithelial ligand, MHC class I molecule A (MICA) on intestinal epithelial cells (Hüe et al. 2004, Mention et al. 2003, Meresse et al. 2004). Interaction of NK2GD and MICA drives epithelial cells to apoptosis at an increased level (Hüe et al. 2004) and thus enables defects in barrier function and leaking of the epithelium.

IL-15 has been suggested to be the main factor in the activation and selective expansion of IELs (Meresse et al. 2009, Meresse et al. 2004). Activation of cytotoxic IELs might also be induced by gluten-specific $CD4^+$ T cells through IL-21 (Kasaian et al. 2002, Monteleone et al. 2001) and interferon-γ (IFN-γ) (Nilsen et al. 1998, Perera et al. 2007). In addition, IL-15-activated T cells secrete cytokines such as IFN-γ, which is further promotive of the inflammatory process (Di Sabatino et al. 2006, Nilsen et al. 1998). IL-15 can also affect immune regulatory mechanisms and synergize with cytokines produced by $CD4^+$ T cells to stimulate the expansion of cytotoxic $CD8^+$ T cells (Abadie and Jabri 2014, Korneychuk et al. 2014, Meresse et al. 2012).

Toll-like receptors (TLRs), which are involved in the recognition of bacteria and other microbes, may also have a role in coeliac disease. A few studies have shown increased levels of TLR2 and TLR4 in coeliac disease patients, but altogether the results are controversial (Cseh et al. 2011, Eiró et al. 2012, Kalliomäki et al. 2012, Szebeni et al. 2007).

2.3.2 Adaptive immunity

Once immunogenic gliadin peptides such as gliadin peptide p57-68 and the 33mer reach the lamina propria they can activate an adaptive immune response in the coeliac disease patient's small intestine (Figure 2) (Arentz-Hansen et al. 2000, Qiao et al. 2004, Shan et al. 2002). These peptides are excellent substrates for TG2 (Bruce et al. 1985, Piper et al. 2002), which converts distinct glutamine residues to negatively charged glutamate in a process known as deamidation (Molberg et al. 1998, van de Wal et al. 1998). Thus the disease-causing immune response in coeliac disease is not to regular gluten antigen, but to posttranslationally modified gluten. This process enables deamidated gliadin to form complexes with HLA-DQ2 or -DQ8 molecules on antigen-presenting cells (APC) such as dendritic cells (Molberg et al. 1998, van de Wal et al. 1998). Prior to presenting gliadin peptides, APCs induce activation of naïve $CD4^+$ T cells (Meresse et al. 2012, Qiao et al. 2012) and the activated gluten-reactive $CD4^+$ T cells are thus able to contribute to the coeliac pathology by secretion of proinflammatory cytokines such as IFN-γ (Nilsen et al. 1998, Perera et al. 2007) and IL-21 (Kasaian et al. 2002, Monteleone et al. 2001). These cytokines can induce mucosal deterioration alongside other inflammatory mediators such as tumour necrosis factor (TNF)-α (Bajaj-Elliott et al. 1998, Deem et al. 1991). However, findings from an *in vivo* study by de Kauwe and associates (2009)

indicate that $CD4^+$ T cells, even together with HLA-DQ2 molecules and gluten ingestion, are not sufficient to cause a full-blown coeliac-like enteropathy. In most coeliac patients, the activation of $CD8^+$ cytotoxic IELs induced by IL-15 is also required for the onset of tissue damage as described in the previous section (Abadie and Jabri 2014).

How gluten-reactive T cells could provide help to TG2-specific B cells to produce autoantibodies is not completely understood. In the early 1990s Mäki

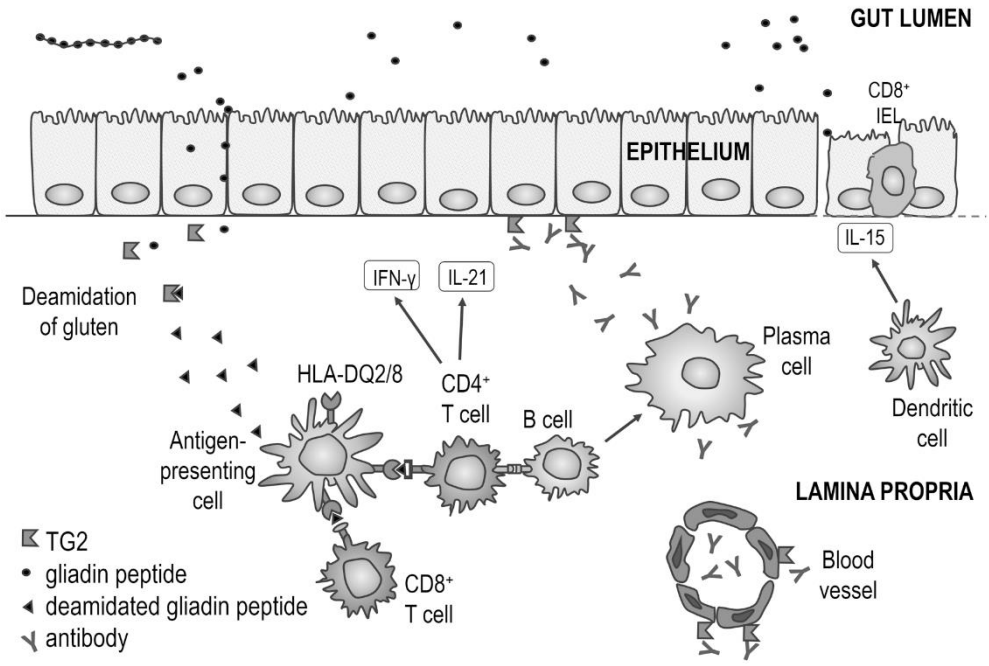

Figure 2. Summary of underlying innate and adaptive immunity system responses in coeliac disease pathogenesis (adapted from Meresse et al. 2012). Poorly digested gluten peptides are transferred from gut lumen to lamina propria either through intra- or paracellular route. Toxic gliadin peptides can induce an innate immune response mediated mainly by interleukin (IL)-15 leading to intestinal epithelial damage. On the other hand, immunogenic gliadin peptides can activate an adaptive immune response by entering the lamina propria. Once transglutaminase 2 (TG2) deamidates gluten peptides, deamidated peptides can form complexes with human leukocyte antigen (HLA)-DQ2 or -DQ8 molecules on antigen-presenting cells (APC) such as dendritic cells. This enables presentation of peptides to gluten-reactive T cells, which provide help to B cells to produce antibodies. Activated gluten-specific T cells secrete cytokines such as IL-21 and interferon-γ (IFN-γ), which are able to activate cytotoxic $CD8^+$ intraepithelial lymphocytes (IELs) together with IL-15 and further promote the inflammatory process.

suggested that coeliac autoantibodies could be generated in a mechanism involving the hapten-carrier model (Mäki 1994). Some years later it was further proposed that B cell receptors (BCR) on TG2-specific B cells form covalent complexes of TG2 and gluten peptides (Sollid et al. 1997). Furthermore, *in vitro* studies have shown that such TG2-gluten complexes are indeed formed (Fleckenstein et al. 2004) and that B cells expressing HLA-DQ2 and a TG2-reactive BCRs present complexes to gluten-reactive T cells (Di Niro et al. 2012).

Recently an alternative model has also been proposed for gluten-dependent TG2 autoantibody production by B cells (Iversen et al. 2015). In this model, TG2-mediated transamidation can cause cross-links between BCRs and gluten peptides (Iversen et al. 2015). Interestingly, especially TG2-specific IgD molecules are able to serve as substrates to TG2 and can become cross-linked to themselves or to gluten peptides (Iversen et al. 2015). Furthermore, this model suggests that B cells could take up gluten peptides with TG2 via BCR and subsequently release deamidated peptides and present them to gluten-reactive T cells (Iversen et al. 2015).

2.3.3 The coeliac disease autoantigen TG2

TG2 is an important player in the adaptive immune response, as it is a target for coeliac disease-specific autoantibodies (Dieterich et al. 1997). TG2 is a protein, which consists of an N-terminal β-sandwich domain, a core domain and two C-terminal β-barrel domains. Fibronectin and integrin binding sites are located in the N-terminal, whereas the regulatory and catalytic areas are in a core domain (Liu et al. 2002, Pinkas et al. 2007). TG2 is ubiquitously expressed throughout the body, for instance in liver, heart and intestine (Klöck et al. 2012). The cell types expressing TG2 include endothelial cells, fibroblasts and smooth muscle cells (Nurminskaya and Belkin 2012). TG2 has an important role in several processes such as apoptosis, wound healing and angiogenesis (Haroon et al. 1999, Jones et al. 2006, Telci and Griffin 2006). This structurally and functionally complex protein has both intracellular and extracellular locations. Among other tasks, on the cell surface TG2 contributes to cell adhesion, and within the cell it acts as a G-protein (Jones et al. 1997). Intracellular TG2 is usually in closed conformation and catalytically inactive, but during stress or injury is transiently activated and released outside cells (Siegel et al. 2008, Stamnaes et al. 2010). This enzymatic activation, which changes TG2 to an open conformation, is regulated by Ca^{2+} and guanine nucleotides (Pinkas et al. 2007, Smethurst and Griffin 1996).

However, extracellular TG2 is also mostly in an inactive although open state due to the oxidizing cellular environment (Stamnaes et al. 2010).

The enzymatic activity of TG2 is able to catalyze post-translational modification of glutamine residues. In the coeliac disease pathogenesis TG2 has a crucial role, as it deamidates distinct glutamine residues of gliadin peptides into glutamic acid. Deamidation increases the affinity of gliadin peptides towards HLA-DQ2 and -DQ8 and the stability of peptides bound to HLA molecules (Molberg et al. 1998, Xia et al. 2005). This process enables a stronger antigen presentation and an inflammatory process and leads to secretion of coeliac disease-specific TG2-targeted autoantibodies (Di Niro et al. 2012, Molberg et al. 1998). Moreover, TG2 catalyzes the formation of ε(γ-glutamyl)lysine isopeptide bonds between a glutamine residue on one substrate and a lysine residue on another. These crosslinks between various proteins, including fibronectin and collagen, are highly resistant to proteolysis and important concerning for instance the organization of the extracellular matrix (Aeschlimann and Thomazy 2000, Griffin et al. 2002). TG2 is also able to crosslink gluten peptides to itself (Fleckenstein et al. 2004), thus possibly enabling the production of the coeliac disease-specific TG2 autoantibodies.

2.4 Antibodies in coeliac disease

In coeliac disease, a variety of different antibodies might be present in the serum of the patients. However, only autoantibodies against TG2 and antibodies against gluten-derived deamidated gliadin peptides (DGP) are gluten-dependent and specific for coeliac disease (Korponay-Szabó et al. 2015). Both immunoglobulin (Ig) A and IgG antibodies against DGP can be detected in coeliac patient serum, while TG2 autoantibodies are predominantly of IgA (Giersiepen et al. 2012, Kaukinen et al. 2007, Lewis and Scott 2010). Patients with selective humoral IgA deficiency produce IgG class autoantibodies against TG2, or IgM class autoantibodies locally in the gut and in secretions (Borrelli et al. 2010, Korponay-Szabó et al. 2003a). Selective humoral IgA deficiency is more common in coeliac patients than in healthy individuals (Cataldo et al. 1997, Collin et al. 1992) and is coupled to an HLA-DQ2 background (Korponay-Szabó et al. 2003a). Regardless of differences in class of TG2-targeted autoantibodies, the clinical presentation of coeliac disease in IgA deficient patients is similar to that in IgA competent patients (Cataldo et al. 1998, Korponay-Szabó et al. 2003a).

Following chapters will describe coeliac antibodies in serum, serological tests and small-intestinal autoantibody deposits in more detail.

2.4.1 Serum antibodies

The first autoantibodies in coeliac disease, antireticulin antibodies (ARA), were described by Seah and colleagues in the 1970s (Seah et al. 1971). These autoantibodies recognize reticular fibres of the endomysium in rodent connective tissue. In 1983, a new autoantibody against the endomysium of the monkey oesophagus was discovered (Chorzelski et al. 1983), and around a decade later it was observed that the monkey oesophagus can be replaced by human umbilical cord when testing for these endomysial antibodies (EmA) from patients' sera (Ladinser et al. 1994). An important finding was made in 1997, when the autoantigen of EmA was recognized to be TG2 (Dieterich et al. 1997). Autoantibodies against TG2 can be measured in coeliac patients' sera by enzyme-linked immunosorbent assay (ELISA), using either human or guinea pig TG2, and this assay has high sensitivity and specificity for the disease (Collin et al. 2005, Fabiani et al. 2004, Sulkanen et al. 1998b). Of note, Korponay-Szabo and associates have shown that ARA also target TG2 and that autoantibodies against TG2 are responsible for ARA and EmA tissue binding in coeliac patient sera (Korponay–Szabó et al. 2000, Korponay-Szabó et al. 2003b).

Nowadays highly sensitive and specific tests are available for EmA and TG2 autoantibodies, but have certain limitations. The EmA test is a semiquantitative and subjective method and thus the result may vary depending on the laboratory in question (Rostom et al. 2005). Also TG2 autoantibody results from ELISA have been somewhat heterogeneous between laboratories depending on the exact methodology used in the tests (Hopper et al. 2007). However, in validated and well-controlled conditions these methods show good accuracy (Rostami et al. 1999). TG2 autoantibodies can also be tested by a coeliac disease rapid test developed for point-of-care detection. This test requires only a fingertip blood sample and shows high accuracy for coeliac disease (Korponay-Szabó et al. 2005, Raivio et al. 2006).

Interestingly, IgA or IgG class autoantibodies against TG2 might also be detected in patients presenting with inflammatory bowel disease (Farrace et al. 2001, Lidar et al. 2009), viral infection including HIV (Pereda et al. 2001) or end-stage heart failure (Peracchi et al. 2002). Furthermore, TG2 autoantibodies have been detected in patients with other autoimmune disorders than coeliac disease

(Sardy et al. 2002, Szondy et al. 2011). However, these autoantibodies are not recognized in immunofluorescent EmA stainings performed with frozen sections of human umbilical cord (Sárdy et al. 2007). In this test fibronectin-bound TG2 antigen catches coeliac-specific TG2 autoantibodies, and thus non-coeliac TG2 autoantibodies, which often target the other TG2 epitopes, may not bind and are usually EmA negative (Szondy et al. 2011). Recently it has been shown that the epitopes of TG2 autoantibodies in coeliac patients are very conservative, and four distinct major epitopes have been identified in TG2 (Iversen et al. 2013, Simon-Vecsei et al. 2012). It is thus plausible that TG2 autoantibodies in the patients present with conditions such as inflammatory bowel disease, viral infection or end-stage heart failure indeed target different epitopes than those recognized by coeliac patient autoantibodies (Simon-Vecsei et al. 2012). Although suggested by Simon-Vecsei and associates (2012), this conception has not yet been applied to diagnostic ELISA tests.

The role of antibody testing is becoming increasingly important in coeliac disease diagnostics. Until 2012, histological evaluation of small-bowel mucosal biopsy was always required and diagnosis was based on the finding of mucosal deterioration, implicating crypt hyperplasia and villous atrophy, as instructed by the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN). Coeliac disease-specific autoantibodies were considered only supportive for the diagnosis (Walker-Smith et al. 1990). However, the diagnostic criteria were recently revised (Husby et al. 2012) and now there is an option to diagnose genetically predisposed symptomatic children without biopsy. This is possible if the patient's serum TG2 autoantibody levels are 10 times greater than the upper limit of normal, the patient is EmA positive and responds well to a gluten-free diet.

Most commonly, serum autoantibodies against TG2 are measured for the diagnostics of coeliac disease, but also tests against anti-gliadin antibodies (AGA) and DGP antibodies have been used (Kaukinen et al. 2007). However, AGA has also been detected in other gastrointestinal diseases, other disorders and in healthy individuals, and thus the specificity is not sufficient for diagnostical purposes in coeliac disease (Kaukinen et al. 2007, Mäki 1995). On the other hand, antibodies against DGP are useful in the diagnostics as it has been shown that they have higher diagnostic accuracy than conventional AGA (Kaukinen et al. 2007, Schwertz et al. 2004). These antibodies might actually be the first serological markers detected in coeliac disease patients in early-stage disease, even before the appearance of villous atrophy (Kurppa et al. 2011, Simell et al. 2007).

Furthermore, several autoantibodies such as antibodies against actin (Clemente et al. 2000), calreticulin (Tučková et al. 1997), desmin (Teesalu et al. 2001) and a 90 kDa dermal glycoprotein (Teppo et al. 1987) can also be found in coeliac disease patients. However, they have only a restricted role in the diagnosis of the disease (Alaedini and Green 2008). Interestingly, serum anti-actin autoantibodies seem to correlate with the presence of villous atrophy (Clemente et al. 2000).

Autoantibodies against transglutaminase 3 (TG3) have been identified in the serum of dermatitis herpetiformis patients (Sardy et al. 2002). In addition, coeliac disease patients without rash may have autoantibodies reacting with TG3, but these, unlike TG2 autoantibodies are not gluten-dependent (Salmi et al. 2016). In addition, autoantibodies against transglutaminase 6 (TG6) have been found in patients with neural involvements such as gluten ataxia (Hadjivassiliou et al. 2013).

2.4.2 Small–bowel mucosal autoantibodies

In coeliac disease, the lesion in a patient's small-intestinal mucosa is characterized by an increased number of plasma cells in the lamina propria, as mentioned before (Baklien et al. 1977). Marzari and associates (2001) have shown, by isolating coeliac disease-specific TG2 autoantibodies from intestinal lymphocyte libraries, that these plasma cells actually produce TG2-specific autoantibodies. More recently Sollid's group visualized both DGP and TG2 positive plasma cells in coeliac lesions and found that on average 10 % of IgA producing plasma cells are specific for TG2 and only around 1 % for DGP (Di Niro et al. 2012, Steinsbø et al. 2014). However, IgA plasma cells seem to disappear within months on a gluten-free diet (Sugai et al. 2006, Sulkanen et al. 1998b).

IgA deposits were already observed in the coeliac patients' small-intestinal mucosa some decades ago (Shiner and Ballard 1972) and much later it was shown that they are targeted to extracellular TG2 (Korponay-Szabó et al. 2004). The deposits are located in the small-bowel mucosa under the epithelial cell layer at the basement membrane and around blood vessels (Korponay-Szabó et al. 2004, Koskinen et al. 2008, Lancaster-Smith et al. 1976). TG2-targeted IgA deposits can be detected in practically all coeliac disease patients with villous atrophy (Koskinen et al. 2008, Kurppa et al. 2010, Tosco et al. 2008), and their specificity is high (Kaukinen et al. 2005, Koskinen et al. 2008, Tosco et al. 2008). In fact,

such mucosal autoantibody deposits also precede and seem to predict the development of villous atrophy (Kaukinen et al. 2005, Salmi et al. 2006a, Tosco et al. 2008). TG2-targeted autoantibody deposits have been shown to be present in the mucosa even in advance of measurable levels of circulating serum autoantibodies (Salmi et al. 2006b). Taking all this into consideration, detection of the IgA deposits in the mucosa could be a valuable tool in the diagnosis of early-stage coeliac disease and in seronegative cases.

In addition to being present in the small-intestinal mucosa, IgA class TG2 autoantibodies have extraintestinal target sites for example in the liver, muscles, kidney and brain (Hadjivassiliou et al. 2006, Korponay-Szabó et al. 2004). Dermatitis herpetiformis patients may have IgA deposits in the subepidermal region of the skin and these deposits contain TG3, not TG2 (Sardy et al. 2002). If the patient is IgA deficient with TG2-targeted IgG autoantibodies in the serum, IgG deposits might be found in the liver and kidney and IgM deposits in the small bowel, as mentioned before (Borrelli et al. 2010, Korponay-Szabó et al. 2004).

2.4.3 Antibodies in the pathogenesis of coeliac disease

During the adaptive immune response, antibodies are released to the intestinal lumen and the circulation (Meresse et al. 2012) and can be found as deposits on the small-intestinal mucosa in untreated coeliac disease patients (Korponay-Szabó et al. 2004). The production of these TG2-specific autoantibodies would thus appear to be tightly connected to the development of coeliac disease. Despite their important role in diagnostics, there is debate as to whether the anti-TG2 response plays any part in the pathogenesis of coeliac disease. The biological effects of TG2 autoantibodies have been tested in *in vitro* studies, where cell cultures have been supplied either with coeliac patient serum IgA or recombinantly produced IgG-class anti-TG2 autoantibodies.

Some studies clearly show that coeliac disease-specific TG2 autoantibodies affect the epithelial cell biology characteristic of coeliac disease. It has been shown, for instance, that both treatments, coeliac IgA and monoclonal TG2 autoantibodies, inhibit the differentiation of T84 intestinal crypt epithelial cells (Halttunen and Mäki 1999) and that monoclonal autoantibodies induce proliferation of intestinal epithelial cells (Barone et al. 2007). Furthermore, coeliac patients' sera have also been found to induce monocyte activation and increase the transepithelial permeability of intestinal epithelial cells (Zanoni et al. 2006). In accord with the latter effect, there are studies suggesting that coeliac

patient-derived IgA enables translocation of gluten-derived gliadin peptides across the epithelial barrier (Lebreton et al. 2012, Matysiak-Budnik et al. 2008, Ménard et al. 2012, Rauhavirta et al. 2011). Interestingly, sera or purified TG2-specific autoantibodies from coeliac patients are also able to reduce the attachment of epithelial cells to the TG2-fibronectin matrix (Teesalu et al. 2012).

Furthermore, coeliac antibodies also seem to affect the endothelial cell biology. It has been shown that coeliac IgA fraction and IgG class TG2 autoantibodies disturb angiogenesis in several steps *in vitro* (Myrsky et al. 2008) as well as increase vascular permeability (Myrsky et al. 2009a). The underlying mechanism might involve the redox sensor protein thioredoxin, a novel regulator of extracellular TG2, as its secretion is promoted by coeliac IgA in endothelial cell cultures and it is needed to keep TG2 in a conformation suitable for antibody binding and activation (Nadalutti et al. 2013). In addition, due to the presence of coeliac patient-derived IgA, endothelial cells attach weakly and they also have high susceptibility to detach from fibronectin (Nadalutti et al. 2014). All these afore-mentioned effects might contribute to the abnormalities observed in coeliac patient small-intestinal mucosa (Cooke and Holmes 1984).

Recently, the binding of autoantibodies to TG2 has been investigated. Simon-Vecsei and co-workers (2012) showed that TG2 autoantibodies from different coeliac patients recognize the same conformational TG2 epitope formed by spatially close amino acids in three adjacent domains in core areas. Subsequently it was noted by Sollid's group that in addition to the epitope already found, autoantibodies also recognize at least three other conformational epitopes (Iversen et al. 2013). Of note, it has recently been demonstrated that binding of coeliac autoantibodies to TG2 seems to affect the structure of TG2. This would suggest that autoantibodies may have a pathogenetic role in coeliac disease, since by having an impact on the function of enzyme they are able to interfere for instance with the binding between TG2 and its substrates (Iversen et al. 2014). Also the enzymatic activity of TG2 could play a role in the pathogenesis, but results reported so far are contradictory. Two studies have shown coeliac patient-derived antibodies to increase TG2 activity (Király et al. 2006, Myrsky et al. 2009a), whereas a few have reported inhibition of activity (Byrne et al. 2010, Dieterich et al. 2003, Esposito et al. 2002), and in one study coeliac antibodies had no effect on TG2 activity (Di Niro et al. 2012).

2.5 The progression of the small-intestinal mucosal damage in the early phases of coeliac disease: synergistic innate and adaptive immune response

The mechanisms of tissue destruction during the progression of coeliac disease have not been fully unraveled. Coeliac disease is a complex systemic disease and thus there are a number of factors contributing to the development of small-intestinal mucosal damage. It has been shown that in early phases of coeliac disease, patients still present with normal small-intestinal mucosal villous structure, but may already have an increased number of IELs (Marsh 1992). Similar results have been seen in animals, as recruitment of IELs was observed in mice expressing either human HLA-DQ2 or -DQ8 (de Kauwe et al. 2009, Verdu et al. 2008). However, these mice did not develop a coeliac-like small-intestinal pathology. The results reported by Setty and colleagues (2015) show that a high number of IELs alone might not be sufficient to induce intestinal damage. They found that seronegative subjects with coeliac disease in the family history evinced signs of epithelial stress and an increased number of activating NK-receptors on cytotoxic IELs like patients with coeliac disease. Strikingly, early coeliac patients seem to lack these characteristics, and in subjects with a coeliac family history, high levels of inhibitory NK receptors prevented the destruction of intestinal epithelial cells. In the study in question, epithelial stress was characterized by ultrastructural changes in the intestinal enterocytes and by the expression of innate epithelial stress markers such as IL-15 (Setty et al. 2015).

In addition to high numbers of IELs, TG2-specific autoantibodies are found in coeliac patients prior to small-intestinal damage (Kaukinen et al. 2001, Paparo et al. 2005, Salmi et al. 2006a). In mouse experiments, mice with humanized HLA-DQ8 did not develop an anti-TG2 autoantibody response irrespective of gliadin sensitization (Verdu et al. 2008), but few of the gluten-immunized HLA-DQ2 expressing mice were positive for TG2 autoantibodies after induction of intestinal inflammation with pertussis toxin (de Kauwe et al. 2009). Of note, only these few cases proceeded to mild enteropathy with elongated crypts and increased cell density in the lamina propria (de Kauwe et al. 2009). It could be suggested that other factors along with TG2 autoantibodies are also necessary to induce full-blown villous atrophy. This is in line with recent results where it was proposed that the presence of immunity to gluten, which is observed in early coeliac patients, is not alone sufficient (Setty et al. 2015).

The production of TG2 autoantibodies, already observed in early phases of coeliac disease, is dependent on the presence of gluten-specific $CD4^+$ T cells recognizing HLA-DQ2 or -DQ8 in the lamina propria (Di Niro et al. 2012). This raises the question whether $CD4^+$ T cells may be required to activate IELs and induce tissue damage. T cells also produce massive amounts of IFN-γ in the untreated coeliac mucosa (Nilsen et al. 1998, Troncone et al. 1998) and the levels are already increased in early phases of the disease (Borrelli et al. 2013). Interestingly, gluten-immunized mice with human $CD4^+$ T cells expressing either human HLA-DQ2 or -DQ8 did not develop villous atrophy (Black et al. 2002, de Kauwe et al. 2009). Anti-gluten T cell immunity may thus not be sufficient to induce villous atrophy.

Furthermore, the contribution of the immunoregulatory system in tissue destruction in coeliac disease has been investigated. The frequency of $CD4^+CD25^+$ regulatory T cells (Tregs) expressing forkhead box P3 transcription factor ($Foxp3^+$) is increased in the coeliac lesion (Vorobjova et al. 2009, Zanzi et al. 2011), as well as in early phases of coeliac disease, albeit in lower numbers than in active disease (Borrelli et al. 2013). Furthermore, $Foxp3^+$ Tregs from early-phase coeliac patients are able to exert suppressive effects on responder T cells ($CD4^+CD25^-$) and IL-15 is not able to impair their activity in terms of proliferation and IFN-γ production (Borrelli et al. 2013). This is in contrast to active coeliac disease, where the suppressive capacity of $Foxp3^+$ Tregs might already be impaired by IL-15 (Zanzi et al. 2011). This difference can be explained by the lower level of IL-15 receptor expression on Tregs in early coeliac patients compared with active coeliac disease (Borrelli et al. 2013). Interestingly, an increased number of $Foxp3^+$ Tregs has also been found in gluten-immunized HLA-DQ8 expressing mice which did not evince villous atrophy, whereas an increased number of IELs was observed (Verdu et al. 2008). Altogether, these results show that regulatory mechanisms have an important role in downregulating the inflammation in the early phase of coeliac disease.

IL-15 is a pleiotropic cytokine at the interface of adaptive and innate immunity (Maiuri et al. 2003, Mention et al. 2003) and the previous paragraphs have already highlighted its role in the epithelial stress and immunoregulatory processes. As previously mentioned, coeliac disease patients overexpress IL-15 in both lamina propria and epithelium (Di Sabatino et al. 2006, Iacomino et al. 2016, Maiuri et al. 2000, Mention et al. 2003), whereas in early coeliac disease the levels are still lower (Borrelli et al. 2013). In fact, Setty and colleagues found that up to 80 % of individuals with TG2 autoantibody positivity had normal levels of IL-15

expression in the small-intestinal epithelium. Interestingly, IL-15 transgenic mice overexpressing IL-15 only in the lamina propria showed no signs of coeliac disease (DePaolo et al. 2011, Fehniger et al. 2001). When these mice were crossed onto humanized HLA-DQ8 transgenic mice, they developed a gluten-specific IFN-γ T cell response with the presence of anti-TG2 autoantibodies as well as an increased number of IELs while on a gluten-containing diet, possibly resembling early coeliac disease. In both models, the mice retained a normal histology in the small intestinal mucosa (DePaolo et al. 2011). This supports the conception that in the absence of the epithelial stress associated with IL-15 overexpression in the epithelium, adaptive anti-gluten immunity is insufficient to induce tissue damage (Setty et al. 2015). Interestingly, when human IL-15 was overexpressed in both epithelium and lamina propria in the small intestine of mice as in active coeliac disease, slowly developing chronic severe enteropathy partially mimicking the pathology of coeliac disease was observed in the mice (Ohta et al. 2002). This was accompanied by an increased number of $CD8^+$ T cells and lamina propria plasma cells, as well as the presence of anti-TG2 autoantibodies in the serum (Korneychuk et al. 2014, Ohta et al. 2002, Yokoyama et al. 2011). In addition, it seems that in the lamina propria IL-15 is able to affect adaptive immune responses by acting on dendritic cells and to promote the development of inflammatory T cell processes to orally ingested gluten in the lamina propria (DePaolo et al. 2011, Mora and Von Andrian 2004). In the epithelium an increased level of IL-15 extensively influences the function of IELs (Abadie et al. 2012, Abadie and Jabri 2014). Taking into account all the above-mentioned results it might be suggested that IL-15 overexpression in both lamina propria and epithelium in the small intestine is necessary for the development of villous atrophy. All in all, it is likely that IL-15, adaptive anti-gluten immunity, and IEL activation induce villous atrophy (Kim et al. 2015).

3 Studying the effects of autoantibodies in autoimmunity

Autoantibodies detected in the patient sera are of importance in the diagnostics of autoimmune diseases. Interestingly, autoantibodies have been shown to have strong predictive value for distinct autoimmune diseases and they have been found in patients' sera already several years before the appearance of clinical symptoms (Lleo et al. 2010). Although it can be stated that the role of autoantibodies in the diagnosis is inevitable, their contribution to pathogenesis *per se*, however, is unclear in many autoimmune diseases (Rowley and Whittingham 2015). Most of the autoimmune diseases have highly complex pathogenesis that involves several pathways. The effects of autoantibodies in those processes have been largely studied using various techniques utilizing mostly patient-derived serum, immunoglobulin fractions, purified autoantibodies and recombinantly produced autoantibodies (Lleo et al. 2010).

One option to investigate the pathogenic role of autoantibodies is to use cell cultures supplemented with autoantibodies, and control antibodies. The biological effects of autoantibodies have been tested for example in coeliac disease with epithelial and endothelial cells as described before (see section 2.4.3), in pemphigus vulgaris with keratinocytes (Ishii et al. 2005) and in rheumatoid arthritis with chondrocytes (Amirahmadi et al. 2004, Amirahmadi et al. 2005), just to mention few. Furthermore, depending on which characteristics are being studied, a wide variety of drugs for instance to induce immune response can be added and different assays performed with cell cultures. Also an *in vitro* model involving cryosections of human skin, for instance, has been used to study, how skin is affected by autoantibodies from patients with subepidermal blistering disease of the skin (Sitaru et al. 2002a, Sitaru et al. 2002b).

Not just cells, but also tissues have been cultured in the presence of distinct autoantibodies. For instance in rheumatoid arthritis, bovine cartilage explants were treated either with disease or control autoantibodies (Crombie et al. 2005, Croxford et al. 2010). Also in coeliac disease small-intestinal biopsy samples have been cultured to study the transport of gliadin peptides through intestinal

epithelium in the presence of coeliac antibody deposits (Lebreton et al. 2012, Matysiak-Budnik et al. 2008, Ménard et al. 2012, Rauhavirta et al. 2011).

Furthermore, the pathogenicity of autoantibodies has experimentally been studied in animals. Several examples show, that passive transfer of plasma, serum, immunoglobulin fractions or autoantibodies from patients with distinct autoimmune disorder can induce features of the given disease in animals. These diseases involve for example pemphigus vulgaris (Anhalt et al. 1982, Roscoe et al. 1985), myasthenia gravis (Toyka et al. 1977) and Sjögren's syndrome (Robinson et al. 1998, Wang et al. 2004). In human subjects, transplacental transfer of IgG from an affected mother to the fetus occurs in a minority of autoimmune diseases (Baumann and Rubin 1973, Chorzelski et al. 1976, Morel et al. 1988). It provides us convincing data on the effects of autoantibodies on the development of the underlying disease.

The present study

4 Aims of the study

The aim of this study was to establish whether coeliac disease patient antibodies and more specifically TG2-targeted autoantibodies have biological effects *in vivo*.

The specific aims were:

1. to assess the pathogenic role of coeliac disease patient antibodies *in vivo* by injecting mice with sera or immunoglobulins from coeliac disease patients (**I**).

2. to show specific *in vivo* effects of TG2-targeted autoantibodies by injecting mice with monoclonal coeliac disease patient-derived recombinant autoantibodies (**II**).

3. to study whether coeliac disease-specific antibodies inhibit angiogenesis *ex vivo* and *in vivo* (**III**).

5 Materials and methods

5.1 Determination of coeliac disease-specific autoantibodies, human IgG levels and serum alanine aminotransferases (I-III)

IgA and IgG class coeliac disease-specific EmA were determined using an in-house indirect immunofluorescence method and human umbilical cord as substrate. More specifically, umbilical cord from a premature baby was used in assessing IgG class autoantibodies. A serum dilution of 1: ≥5 or more was considered positive for IgA class autoantibodies and 1: ≥10 or more for IgG class autoantibodies (Korponay-Szabó et al. 2003a, Ladinser et al. 1994, Sulkanen et al. 1998a). Positive antibody preparations were further diluted 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000, 1:4000, and up to 1:8000, 1:12000 and 1:16000 when necessary. In addition, a commercial ELISA using human recombinant TG2 as antigen was used to measure IgA and IgG class autoantibodies against TG2 (Celikey, Phadia, Freiburg, Germany). Values ≥5 U/ml, and ≥7 U/ml (**I**) or ≥3 U/ml (**II**), respectively, were considered positive according to the manufacturer's recommendations (**I-III**).

Determination of human IgG levels was made with an Immunoglobulin G ELISA Kit specifically recognizing human immunoglobulins (ImmunoDiagnostik, Bensheim, Germany) according to the manufacturer's instructions. The levels of human IgG were given as mg/ml, based on the standards provided by the kit **(I-II)**.

Serum alanine aminotransferase values were measured by standard kinetic methods on an Abbott Architect clinical chemistry analyzer (Abbott Laboratories, Abbott Park, IL, USA) (**I**).

5.2 Patient samples (I, III)

Serum samples from IgA deficient untreated biopsy-proven coeliac disease patients were obtained for the study **I** (Table 1). IgA deficient patient sera were used, since no murine equivalent to Fc-receptor for IgA has been found

Table 1. Sample forms and assays used in each original publication.

Original article	Serum	Purified immunoglobulins	Miniantibodies
I	*In vivo*	*In vivo* (IgG class)	-
II	-	-	*In vivo*
III	-	*In vivo* (IgA class)	*In vitro, ex vivo, in vivo*

IgA/G, A/G class immunoglobulin

and human IgA has been reported to be rapidly degraded in rodents (Bogers et al. 1989, Monteiro and Van De Winkel 2003). Four of the coeliac disease patients were female and six male, with a mean age of 6 years (range 2–13 years). Four of the patients evinced IIIB and the rest IIIC lesion according to the Marsh classification (Marsh 1992). Total serum IgA levels were <0.05 g/l in all patient samples, thus to be regarded as classical selective humoral IgA deficiency while still having normal IgG and IgM levels. All patients were positive for serum IgG class EmA and TG2 autoantibodies (Table 2), but negative for IgA class coeliac autoantibodies. The control sera for study **I** were derived from healthy non-coeliac subjects and were negative for both EmA and TG2 autoantibodies (Table 2).

For the IgA purifications in study **III** (Table 1), serum specimens from three IgA competent biopsy-proven coeliac disease patients on a gluten-containing diet were employed. Sera were positive for both IgA class EmA and TG2 autoantibodies (Table 2). Serum samples from three non-coeliacs were used as controls and all were negative for the above-mentioned autoantibodies (Table 2).

Table 2. Patients and controls.

Patient/Control group	Number (n)	TG2 auto-antibodies (U/ml)	EmA titre	Original article
IgA deficient CD patients	10	>100 (IgG class)	≥1:4000 (IgG class)	I
IgA competent CD patients	3	>100 (IgA class)	≥1:2000 (IgA class)	III
Non-CD controls	7	neg	neg	I, III

CD, coeliac disease; EmA, endomysial antibody; IgA/G, A/G class immunoglobulin; neg, negative; TG2, transglutaminase 2

5.3 Purification of serum immunoglobulins (I, III)

Total IgG fractions were purified from a subset of IgA deficient coeliac disease patients and non-coeliac controls using Protein G Sepharose 4B fast flow (Sigma-Aldrich, St Louis, MO, USA) for study **I** (Table 1). The serum samples were passed through the column and the column was washed with 0.075 M Tris-HCl, pH 8.0, and 0.5 M NaCl. Thereafter IgG was eluted with 0.1 M glycine-HCl in 0.5 M NaCl, pH 2.5 and the collected IgG fractions were neutralized with 1 M Tris-HCl, pH 8.0. Glycine was removed by passing the samples through PD-10 columns (GE Healthcare, Buckinghamshire, UK). The median concentration for TG2 autoantibodies in purified coeliac serum samples was above 100 U/ml (min 88.9 U/ml, max >100 U/ml) and below cut-off value in control samples. Subsequently the IgG samples were lyophilized and resolubilized in phosphate-buffered saline (PBS) to a final concentration of 100 µg/ml.

Total IgA fractions from IgA competent serum samples were purified as previously described (Myrsky et al. 2008) **(III)** (Table 1). In brief, the purification was performed using cyanogen bromide-activated Sepharose 4B (Pharmacia Upjohn, Uppsala, Sweden) coupled with 7 mg/ml rabbit anti-human IgA antibodies (Sigma Aldrich,). The samples then were lyophilized and resolubilized in Hank's balanced salt solution to a final concentration of 100 µg/ml. Purified immunoglobulins were used in the experiments (**III**) at a concentration of 1µg/ml.

5.4 Production of monoclonal miniantibodies (II, III)

IgG class recombinant monoclonal autoantibodies were used in studies **II** and **III** (Table 1). Coeliac patient-derived anti-TG2-specific monoclonal miniantibody clone 4.1 targeting the major coeliac TG2 epitope (referred to in studies **II** and **III** as CD Mab) was used as coeliac antibody. Irrelevant control antibodies were targeted against a Helicobacter pylori antigen (referred to in study **II** as non-CD Mab) or Escherichia coli proteins M5 and M6 (clones 5.1 and 6.2, referred to in study **III** as non-CD Mab) (Marzari et al. 2001, Simon-Vecsei et al. 2012).

Miniantibodies were produced in scFv-Fc format in Chinese hamster ovary cells transfected with pUCOE **(II)** or pCDNA3.1/Hygro(+) (**III**) vector as previously described (Boscolo et al. 2012, Di Niro et al. 2007). For study **II**, miniantibodies were isolated from the medium with an HiTrap Protein A HP column (Bed Volume 5 ml, GE Healthcare Europe, GmbH, Freiburg, Germany).

Prior to isolation the medium was diluted to binding buffer (20 mM sodium phosphate buffer, pH 7). Unbound proteins were removed by washing with binding buffer followed by elution of miniantibody with 0.1 M citric acid (pH 3) into tubes containing neutralization buffer (1 M Tris, pH 9). Elution fractions containing monoclonal miniantibody were pooled and immediately dialyzed against PBS (pH 7.5). Protein concentrations were determined by measuring absorbance at a wavelength of 280 nm. Thereafter, concentrations of miniantibodies were calculated using the molar attenuation coefficient (ε = 135150 $L \cdot mol^{-1} \cdot cm^{-1}$) and molecular weight (MW = 57101.2 g/mol) for the protein. Purified miniantibodies used in the angiogenesis assays (**III)** were received from Professor D. Sblattero and were used in the experiments at a concentration of 1 µg/ml.

5.5 Experimental animals, housing and husbandry (I-III)

For studies **I** and **II**, female Hsd:Athymic Nude-Foxn1nu mice lacking T cells approximately 6 weeks of age were used (**I**: Harlan, Venray, The Netherlands, **II**: Envigo, Gannat, France). All animals were kept in semi-barrier conditions in the facilities of the Central Animal Laboratory, University of Turku, Finland and acclimatized for at least two weeks prior to the commencement of the studies. At the beginning of the experiments, animals were housed in top-filtered stainless steel type II cages (36.5 x 20.7 x 14 cm) with solid base, with nesting material and an igloo as environmental enrichment and Aspen chips as bedding (Tapvei Ltd, Paekna, Estonia). The temperature range in the animal room was 19-23 °C, the relative humidity 42 to 60 %, and artificial illumination with a 12-h light/dark cycle. Throughout the study period, the mice had ad libitum access to a standard gluten-containing mouse chow (Special Diet Services, Witham, Essex, UK) and were provided free access to tap water in polycarbonate bottles. The mice were considered specific-pathogen-free (including free of mouse norovirus and Helicobacter species) based on the results of microbiological screening in the colony, and during the course of the experiments their physical condition was assessed daily on the basis of gross clinical examination. The mice were cared for and used in accordance with the current European (Directive 2010/63/EU) and Finnish (Act 2013/497 and Decree 2013/564) legislation on the protection of animals used for scientific purposes.

For *ex vivo* and *in vivo* angiogenesis studies (**III)**, 4-6-week-old female Balb/c mice (Harlan Laboratories Inc., Horst, the Netherlands) or C57BL/6 wild type or TG2 knockout mice (De Laurenzi and Melino 2001) were used. These studies were performed in the animal facilities of the University of Turku, Finland, and the University of Debrecen, Hungary. The mice were housed at 22 ºC in a 12-hour light/dark cycle with water and standard gluten-containing food freely available. The animals were cared for and used in accordance with the regulations in Finland, Hungary and the European Union (86/609/EC).

5.6 Passive transfer of coeliac disease patient-derived antibodies into athymic mice (I, II)

5.6.1 Study design and experimental procedures (I, II)

Mice were injected with human sera and purified serum total IgG previously collected from untreated coeliac disease patients and healthy controls (**I**), or with monoclonal miniantibodies (CD Mab or non-CD Mab) and PBS (**II**). The single intraperitoneal injections were administered daily during the experiments. Sera from coeliac patients or healthy controls were administered with an injection of 200 µl for either 8 (n=3 mice/group) or 27 days (n=5 mice/group). In another set of experiments, 200 µl of purified serum total IgG from coeliac disease patients or controls for 8 days (n=5 mice/group) were injected into the mice. Non-injected control mice were included in the 27-day serum and the 8-day total IgG experiments (n=2 in each experiment). In addition, CD Mab (n=10 mice), non-CD Mab (n=10 mice) or sterile PBS (n=5 mice) were injected (10 ml/kg mouse) into mice for 8 days. For coeliac disease patient-derived IgG injections the titres of TG2-specific IgG in total IgG fractions were adjusted to equal the levels found in injected coeliac patient serum samples. For control IgG injections, the overall protein concentrations in the control samples were set to the level of those found in coeliac IgG samples. In the CD Mab preparations (protein concentration about 2 mg/ml), the IgG class EmA titres were 1:8000 or higher, and IgG class TG2 autoantibody levels above 100 U/ml, whereas the non-CD Mab was negative for both types of coeliac autoantibodies. The overall protein concentration of non-CD Mab was adjusted to the level of CD Mab.

The body weights of the animals were recorded daily during the experiments. At the beginning of the daily injections, the median weight of the mice in the 8-

day serum experiment was 21.1 g (range 19.6-22.0 g), the 27-day serum experiment 22.9 g (18.7-24.5 g), the total IgG experiment 21.4 g (range 19.4-22.9 g) and the miniantibody experiment 25.2 g (range 21.1-30.3 g). Faecal consistency was evaluated as follows: normal faeces (bullet-like, well-formed faecal pellets), mild diarrhoea (well-formed pellets with clearly moist surface), moderate diarrhoea (very moist surface with loose consistency or viscous, clear diarrhoea with some areas of better consistency) and severe diarrhoea (clearly watery or pasty faeces).

An iohexol test to measure intestinal permeability was made on five non-CD Mab-, CD Mab- and PBS-injected mice (**II**) essentially as described elsewhere (Frias et al. 2009, Frias et al. 2014). The test was carried out two weeks before starting miniantibody and PBS injections and immediately after completion of the 8-day injections. After administration of 10ml/kg of Omnipaque 300 (647 mg iohexol mg/ml Amersham Health, the Netherlands) to the mice by oral gavage, mouse urine was collected in metabolic cages for 24 hours and total urine volume recorded. Thereafter urine samples were stored at –20 °C for iohexol measurements.

At the end of the experiments, Isoflurane was used to anaesthetize the mice. Blood was collected by cardiac puncture, and sera were separated from the blood by centrifugation and stored at –70 °C. After euthanasia tissue samples from the small bowel, heart, kidney, liver, spleen and skeletal muscle were collected and tissue samples either freshly embedded in optimal cutting temperature compound (OCT, Tissue-Tec, Miles Inc, Elkhart, IN, USA), frozen in liquid nitrogen, and stored at –70 °C, or fixed in 10 % phosphate-buffered neutral formalin and embedded in paraffin. During the 8-day miniantibody experiment (**II**), one mouse receiving PBS died at day six after starting the antibody and PBS injections and thus the serum and tissue samples from the animal were collected at this time point.

5.6.2 Laboratory analysis of iohexol in mouse urine (II)

Iohexol levels in mouse urine were determined using the FIT-GFR Iohexol Kit (BioPAL, Inc., Worcester, MA, USA). In the case of incomplete collection or faecal contamination, urine samples were disregarded. Iohexol standards from the kit (0.01 0.03, 0.1, 0.3, 1.0, 3.0, 10.0 µg/ml) were prepared using additional ready-to-use sample diluent (BioPAL) intended for use with the kit. The urine samples were used at a dilution of 1:5000 and the test was thereafter carried out according

to manufacturer's instructions. A plate reader (EnSight™ multimote plate reader, Kaleido™ Data Acquisition and Analysis software) was used to record the absorbance at 450 nm for each sample and data from the standards were fitted to a four-parameter logistic function. The amount of iohexol in mouse urine was finally calculated as described by Frias and colleagues (2014) as follows:

$$\text{Iohexol in urine (\%)} = \frac{\text{iohexol in urine (mg) x TUV (ml)}}{\text{iohexol given orally (mg) x TTSV (ml)}} \text{ x 100, where}$$

TUV, total urine volume; TTSV, total volume of oral solution containing iohexol

The results are presented as percentage of iohexol in urine.

5.6.3 Immunohistochemistry, morphological measurements and cell counting (I, II)

For immunohistochemical stainings, 3-µm-thick well-oriented sections were cut from formalin-fixed proximal parts of mouse small intestines embedded in paraffin. The sections were deparaffinized in xylene and rehydrated in descending ethanol series. Antigen retrieval was accomplished in boiling 0.01 M citrate buffer, pH 6.0, or 1 mM EDTA buffer, pH 8.0, (Table 3) and endogenous peroxidase activity blocked with 0.3 % H_2O_2. Non-specific antibody binding sites were blocked prior to primary antibody incubations at 4 °C overnight and secondary antibody incubations at room temperature for 30 minutes (Table 3). Immunodetection was made using avidin-biotin complex solution (ABC) (Vectastain Elite ABC kit, Vector Laboratories Inc, Burlingame, CA, USA) followed by 3-amino-9-ethylcarbazole (AEC), or 3,3'-diaminobenzidine (DAB) (Table 3). The sections were counterstained with Harris haematoxylin.

Ki-67-stained sections were used to assess the small-intestinal morphology of the mice. Villous heights and crypt depths were measured and Vh/CrD calculated from approximately 15 measurements per mouse. Total amounts of cells and Ki-67-positive proliferative cells per mm^2 of lamina propria were determined by approximately 30 measurements per mouse. Active caspase-3-stained sections were analyzed for apoptosis with a three-grade scale: no staining, single positive cells and continuously stained villous tips. Neutrophil elastase- and F4/80-positive cells were counted per mm^2 of mouse small-intestinal tissue.

Table 3. The following antibodies and other reagents were used in immunohistochemical stainings in the original articles I and II.

Antigen (clone)	Description	Primary antibody		Secondary antibody		Antigen retrieval (boiling + cooling down)	Blocking non-specific binding	Detection
		Producer	Dilution (host)	Producer	Dilution (host), conjugation			
Active caspase-3 (D3E9)	Marker for apoptosis	Cell Signalling Technology[1]	1:250 (rabbit)	Vector Laboratories Inc[2]	1:200 (goat), biotinylation	0.01 M citrate buffer, pH 6.0 (10 min + 30 min)	ImmPRESS Kit*	ABC + AEC
F4/80 (SP115)	Marker for macro-phages	Novus Biologicals[3]	1:100 (rabbit)	Vector Laboratories Inc[2] (ImmPRESS Kit*)	Ready-to-use, HRP	1 mM EDTA buffer, pH 8.0 (20 min + 20 min)	ImmPRESS Kit*	DAB
Ki-67 (SP6)	Marker for cellular proliferation	Novus Biologicals[3]	1:400 (rabbit)	Vector Laboratiories Inc[2]	1:100 (goat), biotinylation	0.01 M citrate buffer, pH 6.0 (20 min + 20 min)	5 % goat normal serum in 5 % milk-5 % BSA-PBS	ABC + AEC
Neutrophil elastase	Marker for neutrophils	Abcam[4]	1:1000 (rabbit)	Biocare Medical[5]	Ready-to-use, HRP	0.01 M citrate buffer, pH 6.0 (7 min + 30 min)	5 % goat normal serum in 5 % BSA-PBS	DAB

*ImmPRESS HRP Anti-Rabbit IgG (Peroxidase) Polymer Detection Kit, Vector Laboratories Inc, Burlingame, CA, USA

[1]Cell Signalling Technology, Danvers, MA, USA; [2]Vector Laboratories Inc, Burlingame, CA, USA; [3]Novus Biologicals, LLC, Littleton, CO, USA; [4]Abcam, Cambridge, UK; [5]Biocare Medical, LLC., Concord, CA, USA

ABC, avidin-biotin complex reagent; AEC, 3-amino-9-ethylcarbazole; BSA, bovine serum albumin; DAB, 3,3'-diaminobenzidine; HRP, horseradish-peroxidase; PBS, phosphate-buffered salin

5.6.4 Staining of human IgG deposits in mouse tissues (I, II)

IgG deposits were stained in 5-µm-thick frozen proximal mouse small-intestinal sections by direct immunofluorescence using DyLightTM594-conjugated goat anti-human IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at a dilution of 1:800 in PBS, pH 7.4. To ascertain co-localization of human IgG and TG2 in the small intestines of mice, the human IgG staining was followed by incubation with polyclonal rabbit antibody to tissue transglutaminase (Zedira GmbH, Darmstadt, Germany) diluted 1:50 in PBS and Alexa Fluor 488-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR, USA) at a dilution of 1:2000 in PBS. Human IgG deposits were stained in other tissues with fluorescein isothiocyanate-conjugated rabbit antibody against human IgG (Dako Denmark A/S, Glostrup, Denmark). The slides were mounted using a Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc.) and images were taken with an Olympus BX60 microscope (Olympus Europa GmbH, Hamburg, Germany).

5.6.5 Investigation of target specificity of small-bowel mucosal IgG deposits (I)

Potassium thiocyanate (KSCN, Merck, Darmstadt, Germany), a substance which disrupts the binding of TG2 to fibronectin (Korponay–Szabó et al. 2000, Upchurch et al. 1991), was used to elute TG2 from frozen mouse small-bowel sections. Unfixed 5-µm-thick cryosections were air-dried for 10 minutes and 2 M KSCN applied to the sections for 30 minutes. PBS was used as control. Thereafter, sections were washed extensively with PBS and incubated with antibodies either for IgG, TG2 or double-stained similarly to IgG deposit staining as described in the previous paragraph. To check that fibronectin remained intact in the tissue sections, human IgG stained sections were incubated with rabbit anti-human anti-fibronectin antibody (Sigma-Aldrich) at a dilution of 1:500 in PBS for 30 minutes. Alexa Fluor 488-conjugated goat anti-rabbit antibody (Molecular Probes) was used as a secondary antibody at a dilution of 1:2000 in PBS for 30 minutes. Finally, the tissue sections were mounted with DAPI-containing Vectashield mounting medium (Vector Laboratories Inc.). After stainings images were taken with an Olympus BX60 microscope (Olympus Europa GmbH).

5.6.6 Quantification of epithelial cell subsets by quantative real-time PCR (II)

Small-intestinal epithelial cells were isolated from five CD Mab- and five non-CD Mab-injected mice. Harvested duodenal fragments were opened longitudinally and washed with cold PBS until the supernatant was clear. Tissue was chopped into approximately 5-mm pieces and incubated in 2mM EDTA-phosphate-buffered saline chelation buffer for 30 minutes rocking at 4 °C. After removal of EDTA-chelation buffer, tissue fragments were vigorously resuspended in PBS and pipetted up and down 20 times in a 10ml pipette. Supernatant containing epithelium from villi and crypts was collected, followed by two washes of the tissue, and supernatants from the washes were pooled. Total RNA was extracted from isolated small-intestinal epithelial cells with TRIzol Reagent (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. RNA was converted to cDNA using an iScript Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) and random primers as instructed by the manufacturer. Quantative real-time PCR (qPCR) was performed using an Evagreen Ssofast supermix kit (Bio-Rad) according to the manufacturers' instructions. Primer sequences are described in the supplementary Table 1 in original article **II**. The housekeeping gene GAPDH was used as normalization control. An average threshold cycle (Ct) value was calculated from three replicate samples per mouse. The messenger RNA (mRNA) levels are expressed in the results relative to those of the housekeeping gene.

5.6.7 Cytokine analysis (II)

Small-intestinal specimens from five CD Mab-treated and five non-CD Mab-treated mice were cut out of frozen Tissue-Tek and weighed. Thereafter, 500 µl of ice-cold H-buffer (150 mM NaCl, 5 mM EDTA, 50 mM potassium phosphate, pH 7.4) containing complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) was added per 100 mg of tissue and the tissues homogenized with Ultra Turrax T8 tissuemizer (IKA Labortechnik, Staufen, Germany). Supernatants were collected after centrifugation at 4 °C and the total protein concentration measured using the Bio-Rad DC Protein Assay (Bio-Rad). Supernatants were then diluted to 10 mg protein/ml with PBS and used in the assay. Eleven cytokines (IL-1β, IL-3, IL-6, IL-15/IL-15R, IL-17A, IL-27, IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α, epithelial neutrophil activating peptide (ENA-78) and macrophage inflammatory protein

(MIP)-2) were tested for protein expression levels with the Bio-Plex 200 system (Bio-Rad) using a ProcartaPlexTM Multiplex Immunoassay (eBioscience, San Diego, CA, USA) in accordance with the manufacturer's instructions. The standard curves were used to calculate the amounts of the cytokines. Values below detection limit were set to zero for the statistical analysis.

5.7 Investigating the effects of coeliac disease antibodies on angiogenesis (III)

5.7.1 *In vitro* angiogenesis assays (III)

Human umbilical vein endothelial cells (HUVECs) purchased from Lonza (Cambrex Bio Science, Walkersville, MD, USA) were used in *in vitro* assays. HUVECs were cultured at 37 °C and 5 % CO_2 in endothelial growth medium-1 (EGM-I; Clonetics, San Diego, CA, USA). EGM-I consists of endothelial cell basal medium (EBM-I; Clonetics) and endothelial cell growth factors provided in the EGM-I Bulletkit (Clonetics).

HUVECs ($2.5x10^5$ cells/well) were mixed with matrigel (BD Biosciences; Bedford, MA, USA) diluted 1:3 in EGM-1 and coeliac patient-derived total IgA fraction (CD IgA) or CD Mab or their respective controls (non-CD IgA and non-CD Mab). An active site-directed irreversible extracellular TG2 inhibitor R281 (Griffin et al. 2008) was used in a subset of experiments at a concentration of 200 µM. In this case, the inhibitor was administered one hour prior to addition of antibodies. After 48 hours, images of different fields were randomly taken using a Zeiss inverted microscope and Axiovision 3.0 program (Carl Zeiss Vision GmbH, München-Hallbergmoos, Germany). ImageJ analysis software (http://rsb.info.nih.gov/ij) (Abràmoff et al. 2004) was used to determine length, branch, area and number of endothelial tubules.

HUVECs embedded in matrigel as described above were also cultured for ten days in Cell-IQ (Chip-man Technologies LTD, Tampere, Finland), which consists of a cell incubator containing an integrated system designed to take and analyze images. Images were taken every five minutes during the assays. Apoptotic and immobile cells were enumerated by Cell IQ software and video editing was done with the software. Cells were tracked using the MTrack tool in ImageJ software for the analysis of cell movements.

5.7.2 *Ex vivo* aorta ring and *in vivo* matrigel plug angiogenesis assays (III)

Mouse aortas, cut into 0.5 mm-thick rings, were embedded in matrigel (BD Biosciences) containing CD Mab or their respective control, non-CD Mab (n=4 aortas/group). For ten days, the aorta rings were cultured in EGM-2 plus endothelial cell growth factors provided in the EGM-2 Bulletkit (Clonetics). Thereafter, images of endothelial sprouts and interconnected capillary tubes were randomly taken using a Zeiss inverted microscope and Axiovision 3.0 program (Carl Zeiss Vision GmbH, München-Hallbergmoos, Germany) and the pictures analyzed using ImageJ software.

In *in vivo* mouse angiogenesis assays, matrigel (BD Biosciences) containing 10 µg/kg of erythropoietin (EPO; Sigma Aldrich, St. Louis, MO, USA) was injected subcutaneously into the backs of the mice. This system is based on the EPO-induced self-production of vessels by cells migrating into the matrigel plugs from the host (Malinda 2009, Norrby 2006, Pagonopoulou et al. 2008, Ribatti et al. 1999). Also CD Mabs or their respective controls were mixed with matrigel (n=8 mice/group). After eight days, some of the animals (n=4 animals/group) were studied for *in vivo* vascular functionality by intravenous injection of Hoechst 33342 (Hoechst; Invitrogen, Carlsbad, CA, USA) into the tail vein five minutes before euthanasia. Finally, the matrigel plugs were removed from all animals, snap-frozen and stored at -80 °C for further analysis.

5.7.3 Positron emission tomography (III)

In this experiment each mouse (n=3 mice) received three matrigel implants containing EPO injected subcutaneously into separate limbs. Each of the three implants was treated with PBS (basal group), non-CD Mab or CD Mab. After eight days, the mice were anaesthetized with isoflurane, followed by intravenous injection of 2-[^{18}F]-fluoro-2-deoxy-D-glucose ([^{18}F]FDG). At 60 minutes post injection, PET imaging was performed using an Inveon Multimodality scanner (Siemens Medical Solutions, Knoxville, TN) for 20 minutes and reconstructed with the ordered-subsets expectation maximization 2D algorithm (OSEM2D). After PET imaging the animals were euthanized and the matrigel plugs excised, weighed and measured for radioactivity using a gamma counter (Triathler 3", Hidex, Turku, Finland), which was cross-calibrated with a dose calibrator (VDC-202, Veenstra Instruments, Joure, the Netherlands). Regions of interest in the matrigel plug areas were drawn and quantitative analysis performed on Vinci

software (version 2.54; Max Planck Institute for Neurological Research, Cologne, Germany). The average radioactivity concentration (kBq/ml) in the regions of interest was used to verify *in vivo* results.

5.7.4 Electron microscopy (III)

Aortas collected from the mice and treated as described above were prepared for electron-microscope analysis. The specimens were cut to 1 mm thickness, fixed in 5 % glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA) in 0.16 M s-collidin buffer (pH 7.4) and post-fixed with potassium ferrocyanide-osmiun tetroxide as previously described (Fröjdman et al. 1992). The samples were embedded in epoxy resin (Glycidether 100, Merck) and cut into thin 70-nm-sections (Fröjdman et al. 1992). The sections were stained with 5 % uranyl acetate and 5 % lead citrate in an Ultrostainer (Leica, Wien, Austria) and examined under a JEM-100SX transmission electron microscope (JEOL, Tokyo, Japan).

5.7.5 Immunofluorescence studies of the matrigels (III)

Frozen sections from mouse matrigel plugs were fixed in 4 % paraformaldehyde and processed to 5-7-µm-thick sections. The matrigel implants were studied for the presence of vessels by immunofluorescent staining with an anti-von Willebrand factor (vWF) antibody (1:200, Dako Denmark A/S) followed by secondary antibody labeled with Alexa-488 (1:1000). Nuclei were stained with Vectashield mounting medium containing DAPI (Vector Laboratories Inc.). Images were taken with an Olympus BX60 microscope (Olympus Europa GmbH). The number and diameter of vWF positive blood vessels as well as the total number of cells per matrigel area were analyzed in digitalized images using ImageJ software.

5.7.6 Western blot (III)

First, 100 µl of matrigel (BD Biosciences) was jellified and mixed with 100 µl of Laemmli buffer. After ten minutes the samples were sonicated, centrifuged at 12 000 rpm at 4 °C for ten minutes and concentrated with acetone. The Bradford

method was used to measure protein concentrations and 20 µg of total protein was loaded on 10 % polyacrylamide gels (1.5 M Tris–HCl, pH 8.8; sodium dodecyl sulfate, 12 %; acrylamide/bis-acrylamide, 30 %; APS, 10 %; and TEMED), followed by transfer to a Hybond-P membrane (Amersham Biosciences, Little Chalfont, Bucks, UK). After blocking in 5 % milk, the membrane was incubated with mouse monoclonal antibody against TG2, CUB7402 (1:200, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) and secondary rabbit antibody conjugated with horseradish peroxidase (Dako Denmark A/S). The ECL PlusWestern Blotting Detection System (GE Healthcare Biosciences, Pittsburgh, PA, USA) was used to detect the protein bands.

5.8 Statistics (I-III)

Statistical analysis was made using statistical analysis software (IBM SPSS Statistics, SPSS Inc., Chicago, IL, USA). Data are presented as either means or medians combined with standard error of means (SEM) or ranges, a p-value ≤ 0.05 being considered significant. Kruskal-Wallis test followed by Mann-Whitney U test were used to compare differences between the groups (**I-III**) and Wilcoxon test to compare changes within the groups (**II**). Correlations were studied using Spearman's correlation test in study **II**, and data on Vh/CrD and lamina propria cell infiltration from the previously published study **I** were also included. These data include Vh/CrD and lamina propria cell infiltration values from all mice injected with coeliac disease patient or control individual sera as well as total serum IgG from coeliac patients or control individuals, and non-injected mice. In substudy **III**, when the data was homogenic and there were three or more groups involved in an experiment, one-way ANOVA analysis was used. A two-way ANOVA within subjects was used to compare the effect of TG2 inhibitor with respect to each treated group. In both cases a Student Newman Keuls (SNK) test was applied as post hoc analysis.

5.9 Ethical considerations (I-III)

Ethical permission from the Heim Pál Children's Hospital Ethical Committee (license number 20/1996, revised 19.10.2000) was obtained for use of sera from Hungarian patients (**I**). The study protocol for use of human serum samples in the angiogenesis study was approved by the Ethics Committee of Tampere University

Hospital, Tampere, Finland, and written informed consent was received from all subjects (**III**).

The animal experiments were designed with a veterinarian. The persons carrying out the animal experiments were either personnel from animal facilities or persons who had completed an experimental animal course, and thus were competent to perform animal experiments. The guiding principles for more ethical use of animals, involving replacement, reduction and refinement (the 3Rs), were carefully taken into consideration when carrying out the experiments with mice. All procedures such as administration of antibodies, blood sampling, anaesthesia and euthanasia were performed according the recommendations and guidelines given by Animal Experiment Board in Finland. The animal experiments for study of the effects of human sera, total IgG and miniantibodies on mice (**I, II**) were conducted as approved by the National Ethics Committee for Animal Experiments in Finland (license numbers ESHL-2009-04108/Ym-23, ESLH-2009-08347/Ym-23, ESAVI-2010-06223/Ym-23, ESAVI/2723/04.10.03/2011, ESAVI/4279/04.10.07/2013 and ESAVI/781/04.10.07/2015). The protocol for mouse studies in angiogenesis experiments (**III**) was approved by the Finnish and Hungarian authorities, the Turku Central Animal Laboratory (University of Turku, Finland) and the Debrecen University animal facility (Debrecen, Hungary). These experiments (**III**) were conducted after approval by the National Ethics Committee for Animal Experiments in Finland (licence numbers ESLH-2008-04031/Ym-23, ESAVI-2010-06223/Ym-23 and ESAVI/4279/04.10.07/2013).

6 Results

6.1 Coeliac disease-specific autoantibody and human IgG levels in the mouse sera (I, II)

At the end of the serum, total IgG and miniantibody injection experiments, human IgG levels in mouse sera were measured to confirm that the intraperitoneal injections into the mice were successful. Human IgG was detected in the sera of all animals injected with human sera, total IgG or miniantibodies proving that human IgG was transported from the peritoneum to the circulation in the mice. The human IgG levels were lowest in the mice injected with monoclonal miniantibodies and highest in the serum-injected mice. All PBS- and non-injected mice were negative for human IgG (Table 1 in original articles **I** and **II**).

Mouse sera were also tested for IgG class coeliac disease-specific EmA and TG2 autoantibodies and, notably, all mice receiving IgA deficient coeliac disease patient sera or total IgG or CD Mab were positive for both autoantibodies. The medians of IgG class EmA titres in mice injected with coeliac patient serum for 8 or 27 days or with total human IgG or CD Mab for 8 days were 1:200, 1:2000, 1:50 and 1:1000, respectively. The corresponding TG2-IgG titres were 96.6, 150.5, 158.8 and 87 U/ml, respectively. In contrast, none of the non-injected animals or animals injected with control sera or total IgG, non-CD Mab or PBS had detectable levels of these autoantibodies (Table 1 in original articles **I** and **II**).

6.2 Clinical observations and intestinal permeability (I, II)

Mice injected with sera, total IgG or miniantibodies were monitored for weight and faecal consistency during the entire length of the experiments. Injections of coeliac disease patient sera or total IgG affected mouse weight gain, unlike when treating the mice with CD Mab. At the end of the 8-day serum experiment, differences in weight gain between the study and control groups did not reach statistical significance, probably due to the small number of animals, even though

the weight gain of mice injected with coeliac sera was virtually halted (increase 1.2 %) compared to controls (8.7 %) (Figure 1A in original article **I**). In the 27-day serum experiment, a statistically significant delay in weight gain was observed up to day 10 in mice receiving coeliac sera when compared to controls. During the rest of the experiment the weights of the mice injected with coeliac patient sera still remained lower compared to controls (Figure 1B in original article **I**). In the total IgG experiment, a statistically significant difference in weight gain between groups receiving coeliac or control total IgG was observed on day 8 (Figure 1C in original article **I**). In contrast, no significant differences in the increment of body weight were found between CD Mab-, non-CD Mab- and PBS-injected groups in the miniantibody experiment (**II**). Of note, the body weights remained fairly constant within each treatment group during the procedure.

Strikingly, mild diarrhoea was detected in a subset of animals receiving coeliac sera or total IgG, whereas the stool consistency of the control animals was normal in all cases. In the 8-day serum experiment two out of three, in the 27-day serum experiment two out of five and in the IgG experiment three out of five mice presented with mild diarrhoea (Table 1 in original article **I**). During the entire length of the miniantibody experiment (**II**), the stool consistency of all mice was mostly graded as normal, with occasional transient mild diarrhoea in some animals. However, there was no difference in the occurrence of such transient mild diarrhoea between the three study groups: CD Mab, non-CD Mab and PBS (data not shown).

To study intestinal permeability, the percentage of orally administered iohexol was studied in the urine of mice treated with miniantibodies and PBS (**II**). Before antibody and PBS injections, the median percentage of iohexol in the CD Mab group was 1.00 % (range 0.15-2.22 %), the non-CD Mab-group 0.15 % (range 0.14-8.64 %) and the PBS group 0.31 % (range 0.04-1.39 %). After the study period, no significant increase in the percentage of iohexol in urine was observed in any of the groups, the median percentages being 0.33 % (range 0.14-4.31 %), 0.23 % (range 0.13-2.67 %) and 0.43 % (range 0.15-0.96 %) in CD Mab-, non-CD Mab- and PBS-injected mice, respectively.

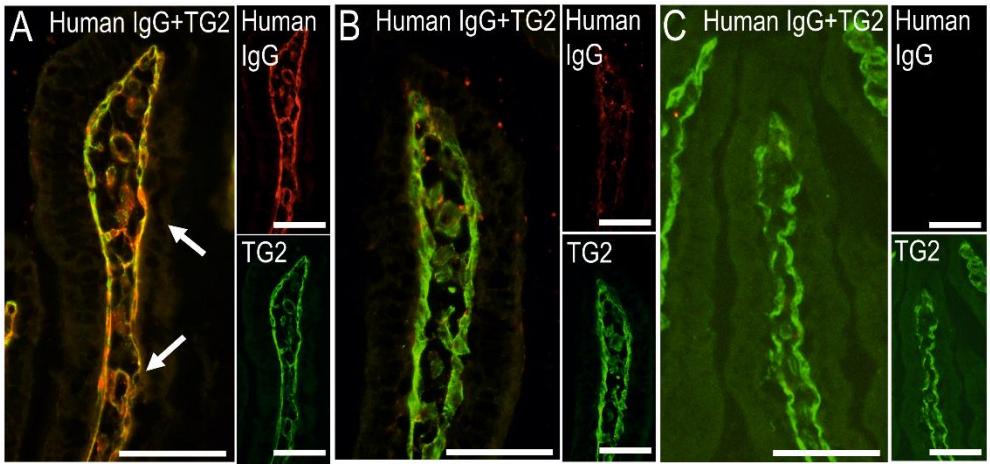

Figure 3. Representative images of small-bowel immunofluorescence stainings of human IgG (red) and transglutaminase 2 (TG2) (green) in mice injected with coeliac disease patient serum (A), healthy control serum (B) or PBS (C) for 8 days. Colocalization of IgG deposits and TG2 can be seen as yellow under the basement membrane and around blood vessels (arrows) in a composite picture (A). Scale bar=100μm.

6.3 Human IgG deposits in mouse tissues (I, II)

All mice receiving coeliac disease patient sera or IgG or CD Mab evinced clear human IgG positivity in the small-intestinal mucosa at the villous and crypt basement membranes and around blood vessels (Figure 3). Notably, the pattern of human IgG positivity co-localized with mouse TG2 at these sites (Figure 3). Human IgG class antibody deposits were also detected in the heart, kidney, liver and skeletal muscle of all mice injected with coeliac disease patient sera or IgG (Figure 5 in original article **I**) or CD Mab (data not shown). None of the control mice in any experiments exhibited such deposits (Figure 3 above and Figure 5 in original article **I**).

To ascertain that human IgG is specifically bound to mouse TG2 in the small-intestinal mucosa, the binding of TG2 to fibronectin was disrupted by KSCN (Korponay–Szabó et al. 2000, Upchurch et al. 1991). Treatment of the frozen tissue sections with KSCN led to the disappearance of both TG2 and human IgG antibody deposits. In contrast, fibronectin staining in the sections remained essentially unchanged (Figure 4 in original article **I**).

6.4 Mouse small-intestinal mucosal morphology (I, II)

The small-intestinal morphology of the mice was studied in the proximal parts of small intestines (Figure 4). In the 8-day serum experiment no significant difference in villous height was observed between groups, whereas mice injected with coeliac patient sera for 27 days or with coeliac patient total IgG had significantly shorter villi than respective controls and non-injected animals (Table 2 in original article **I**). Also mice receiving CD Mab had significantly lower villi than those injected with non-CD Mab or PBS (Figure 1A in original article **II**). Crypts were significantly elongated in mice receiving coeliac sera for either 8 or 27 days compared to their controls, whereas in the total IgG injection experiment no such differences were observed (Table 2 in original article **I**). Crypt depths of CD Mab- and non-CD Mab-injected mice did not differ significantly, but the control mice injected with PBS had significantly shorter crypts than those injected with miniantibodies (Figure 1B in original article **II**). Most importantly, as seen in Figure 4, Vh/CrD was significantly lower in all animals receiving coeliac disease patient-derived antibodies (coeliac serum or total IgG, or CD Mab) in comparison to their respective controls or PBS- and non-injected animals.

6.5 Lamina propria cellular infiltration in mice (I, II)

In order to quantify mucosal lamina propria cellular infiltration, we counted the total number of cells and Ki-67^+ proliferative cells per mm^2 of lamina propria in proximal mouse small intestines. The total number of lamina propria cells was significantly higher in mice injected with coeliac disease patient sera or total IgG or CD Mab when compared to their respective controls in any of the experiments (Figure 5). In addition, the total number of infiltrative cells in non-injected animals in the total IgG experiment and PBS-injected animals differed significantly from that observed in mice receiving coeliac patient-derived antibodies (Figure 5). A parallel finding was made with the number of Ki-67^+ proliferating lamina propria cells (Table 3 in original article **I** and Figure 2B in original article **II**).

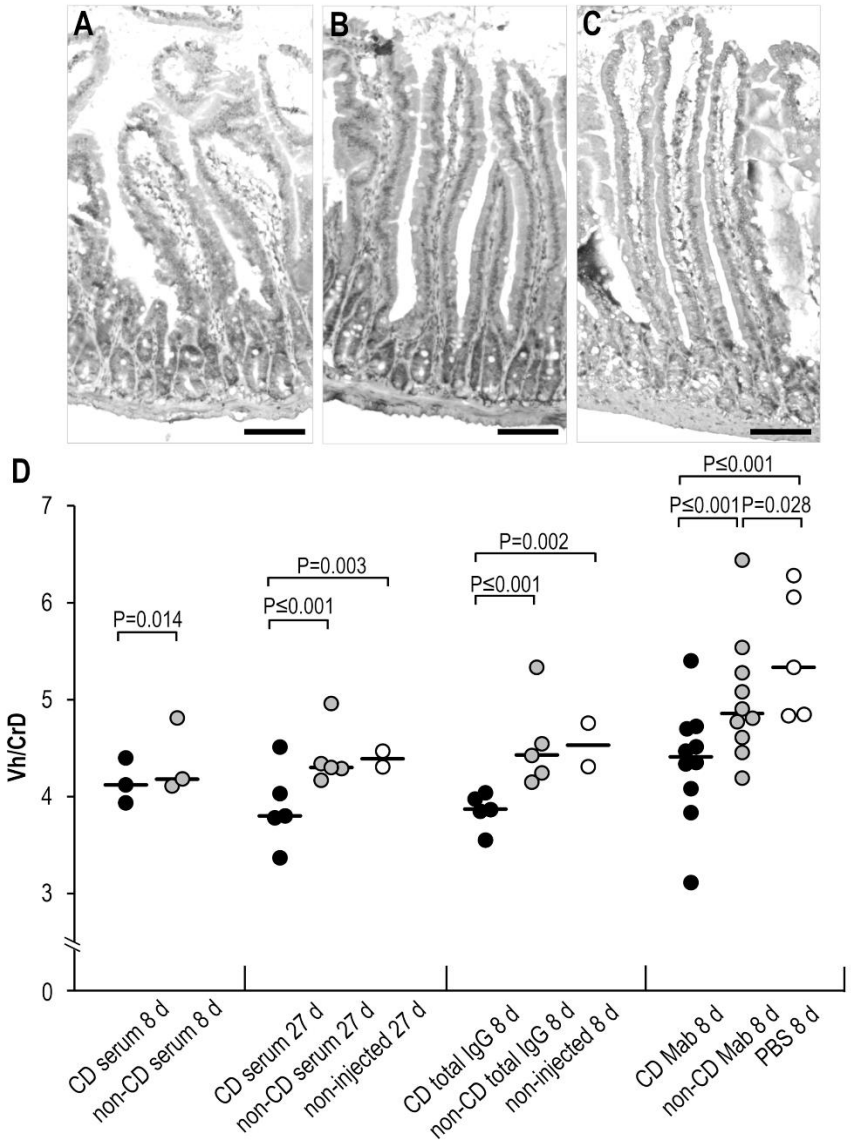

Figure 4. Small-bowel mucosal morphology of mice. A, B and C show representative images of small intestines from coeliac disease-specific anti-TG2 autoantibody-, control antibody- and PBS-injected mice, respectively. Scale bar=50µm. (D) Villous heights and crypt depths were measured and villous height crypt depth ratio (Vh/CrD) calculated in the 8- and 27-day serum experiments as well as in the total IgG and miniantibody experiments. Each dot represents the median value for one mouse calculated from approximately 15 measurements, and black lines indicate the median values of each group. P values ≤0.05 were considered statistically significant and only significant P values are reported in the figure. CD, coeliac disease, IgG, G class immunoglobulin, Mab, recombinantly produced monoclonal antibody; PBS, phosphate-buffered saline.

Of note, a significant negative correlation was observed at first between Vh/CrD and the number of proliferative cells in the lamina propria in the minibody experiment (Figure 3A in original article **II**). As the Vh/CrD data and the number of lamina propria cells were also available from the previous experiments (**I**), the correlation analysis was performed also taking this data set into consideration. In the larger data set, a negative correlation was found between Vh/CrD and the number of proliferative cells in the lamina propria ($P \leq 0.001$) (Figure 3B in original article **II**) and also between Vh/CrD and the total number of cells in the lamina propria ($P \leq 0.001$) (Figure 3C in original article **II**).

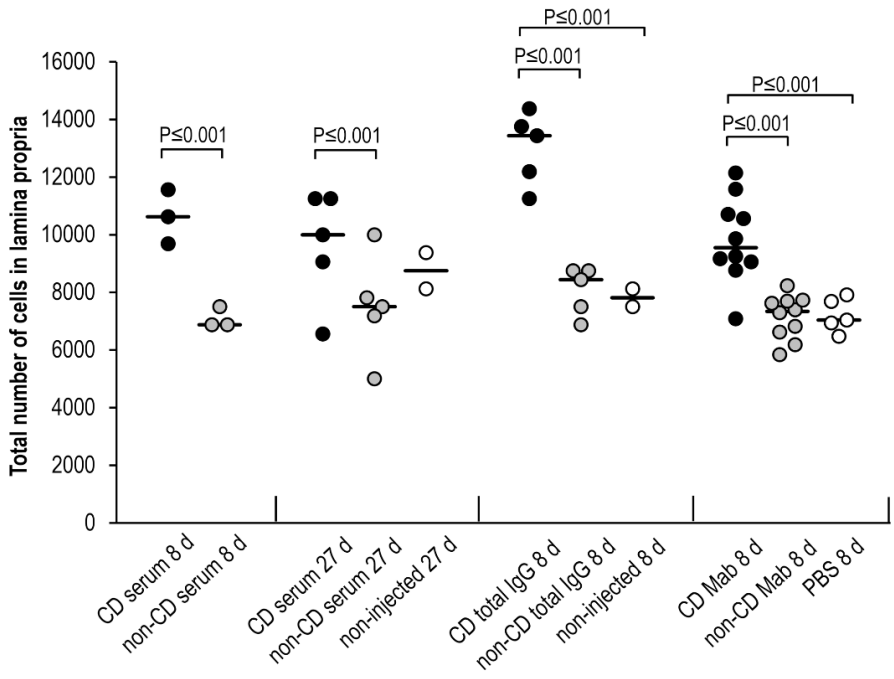

Figure 5. Lamina propria infiltration in the mice. Total amounts of cells per mm^2 of lamina propria were counted in the 8- and 27-day serum experiments as well as in the total IgG and miniantibody experiments. Each dot represents the median value for one mouse calculated from approximately 30 measurements, and black lines indicate the median values of each group. P values ≤0.05 were considered statistically significant and only significant P values are reported in the figure. CD, coeliac disease, IgG, G class immunoglobulin, Mab, recombinantly produced monoclonal antibody; PBS, phosphate-buffered saline.

6.6 Epithelial cell subsets and apoptosis (II)

The mRNA expression of distinct intestinal epithelial cell differentiation markers was evaluated in the miniantibody experiment by qPCR. This was done to assess whether the observed changes in the small-intestinal mucosal morphology of the CD Mab-injected mice could be due to altered differentiation of epithelial cells. However, the mRNA expression level of the enterocyte marker ALP1, the enteroendocrine marker CHGA, the Paneth cell marker LYZ1, the goblet cell marker MUC2 and the stem cell marker LGR5 were similar in CD Mab- and non-CD Mab-injected mice (Supplementary Figure 1 in original article **II**).

Regarding apoptosis, mostly single epithelial cells in the villous tips were positive for active caspase-3. Only rare caspase-3^+ cells were detected in the lamina propria. Finally, no differences were observed between CD Mab and the control groups in the level of apoptosis (Supplementary Figure 2 in original article **II**).

6.7 Cytokine expression levels in the small intestine of mice (II)

Small-intestinal tissues from mice receiving CD Mab or non-CD Mab were studied for the expression of 11 cytokines. Protein levels of IL-3, IFN-γ, IL-17A and GM-CSF were below the detection limit range in tissue homogenates of all mice and thus excluded from further analysis. IL-1β, IL-6, IL-15, IL-27, ENA-78, MIP-2 and TNF-α were chosen for further evaluation, but no statistically significant differences were found in the expression levels of these cytokines between CD Mab-treated and non-CD Mab-treated mice. However, three out of five CD Mab-injected mice had detectable levels of IL-27 (median 4.97 pg/ml in the samples above detection limit), while in all five non-CD Mab-injected mice the level of IL-27 was below the detection limit. In addition, four out of five CD Mab-injected mice had measurable levels of intestinal TNF-α (median 4.2 pg/ml in the samples above detection limit), in contrast to only one non-CD Mab-injected mouse (3.9 pg/ml).

The amount of intestinal IL-27 correlated significantly with the number of Ki-67 positive proliferating cells in the lamina propria (P=0.042), and a similar trend was observed between IL-27 and total amount of infiltrative cells in the lamina propria (P=0.056) (Table 2 in original article **II**). In addition, TNF-α levels correlated with both the Ki-67 positive cells in the lamina propria and the total

number of infiltrative cells (P=0.041 and P=0.036, respectively). A positive correlation was also found between the levels of TNF-α and IL-27 (P=0.018) and the levels of TNF-α and IL-1β (P=0.041) (Table 2 in original article **II**).

6.8 Effects of coeliac disease antibodies on angiogenesis *ex vivo* and *in vivo* (III)

Angiogenic effects of coeliac disease autoantibodies were studied by *ex vivo* aorta ring experiments. This system recapitulates all the key steps in angiogenesis, including matrix degradation, migration, proliferation and reorganization. Importantly, the number of the tubules, the microvascular area and the maximum microvessel outgrowth were significantly reduced in the presence of CD Mab as compared to non-CD Mab (Table 4). In *in vivo* experiments, where matrigel was implanted in the mouse backs, parallel results were obtained. Both the number and diameter of the vessels inside the matrigel were significantly decreased in the presence of CD Mab (Table 4).

Transmission electron microscope images were taken from *ex vivo* mouse aorta ring assays to gain further insight into the effects of coeliac autoantibodies. In control cultures, pseudopodia were observed in the protruding leading edges of cells and pericellular areas free of ECM (Figure 2A in original article **III**). In contrast, in the presence of CD Mab cells outgrowing from mouse aortas were round and did not exhibit cellular processes characteristic for the leading edge during migration. The migration was also studied in the mouse matrigel implants, where cells migrate from the mouse to the plug. In the basal group and in the group treated with non-CD Mab, cells migrated from the animal even to the centre of the matrigel and were evenly distributed inside the matrigel implant, whereas in the presence of CD Mab cells were mainly located in the border of the matrigel (Figure 2B in original article **III**). Thus it seems that the presence of coeliac disease autoantibodies alters the behaviour of endothelial cells.

The results from the aorta ring and mouse matrigel experiments suggested that defective migration could contribute to the anti-angiogenic effects exerted by coeliac autoantibodies. To study this further, videos were taken of HUVECs growing inside matrigel. In the presence of coeliac antibodies (both total CD IgA and CD Mab) the formation of tubule-like structures, measured by several angiogenic parameters, was inhibited when compared to controls during the 48-hour time course (Table 4 and Figure 3A, Supplementary Figure 1 and Video S1

in original article **III**). Tracking of cells grown inside matrigel showed an increased number of immobile cells in the presence of coeliac antibodies. Despite the substantial variability among the experimental groups, the moving cells treated with coeliac antibodies moved only half of the distance of those cultured in the presence of control antibodies (Figure 3B, C in the original article **III**). The number of apoptotic cells was quantified, but no increase was found in the cultures treated with coeliac antibodies (Supplementary Figure 2 in the original article **III**), thus excluding the possibility that anti-angiogenic effects exerted by coeliac antibodies are due to increased apoptosis.

Table 4. Effects of coeliac and control antibodies on several angiogenic parameters measured in three angiogenic models. All data is normalized to the basal group without any treatment (100 %). Numbers represent mean values ± SEM.

	non-CD Mab	CD Mab	non-CD IgA	CD IgA
***In vivo* assay**				
Number of vessels (%)	130±20	33±10*	ND	ND
Diameter of vessels (%)	68±6	40±4*	ND	ND
***Ex vivo* assay**				
Number of tubules (%)	85±11	55±6*	ND	ND
Microvascular area (%)	86±7	60±5*	ND	ND
Maximum outgrowth (%)	82±6	36±9*	ND	ND
***In vitro* assay**				
Tubule length (%)	72±2	39±1*	72±3	24±1*

* represents P≤0.001 statistical difference when compared to the control antibody-treated group. CD IgA, coeliac disease patient-derived immunoglobulin A; CD Mab, coeliac disease-specific transglutaminase 2-targeted miniantibody; ND, not determined; non-CD IgA, healthy control-derived immunoglobulin A, non-CD Mab, irrelevant control miniantibody; SEM, standard error of mean.

6.9 TG2-specific coeliac autoantibodies induce changes in *in vivo* vascular functionality (III)

Capillary perfusion was studied in *in vivo* matrigel implants using an intravenous tail vein injection of Hoechst (Smith et al. 1988). The vessels inside the matrigel were functional in all treatment groups, since the injected dye was located around blood vessels (Figure 4A in original article **III**). Although the percentage of the Hoechst positive area was similar in all groups, the area occupied by vWF-staining was significantly reduced in the CD Mab-treated group, this resulting in 2.6 times higher Hoechst/vWF ratio compared to controls (Figure 4B in original article **III**).

PET was used to study the functionality of the vessels formed inside *in vivo* matrigel implants (Video S2 in original article **III**) and it revealed decreased uptake of [^{18}F]FDG in the matrigel treated with CD Mab in comparison with non-CD Mab but not with the basal group. Taking into account that the matrigel implants treated with CD Mab contained significantly fewer vessels than controls, the ratio of [^{18}F]FDG uptake and vessel area was increased more than two-fold in CD Mab-treated matrigel implants compared to the controls (Figure 5A, B in original article **III**).

6.10 Extracellular TG2 contributes to the anti-angiogenic response (III)

Whether cellular or extracellular TG2 mediates the anti-angiogenic response by coeliac disease antibodies was studied next, as it has been demonstrated that extra- and intracellular TG2 are involved in distinct endothelial cell processes (Nurminskaya and Belkin 2012). Notably, in our experiments with HUVECS the cell-impermeable TG2 inhibitor R281 was able to restore the tubule length of CD IgA- or CD Mab-treated cells to control level (Figure 6A in original article **III**). To confirm the contribution of extracellular TG2, we performed experiments using TG2 knockout mice, which do not express cellular TG2 (De Laurenzi and Melino 2001). In contrast, the matrigel used in the experiments contains significant amounts of TG2 (Figure 6B in original article **III**). Both *ex vivo* aorta ring and *in vivo* matrigel plug assays, performed with TG2 knockout mice, showed decreased levels of angiogenic parameters in the presence of CD Mab compared to controls. However, in the *in vivo* experiment the difference became statistically significant. It is of note that the overall angiogenic response in TG2

knockout mice was reduced when compared to wild type animals of the same mouse strain (Figure 6 here and Figure 6C, D in original article **III**).

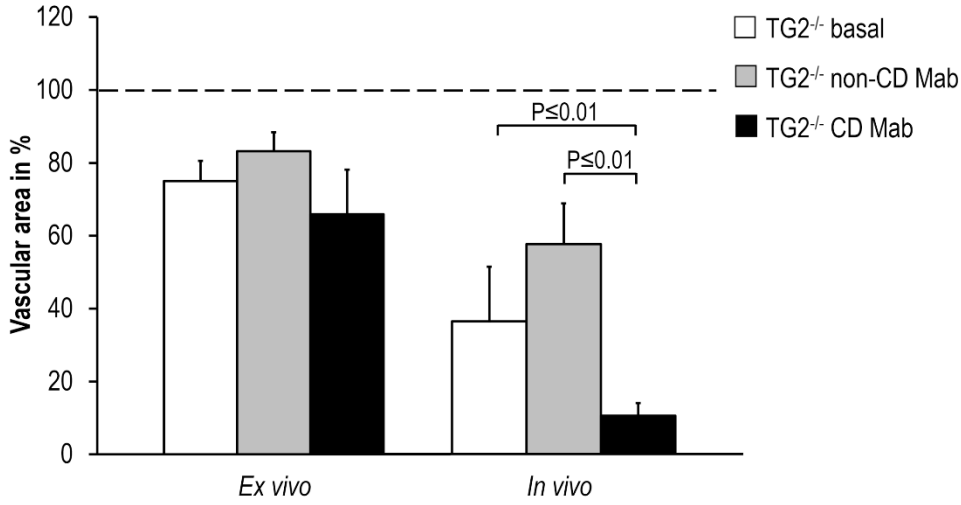

Figure 6. The contribution of extracellular transglutaminase 2 (TG2) in the anti-angiogenic response exerted by coeliac autoantibodies. In the *ex vivo* experiment, mouse aorta rings derived from TG2 knockout mice ($TG2^{-/-}$) were cultured inside TG2-containing matrigel for ten days without supplementation (basal) or in the presence of recombinantly produced coeliac TG2 autoantibody (CD Mab) or control antibodies (non-CD Mab). After the culture period the vascular area was measured (n=5 aortas per group). Also *in vivo* angiogenesis assays were performed in $TG2^{-/-}$ mice. Matrigel containing endogenous TG2 was supplemented with CD Mab or non-CD Mab and injected into the mice. After eight days the implants were removed and the vascular area determined by anti-von Willebrand factor (vWF)-antibody staining (n=8 animals per group). Bars in the charts represent the average value as percentage + SEM. All data were normalized to the wild type mouse basal group (dotted line).

7 Discussion

The role and significance of the coeliac disease-specific TG2-targeted autoantibodies in the progression of coeliac disease in patients has long been a controversial issue. Autoantibodies have been regarded more as bystanders despite their usefulness in diagnostics and convincing results at *in vitro* level. Both IgA and IgG class antibodies have been shown to exert multiple biological impacts in studies performed with cell cultures and small-intestinal biopsies. This present work aimed to demonstrate the biological effects of coeliac disease patient antibodies especially *in vivo* using coeliac disease patient-derived antibodies in mice.

7.1 Signs of early developing coeliac disease in mice

7.1.1 Clinical features of coeliac disease in mice and changes in small-intestinal mucosa

In order to confirm that the injections of sera, total IgG and miniantibodies were successfully performed, human IgG levels were measured in the mouse sera after each experiment. Importantly, human IgG was detected in all animals injected with coeliac or control sera, total IgG or miniantibodies, whereas PBS- and non-injected mice were negative for human IgG. This showed that human IgG was able to reach the circulation in the mice after injections. Human IgG levels were comparable between mice injected with coeliac antibodies and control antibodies in all other experiments, but in the miniantibody experiment CD Mab-injected mice had clearly lower levels of human IgG compared to those injected with non-CD Mab.

Interestingly, all mice (**I**, **II**) injected with IgG class coeliac disease patient-derived antibodies, i.e. sera, purified immunoglobulins or miniantibodies, evinced slight albeit significant morphological alterations in the small-intestinal mucosa when compared to control mice (Figure 4). In addition, the density of infiltrative cells in the lamina propria was increased in all mice receiving coeliac

antibodies (Figure 5). In humans, changes in the composition of different cell populations in the lamina propria typically are signs of inflammatory process (De Winter et al. 2012, Fournier and Parkos 2012, Serra and Jani 2006) and thus the increased lamina propria infiltration in mouse small intestines might imply an inflammatory reaction in response to the injected coeliac antibodies.

Perhaps the most significant finding in study **I** was the development of gastrointestinal symptoms coinciding with mild enteropathy in mice following the serum or immunoglobulin injections. A subset of mice receiving coeliac antibodies evinced mild diarrhoea at the end of the serum and total IgG experiments, while none of the control mice had diarrhoea. Importantly, also patients with early developing coeliac disease might have for example abdominal pain and diarrhoea prior to the development of villous atrophy and crypt hyperplasia (Kaukinen et al. 2001, Kurppa et al. 2009). Furthermore, coeliac disease patients may have growth problems in childhood (Kaukinen et al. 2010), and a parallel phenomenon was observed in mice injected with coeliac patient-derived sera and immunoglobulins, as the mice had delayed weight gain.

Surprisingly, the injection of coeliac patient-derived coeliac miniantibodies did not affect the increment of body weight or the occurrence of diarrhoea in study **II**. In fact, the mouse body weights remained fairly constant throughout the study period in all groups. This was presumably because the weights at the beginning of the injection period had for some reason already reached that of adults, as reported by the company the animals were purchased from. Thus, based on study **II**, it is not evident whether other TG2 autoantibody epitopes or antibody populations are necessary for delayed weight gain. However, it was seen in study **I** that the mice injected with coeliac total IgG did not evince as much delayed growth as the coeliac serum-injected mice, suggesting that other factors are needed. Also the difference in the occurrence of mild transient diarrhoea between study **I** and **II** might be contributed to by the above-mentioned factors. The TG2-targeted autoantibodies injected into mice in study **II** were representative only of a single previously identified classical coeliac epitope, while three other major TG2 epitopes also exist in coeliac patient serum autoantibodies (Iversen et al. 2013, Simon-Vecsei et al. 2012). In addition to the TG2 autoantibodies, also other antibody populations such as DGP antibodies were very likely present in the injections in study **I**. It is thus reasonable to assume that a combined action of TG2 autoantibodies targeting different epitopes and/or other antibody populations such as DGP antibodies might be necessary to induce delayed weight gain and the development of diarrhoea in the mice. However, it must be noted that also

patients presenting with early-developing coeliac disease do not always present with gastrointestinal symptoms, including diarrhoea (Kurppa et al. 2009, Salmi et al. 2006a).

7.1.2 The presence of coeliac disease-specific autoantibodies in mouse tissues

Importantly, all mice receiving antibodies from coeliac patients (**I**, **II)** evinced clear human IgG positivity in the small-intestinal mucosa at the basement membrane and around blood vessels, and the IgG staining pattern co-localized with mouse TG2 (Figure 3). The injected antibodies were also detected in the mouse sera.

Korponay-Szabó and colleagues (2000) have investigated with human umbilical cord and monkey oesophagus tissues the binding of coeliac patient sera to TG2 by removing TG2 from tissue sections. We applied the same approach using KSCN to disrupt the binding of TG2 to fibronectin (**I**), as fibronectin is one of the major proteins anchoring TG2 to matrix structures (Korponay–Szabó et al. 2000, Upchurch et al. 1991). Indeed, elution of TG2 from mouse intestinal tissue sections led to the disappearance of human IgG antibody deposits along with the TG2 signal, while fibronectin staining remained essentially unchanged. This finally confirmed that coeliac disease-specific autoantibody deposits in the mouse small-intestinal mucosa did indeed target murine TG2 in the present experiments and are comparable to those found in coeliac patients' mucosa.

In addition to IgA autoantibody deposits in the small-intestinal mucosa, coeliac disease patients may have deposits in extraintestinal tissues such as liver, muscles, kidney and brain (Hadjivassiliou et al. 2006, Korponay-Szabó et al. 2004). IgG deposits might be found in the liver and kidney of IgA deficient coeliac patients (Korponay-Szabó et al. 2004). In the present studies **I** and **II**, the mice were injected with IgG class antibodies and thus human IgG deposits were found in a number of tissues: liver, kidney, heart, spleen and skeletal muscle. The presence of autoantibodies in several tissues could actually account for the lower levels of human IgG observed in the circulation of the mice injected with CD Mab compared to controls, as there were no other IgG antibody populations present in those injections. Furthermore, autoantibody deposits have also been reported in previous studies addressing the effects of antibodies from coeliac disease patients in mice. There deposits were observed not in the small intestine but for instance in liver, muscle fibres and brain (Boscolo et al. 2007, Boscolo et al. 2010, Di Niro et al. 2008), which are actually also target sites for extraintestinal manifestations

in coeliac disease (Hadjivassiliou et al. 2006, Korponay-Szabó et al. 2004). Although in the present series (**I**) no statistically significant differences were found in the serum alanine aminotransferase values of mice injected with coeliac patient sera or total IgG and controls (data not shown) associated with the liver health, it is nonetheless intriguing to speculate that coeliac autoantibodies may play a pathogenetic role in coeliac disease and also its extraintestinal manifestations.

7.2 Vascular biology and coeliac antibodies

It is known that coeliac disease-specific TG2 autoantibodies form deposits in the small-intestinal mucosa of coeliac patients and deposits can also be found around blood vessels (Korponay-Szabó et al. 2004, Koskinen et al. 2008, Lancaster-Smith et al. 1976). The autoantibodies have been reported to fulfil several biological functions and their possible role in vascular deterioration has also been described in a few studies (see section 2.4.3).

Previous studies have demonstrated that coeliac disease antibodies, both total IgA fraction and IgG class TG2 autoantibodies purified from coeliac patient serum, disrupt angiogenesis *in vitro*. In a two-dimensional cell culture model, binding of TG2-targeting coeliac autoantibodies to vascular cells cultured on collagen, disturbed endothelial sprouting and the migration of both endothelial and vascular mesenchymal cells. In addition, after treatment with coeliac antibodies, actin disorganization has been observed in both cell types, and this might negatively affect cellular migration (Myrsky et al. 2008). Supportive results have also been obtained from other cell culture studies (Caja et al. 2010, Simon-Vecsei et al. 2012), where endothelial cells have grown in matrigel, which is a commercial basement membrane preparation and supports endothelial cells in forming capillary-like structures with a lumen in this particular assay (Kleinman and Martin 2005). This three-dimensional assay recapitulates all the key steps in the angiogenic process, including matrix degradation, migration, proliferation and reorganization (Kleinman and Martin 2005).

In the present study (**III**), the results from above-mentioned previous studies with endothelial cells grown in matrigel were confirmed with both coeliac total IgA fraction and recombinantly produced IgG-class miniantibodies. Moreover, the studies were extended to *ex vivo* and *in vivo* systems to address the question whether similar effects would be seen than at *in vitro* level. The results clearly

demonstrated that coeliac disease-specific TG2 autoantibodies are anti-angiogenic also in three-dimensional conditions, where mouse aorta rings were grown in matrigel, but importantly also in matrigel plugs implanted in the backs of mice (Table 4). In matrigel implanted into mice EPO induces self-production of vessels by cells migrating into the matrigel from the host (Malinda 2009, Norrby 2006, Pagonopoulou et al. 2008, Ribatti et al. 1999). In both assays, several angiogenic parameters were decreased in the presence of coeliac antibodies compared to controls. Vessel functionality was studied *in vivo* by intravenous injections of Hoechst, a fluorescent dye used to stain DNA, and PET scans. The vessels formed in matrigel were able to transport blood in all implants; however, the vascular area was smaller in the cases treated with coeliac TG2 autoantibodies, which would suggest impaired vessel functionality. This could be due either to increased permeability or immaturity of the vessels. Interestingly, it has previously been suggested that coeliac autoantibodies would affect endothelial permeability (Myrsky et al. 2009a). However, because the number of vessels and their diameter were diminished in the presence of coeliac autoantibodies, it is reasonable to assume that immaturity of the vessels could explain the lack in functionality. Already previously a similar state of immaturity, a lack of a sufficient smooth muscle supportive layer around vessels, has been demonstrated in the small-intestinal mucosa of coeliac disease patients (Myrsky et al. 2009b). This again might be a consequence of the problems in vascular mesenchymal cells observed in the presence of coeliac autoantibodies (Myrsky et al. 2008).

Breakdown of the ECM is the prerequisite for proper cell movement during the angiogenic process, when cells migrate (Guedez and Stetler-Stevenson 2008, Papetti and Herman 2002). However, such areas of proteolytic degradation were not seen in aorta ring cultures supplemented with coeliac miniantibodies; instead, the cells were surrounded by electron-dense areas of ECM. Both observations may indicate that coeliac autoantibodies disturb the degradation or remodeling of ECM, which could subsequently affect the mobility of the cells. In fact, when endothelial cells grown in matrigel were recorded for ten days, they were less mobile when treated with coeliac total IgA fraction and IgG-class miniantibodies compared to controls. It seemed that these cells were also not able to establish cell-cell contacts and their capacity to form sprouts was reduced, and thus both IgA and IgG class autoantibodies were able to reduce the mobility of the cells.

Of note, results obtained by Di Simone and associates (2013) are parallel to the data from the present study (**III**). The group investigated the effects of anti-

TG2 autoantibodies on the process of endometrial angiogenesis *in vitro* and *in vivo*, as several studies suggest a possible association between coeliac disease and an increased risk of pregnancy failure or infertility (Alstead and Nelson-Piercy 2003, Khashan et al. 2010, Rostami et al. 2001). The results from both angiogenesis assays, an *in vitro* experiment with human endometrial endothelial cells and an *in vivo* murine model with matrigel, showed that human endometrial angiogenesis is impaired in the presence of a total fraction of serum IgA or IgG derived from coeliac women on a gluten-containing diet. Immunoglobulin fractions also contain other factors and antibodies in addition to TG2-specific autoantibodies, but the results were confirmed by commercial monoclonal anti-TG2 autoantibody and down-regulating TG2 with siRNA in the endothelial cells. A particularly intriguing finding was that also in the study in question anti-TG2 autoantibodies seemed to inhibit angiogenesis by affecting degradation of ECM and TG2-dependent migration of the cells (Di Simone et al. 2013).

Simon-Vecsei and colleagues (2012) have shown that placenta specimens from mothers presenting with coeliac disease contain high amounts of maternal IgA autoantibodies bound to TG2 for instance on the blood vessel walls. Interestingly, they also showed that maternal coeliac autoantibodies have biological effects as transferred into newborns. These maternal IgG class autoantibodies were detected in the umbilical cords and sera of the newborns, and the autoantibodies were against the same specific epitope as in the mothers. In addition, HUVECs isolated from coeliac mothers displayed abnormalities similar to those previously observed in normal HUVECs treated with coeliac IgA (Myrsky et al. 2008). Thus these two studies together suggest that coeliac autoantibodies may have for example a role in unsuccessful embryo implantation and placentation via inhibited angiogenesis. In addition, the data show that autoantibodies might also contribute the other problems in pregnancy, for example early pregnancy losses and intrauterine growth retardation related to coeliac disease. Considering that coeliac autoantibodies may disturb several steps in this angiogenic process and that in the normal condition, intestinal vessels are constantly lost and gained at an equal rate (Stappenbeck et al. 2002, Unthank et al. 1990), these results together with those of the present series strongly suggest that autoantibodies may have a role in pathogenesis of coeliac disease.

The *in vitro* experiments in the present study (**III**) were performed with total IgA fraction and IgG class miniantibodies and results were parallel to each other. The *ex vivo* and *in vivo* results were obtained only with miniantibodies leaving the question open whether coeliac IgA autoantibodies would have had anti-

angiogenic effects in mouse tissues. However, the studies mentioned in the section 7.2 emphasize that both IgA and IgG class antibodies seem to have similar effects on angiogenesis and thus it is possible that IgA class antibodies might also disturb angiogenesis in mice.

7.2.1 Involvement of TG2 in the inhibition of angiogenesis

TG2 is the autoantigen for coeliac autoantibodies, and it seems apparent that this enzyme might be an important factor in the process of inhibited angiogenesis exerted by coeliac autoantibodies, as previous publications have also suggested (Caja et al. 2010, Myrsky et al. 2009a). In the present study (**III**), results from experiments performed with TG2 knockout mice showed that in the *in vivo* matrigel plug angiogenesis assay, the angiogenic response was significantly decreased after coeliac miniantibody treatment when compared to control treatments (Figure 6). In this case, only the extracellular TG2 supplied by the matrigel itself was present. In the aorta ring experiments a trend towards reduced microvessel (Figure 6) and maximum outgrowth areas was observed in the presence of coeliac autoantibodies, though the change was not statistically significant. The difference in the level of angiogenic response between these two experiments in the presence of coeliac autoantibodies could be partly explained by the fact that in the TG2 knockout mice the overall angiogenic response in matrigel was greatly reduced compared to wild type animals, while in the aorta ring experiment the reduction was not so extensive. This can be affected by the differences in experimental settings, as in the matrigel plug assay the cells first have to migrate to the plug to form vessels, whereas in the aorta ring assay cells can initiate the angiogenic process immediately. Also other factors such as the proportion of endogenous ECM secreted by the aortic tissue or individual migrating cells might possibly contribute to the discrepancy.

It has been shown that accumulation of ECM and defective angiogenesis *in vitro* can be caused by the administration of catalytically active TG2 (Jones et al. 2006). Together this and the finding in study **III** that cell-impermeable TG2 inhibitor R281 prevented the anti-angiogenic effects of coeliac antibodies, would suggest the involvement of extracellular TG2 activity in the impaired angiogenic response in the presence of coeliac antibodies. Another possible explanation as to how R281 could rescue impaired angiogenesis is prevention of the binding of the coeliac autoantibodies to TG2. According to previous data (Myrsky et al. 2009a) this would not seem to be the case. Furthermore, inhibitor R281 could affect the

proteins interacting with TG2 and thus prevent effects exerted by coeliac autoantibodies. However, some previous studies have shown increased TG2 activity in the presence of coeliac autoantibodies (Király et al. 2006, Myrsky et al. 2009a), and thus this explanation is more probable, although TG2 activity was not addressed in the present study.

7.3 Possible contributors to the deterioration in the small-intestinal mucosa

Korponay and associates (2004) report that autoantibody deposits are present in the small-intestinal mucosa before they are detectable in the circulation. It is also known that TG2-targeted IgA deposits, which form a certain staining pattern under the epithelial cell layer and also around blood vessels, precede deterioration in the mucosa (Korponay-Szabó et al. 2004, Koskinen et al. 2008, Salmi et al. 2006a). In addition, the present findings show that the coeliac disease-specific autoantibodies interfere with angiogenesis *in vivo*. It would thus appear conceivable that autoantibodies against TG2 might contribute to the pathogenesis of mucosal deterioration. In point of fact, deposits could be anti-angiogenic and lead to altered small-bowel mucosal vasculature. Such an abnormal vascular network would no longer be able to provide mechanical support to the villi and would thereby contribute to the development of villous atrophy. A recent paper by Rubio-Tapia and colleagues (2012) supports the conception of an association between morphological changes in the intestinal mucosa and angiogenesis. They note that the use of olmesartan as a medicine for unexplained severe sprue-like enteropathy was found to be associated with unexplained chronic diarrhoea and small-intestinal villous atrophy in patients taking the drug. Olmesartan is one of several angiotensin II receptor antagonists used in the management of hypertension, and it has also been described to reduce angiogenesis in mice (Cheng et al. 2011, Rubio-Tapia et al. 2012).

The changes in the small-bowel mucosal vasculature in coeliac disease may also contribute to the pathogenesis in other ways, not only affecting the mucosal architecture. The coeliac autoantibodies together with other factors, including inflammatory mediators (Abadie et al. 2011), could also increase the permeability of vessels and thus potentiate the small-bowel mucosal inflammatory response. Unfortunately angiogenesis was not assessed in studies **I** and **II**. However, an inflammation-like condition was observed in the small-intestinal mucosa of mice

injected intraperitoneally with coeliac antibodies, as there was an increased density of infiltrating cells in the lamina propria. A significant negative correlation was found between Vh/CrD and the number of infiltrative cells in the lamina propria, indicating that the infiltrative cells in the lamina propria could actually be involved in the process of mucosal deterioration. However, this is probably not attributable to neutrophils or macrophages, as the expression of these specific cell types was equal between groups in study **II** (data not shown). It still needs to be determined which cells are responsible for the increased number of inflammatory cells in the mice lacking T cells.

According to the results from study **II**, the presence of coeliac TG2-specific autoantibodies did not increase the level of apoptosis, which is in keeping with earlier findings (Barone et al. 2007). Furthermore, TG2 autoantibodies did not affect the proportions of different intestinal epithelial cell lineages, which together with unaffected apoptosis might thus partly account for the unchanged intestinal permeability seen in study **II**. Previously it has been reported that gluten-sensitive individuals with normal small-intestinal mucosal morphology already have decreased expression of distinct junctional proteins (Rauhavirta et al. 2014) and increased permeability (Smecuol et al. 2005). It might thus be presumed that other factors along with TG2-targeted autoantibodies are needed for the induction of epithelial permeability in the early phases of coeliac disease development. On the other hand, the injections of TG2-targeted autoantibodies in mice induced elongation of crypts in study **II,** suggesting that crypt epithelial cell proliferation could be increased. Indeed, one previous study has shown that autoantibodies against TG2 are able to induce the entry of epithelial cells into S-phase (Barone et al. 2007). However, in the present study, changes in both crypt depth and also villous height in mice were milder than those observed in untreated overt coeliac patients presenting with considerable villous atrophy and large-scale crypt hyperplasia, and there might be several reasons for this. For instance, the mice used in the study lacked T cells and also the correct MHC molecules, both of which are relevant for the adaptive immune response in the coeliac disease pathogenesis (du Pré and Sollid 2015). In addition, innate immune activation (Kim et al. 2015), structural changes in the epithelial cell layer (Hüe et al. 2004) and epithelial stress (Setty et al. 2015) are involved in the development of the small-bowel mucosal damage in coeliac patients. Therefore, these factors, along with coeliac disease antibodies, might be necessary for the development of both total villous atrophy and increased permeability.

Although the mice used in the present series did not have T cells, the levels of 11 cytokines were studied (**II**). It was not surprising that the entire small-intestinal cytokine profile of the mice treated with coeliac miniantibodies did not resemble that observed in coeliac disease patients (Garrote et al. 2008, Lahat et al. 1999, Iacomino et al. 2016). For instance, no increased level of IL-15, the innate immunity cytokine overexpressed in coeliac patient small-intestinal epithelia and lamina propria (Di Sabatino et al. 2006, Iacomino et al. 2016, Maiuri et al. 2000, Mention et al. 2003), was observed in animals receiving coeliac miniantibodies. Also the levels of other pro-inflammatory cytokines such as IFN-γ, IL-1β and IL-6, which have been found to be increased in active coeliac patients in a number of studies (Fornari et al. 1998, Kontakou et al. 1994, Manavalan et al. 2010, Nilsen et al. 1998), were not elevated. However, the levels of IL-27 and TNF-α were slightly although not significantly increased in coeliac miniantibody-injected mice compared to controls. In addition, it was shown that lamina propria infiltration parameters correlated with these protein levels. This finding is of interest, as it has been reported that the expression of IL-27 mRNA is increased in active coeliac disease patient biopsy specimens (Garrote et al. 2008) and numerous studies report elevated levels of TNF-α in coeliac patients (Cataldo et al. 2003, Kontakou et al. 1995, Manavalan et al. 2010, Przemioslo et al. 1994, Westerholm-Ormio et al. 2002).

7.4 Animal studies demonstrating the biological role of coeliac autoantibodies

The role of autoantibodies in disease induction has long been under debate and has been investigated by a classical and widely used approach in which patient serum or purified antibodies specific to culprit autoantigens are injected into healthy individuals (Harrington et al. 1951). In several other autoimmune disorders, for example arthritis, narcolepsy and some skin blistering diseases, passive transfer of plasma, serum or IgG class antibodies from patients with a distinct autoimmune disorder characterized by specific IgG class antibodies has been a successful means of inducing features of the disease in mice (Anhalt et al. 1982, Katzav et al. 2013, Petkova et al. 2006, Roscoe et al. 1985, Toyka et al. 1977). These studies suggest that pathological changes consistent with the disease in question can be induced by humoral autoimmunity. The results from the present study are in line with such a view, since sera, total IgG and miniantibodies

derived from IgA deficient coeliac patients induced features of early developing coeliac disease in mice.

The effects of autoimmunity against TG2 in coeliac disease have apparently been assessed in only two previous *in vivo* studies, and neither reports any signs of diarrhoea, effects on weight gain or intestinal permeability (Di Niro et al. 2008, Freitag et al. 2004). The first study by Freitag and co-workers induced a humoral response against TG2 by immunizing mice with exogenous TG2 (Freitag et al. 2004). In contrast to our observations, no histological changes in the small intestine were observed despite the high levels of anti-TG2 autoantibodies in mouse sera. Though not reported in the article, it is very likely that the epitopes of the murine TG2 autoantibodies deviate considerably from those of coeliac patients used in the present study, thus providing a possible explanation for the differences in the findings. In the second study, by Di Niro and associates, adeno-associated virus vectors were utilized to express TG2 miniantibodies in immune-competent mice (Di Niro et al. 2008). Using a coeliac patient-derived single-chain TG2 miniantibody similar to the one used in study **II**, they observed no morphological alterations in small-bowel mucosa, although the autoantibodies were present in mouse serum. Interestingly, a strong anti-idiotypic response was apparent in the mice and this may explain why no changes were seen in the small-bowel mucosal morphology. In addition, no antibody deposits were formed in most tissues, including the small intestine, that might have accounted for the presence of idiotypic antibodies and their competitive binding with TG2. In light of these results, it is conceivable that autoantibodies deposited in the small intestine might be one factor inducing decreased Vh/CrD.

Two studies by Boscolo and associates (2007, 2010) have emphasized the role of TG2 autoantibodies in gluten ataxia, a neurological condition recognized in gluten sensitivity patients. In the first study they demonstrated that sera from patients with gluten sensitivity contain antibodies against gliadin or TG2, or/and neural antibodies capable of causing ataxia when injected into the lateral ventricle of the mouse brain. In addition, in both studies recombinantly produced TG2 miniantibodies were transferred into the mouse central nervous system and significant impairment in motor function occurred after injections. Moreover, TG2 autoantibody deposits were found in the brain vessels of these mice. Both studies would thus suggest that TG2 autoantibodies can induce ataxia in mice, but may however act in concert with other autoantibodies to cause neuronal problems in patients. The conclusion was thus similar to that reached in this present study:

TG2 autoantibodies are able to induce features of distinct disease, but most probably other antibodies are needed to develop full-blown dysfunction.

An interesting addition to the above discussion is the hypotheses which proposes that autoimmune disorders could be an indolent lymphoproliferative disease of B cell origin (McQueen and Elliott 2010). It further emphasizes the role of both B cells and autoantibodies in the onset of autoimmune disorders (McQueen and Elliott 2010) and supports the conception underlying the studies mentioned above.

7.5 Strengths and limitations of the present study

So far, a series of *in vitro* studies have shown biological effects induced by coeliac disease-specific TG2-targeted autoantibodies (see section 2.4.3). In the present study, the most intriguing results are from *in vivo* experiments performed in mice. Over the last few decades, the laboratory mouse has been widely used as a model organism of choice for studying diseases of humans, as 99 % of mouse and human genes are shared. In addition, mice and humans have great similarities for example in nervous, cardiovascular, endocrine and immune systems (Rosenthal and Brown 2007). Although the overall structure of the immune system in mice and humans is quite similar, and in many respects mice mirror human biology remarkably well, significant differences prevail between mice and humans in the immune system, as reviewed in detail by Mestas and Hughes (2004). Thus special caution is needed in interpreting data obtained on mice. Nonetheless, mice will probably continue to be the most widely used *in vivo* model for human diseases and be thereby essential for progress in understanding the immune system function in humans (Mestas and Hughes 2004).

Mice with various immunodeficiencies are used for engrafting normal or malignant human tissue, and T cell deficient mice have proved to be one of the most popular such mouse models (Rosenthal and Brown 2007). Immunodeficient mice lacking T cells were also used in the present studies **I** and **II**, since the aim was to address specifically the effects exerted by patient-derived antibodies, and with this model it was possible to avoid the development of an anti-idiotypic response in mice (Di Niro et al. 2008). On the other hand, T cell deficient mice are not able to mount the cytokine response characteristic of T cell mediated adaptive immune activation which occurs in coeliac disease. Thus, for instance,

the difference in the cytokine profile between coeliac patients and coeliac miniantibody-injected mice is not surprising.

An important aspect in the current series was that coeliac disease patient-derived antibodies were used in all experiments. The miniantibodies used in studies **II** and **III** were made by recombinant technology, but they are also originally derived from patients and these specific miniantibodies target a previously identified classical coeliac epitope (Simon-Vecsei et al. 2012). Although this present study focused on the effects of TG2-targeted autoantibodies, the injections of whole sera and purified IgGs into mice in study **I** offered an insight into a situation resembling that in coeliac patients, where also other antibodies and factors are present. Combining these results to those obtained using only TG2 autoantibodies in studies **II** and **III** creates an excellent opportunity to broaden the understanding of the role of autoantibodies in coeliac disease.

In study **I**, it was anticipated that human IgA class anti-TG2 autoantibodies from IgA competent coeliac disease patients might not be efficiently transported to the mouse circulation due to the absence of IgA-Fc-receptor expression in mice (Monteiro and Van De Winkel 2003). In addition, human IgA has been reported to be rapidly degraded in the circulation of rodents (Bogers et al. 1989). Hence sera and purified serum total IgG from IgA deficient coeliac disease patients were used, as they contain IgG class instead of IgA class antibodies. In line with this, IgG class miniantibodies were utilized in the other studies. In the few angiogenesis experiments (**III**) IgA class antibodies were also used, but only in *in vitro* studies, where cells were cultured in the matrigel mixed with antibodies. Importantly, the results were on an equal level whether the cells were supplied with IgA class antibodies or IgG class TG2-targeted autoantibodies as also previously shown (Myrsky et al. 2008, Myrsky et al. 2009a). As mentioned before, TG2-targeted autoantibodies have been shown to disturb angiogenesis *in vitro* also in previous studies, but the present study describes such effects for the first time in three-dimensional *ex vivo* experiments and also importantly *in vivo* in mice.

In coeliac patients, the development of overt villous atrophy and crypt hyperplasia may take years or even decades (Lähdeaho et al. 2005, Mäki et al. 1990). This would induce one to suggest that longer exposure to coeliac patient antibodies and/or additional factors might be required to develop even more severe condition in mice than those observed in the present study. Mostly the amount of sera received from IgA deficient coeliac patients but also

recombinantly produced autoantibodies was the factor limiting to the length of the studies. With higher amounts of antibodies the number of animals could also have been increased.

8 Summary, conclusions and future perspectives

The present study is the first to report coeliac disease-specific TG2-targeted autoantibody deposits in the small-intestinal mucosa of mice. Deposits were found below the epithelial cell layer and around blood vessels. Furthermore, it has not previously been shown that intraperitoneally injected TG2 autoantibodies are sufficient for the induction of mild enteropathy in mice. In addition, an increased number of infiltrative cells was observed in the lamina propria of these mice.

Noteworthily, the condition of mice receiving TG2 autoantibodies resembled early-phase disease in coeliac patients, when already many features of the disorder can be observed. Several publications show that early developing coeliac patients with normal morphology or mild changes in the small-intestinal mucosa already have autoantibodies as deposits targeted to TG2 in the small bowel (Kaukinen et al. 2001, Kurppa et al. 2009). It has to be emphasized that the presence of IgA deposits in the small intestine seems to predict forthcoming coeliac disease (Salmi et al. 2006a, Tosco et al. 2008).

In the present study, several factors may have contributed to the morphological changes seen. Autoantibodies against TG2 were found as deposits in the small-intestinal mucosa of the mice and it has been suggested that such deposits might have a role in the coeliac disease pathogenesis (Lindfors et al. 2010). In fact, in the present study TG2 autoantibodies were found to inhibit angiogenesis *in vitro*, *ex vivo* and *in vivo*. Data suggested that the anti-angiogenic mechanism of the coeliac disease-specific autoantibodies involves extracellular TG2 and inhibited endothelial cell mobility. It would thus seem plausible that such deposits may contribute to the development of villous atrophy, as they may lead to abnormal intestinal vascularity and thus diminish the mechanical support of the villi.

Increased cellular infiltration and slightly increased levels of TNF-α and IL-27 were also observed in conjunction with morphological alterations in the mice. Although the precise mode of action exerted by the autoantibodies remains to be established, the mechanism might involve cellular infiltration and these cytokines. However, further research is necessary to determine which cell types

are responsible for the increased infiltration in these mice and what is the role of the above-mentioned cytokines in the small-intestinal deterioration.

Interestingly, only injections of coeliac sera or serum total IgG fractions to mice, contrary to TG2 miniantibodies targeting the major coeliac epitope, induced an increased occurrence of mild diarrhoea and delay in weight gain. This would imply that the development of such clinical features requires TG2 autoantibodies targeting other epitopes than in study **II**, entirely other antibody populations and/or longer exposure to the antibodies. Indeed, the development of overt small-bowel mucosal villous atrophy with crypt hyperplasia and extraintestinal manifestations may likewise require even longer exposure to coeliac disease patient antibodies and/or additional factors. One possibility to overcome problems with the limited amount of antibodies would be to design a transgenic mice expressing coeliac autoantibodies. This model would not only provide a great advantage to study the effects of coeliac antibodies for longer time period, but also there would be no longer need for autoantibody production in laboratory or antibody purification from patient samples.

Altogether, this study presented novel data on the biological effects of coeliac disease-specific autoantibodies *in vivo* and demonstrated that indeed, these autoantibodies can induce a condition in mice which resembles early developing coeliac disease in humans. The abovementioned transgenic mouse model would provide an interesting tool to further study the role of autoantibodies and other factors in the pathogenesis of coeliac disease. Also the effects of different cytokines could be tested in this model by inducing immunity for example with recombinant IL-15 or IL-10, or by blocking with inhibitors. When designing a transgenic mice, also the contribution of coeliac gene background, HLA, could be taken in to consideration as well as other mouse strains than athymic mice. Autoantibodies could also be used in other mouse models, for example with IL-15 transgenic mice which retains a normal histology in the small intestinal mucosa (DePaolo et al. 2011). Another possibility would be to use autoantibodies in conjunction with a drug inducing inflammatory response in the mouse small intestine. In each of these models, it would be interesting to test switching from gluten-containing diet to gluten-free diet and back to study effects of gluten itself in the models.

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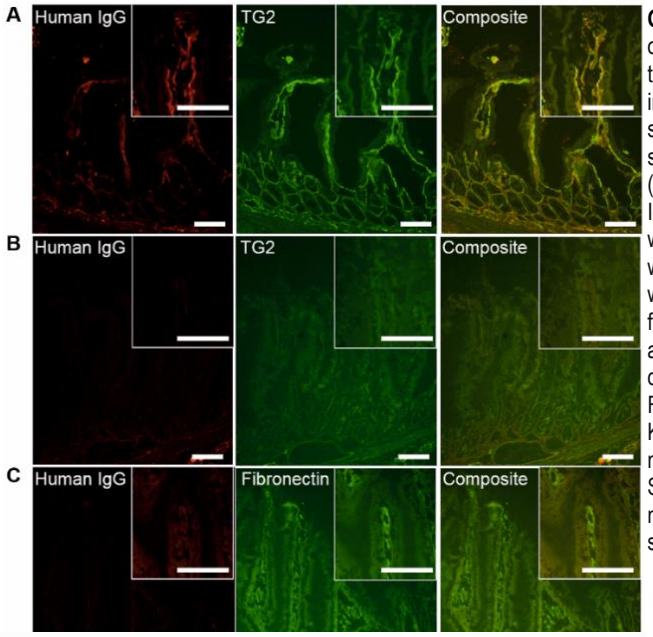
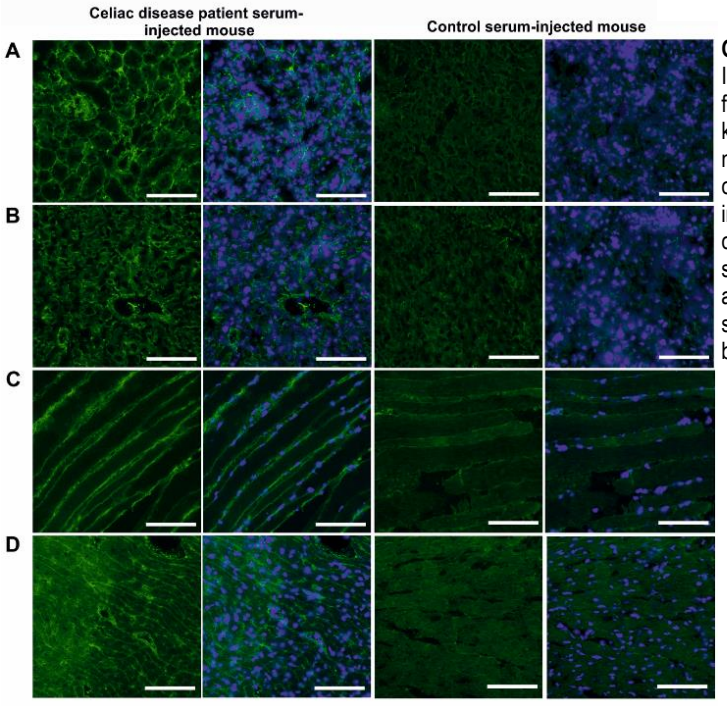
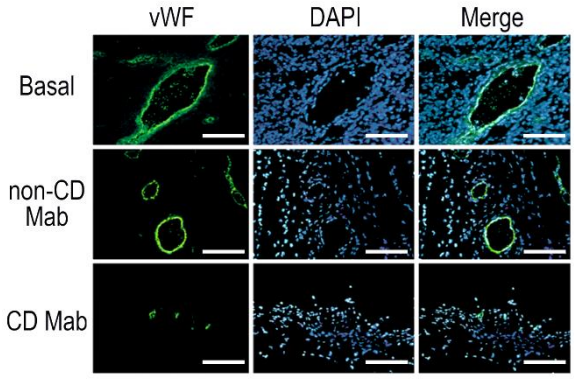
11 Appendix: colour figures from original articles I and III



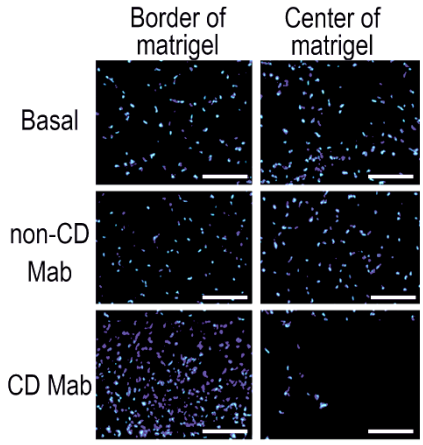
Original article I, figure 4: Specificity of deposited human IgG for transglutaminase 2 (TG2) in mice injected with coeliac disease patient sera or total IgG. (A) In PBS-treated sections where the binding of TG2 (green) to fibronectin is intact, human IgG (red) is detectable and colocalized with TG2 (yellow). (B) After incubation with potassium thiocyanate (KSCN), which disrupts the binding of TG2 to fibronectin, deposited human IgG (red) and extracellular TG2 (green) disappear from the sections. (C) Fibronectin staining was visible after KSCN treatment. The images are from mice injected with sera for 27 days. Scale bar=100 µmin the lower magnification images on the left and scale bar=50 µm in other images.



Original article I, figure 5: Immunofluorescent stainings for human IgG (green) in the kidney (A), liver (B), skeletal muscle (B), and heart (D) of coeliac disease patient serum-injected or control mice. Blue color represents nuclear staining (DAPI). The images are from mice injected with sera for 27 days. Scale bar=100 µm.

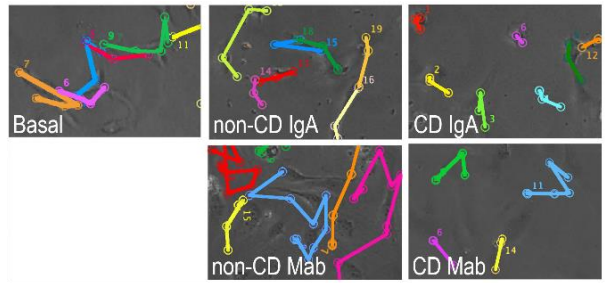


Original article III, figure 1B: Representative pictures of mouse matrigel implants treated with monoclonal coeliac disease-specific transglutaminase 2 antibody (CD Mab) or its respective control (non-CD Mab). After eight days the implants were removed and stained for blood vessels with von Willebrand factor (vWF)-antibody (green) and blue color indicates nuclei (DAPI). Scale bar represents 200 µm.

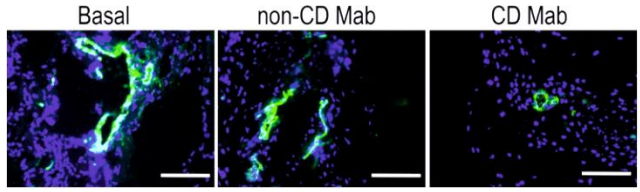


Original article III, figure 2B: Representative images taken from mouse matrigel implants treated with coeliac-specific transglutaminase 2 antibody (CD Mab) or its respective control (non-CD Mab). Nuclei are shown as blue (DAPI) from the border and center of the implants. Scale bar represents 300 µm.

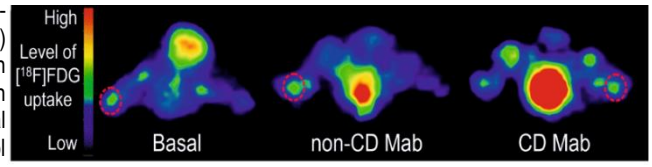
Original article III, figure 3B: Human umbilical vein endothelial cells (HUVEC) were tracked for 48 hours and cellular mobility during that time course is indicated in representative pictures. Each color represents an individual cell. CD IgA, coeliac patient-derived total IgA (CD IgA) or monoclonal antibodies (CD Mab), or their relevant controls (non-CD IgA or non-CD Mab).



Original article III, figure 4A: Representative images of mouse matrigel implants treated with coeliac disease-specific transglutaminase 2 antibody (CD Mab) or its control (non-CD Mab). Blue color indicates Hoechst-stained nuclei (DAPI), green von Willebrand factor (vWF) and scale bar represents 200 µm.



Original article III, figure 5A: 2-[^{18}F]-fluoro-2-deoxy-D-glucose ([^{18}F]FDG) PET scans of mice implanted with matrigel (basal) or supplemented with coeliac disease-specific monoclonal autoantibodies (CD Mab) or control antibodies (non-CD Mab).



12 Original publications

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Injection of celiac disease patient sera or immunoglobulins to mice reproduces a condition mimicking early developing celiac disease

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KEY MESSAGE

- Celiac disease patient sera or total IgG was injected into athymic mice.
- A significant delay in weight gain and mild diarrhea were observed.
- Mice evinced significantly decreased villus height crypt depth ratios.
- Celiac disease-specific autoantibody deposits were present in several tissues.
- The condition in mice resembles early stage celiac disease in humans.

ABSTRACT

Typical features of celiac disease are small-bowel villus atrophy, crypt hyperplasia and inflammation which develop gradually concomitant with ingestion of gluten. In addition, patients have anti-transglutaminase 2 (TG2) autoantibodies in their serum and tissues. The aim of this study was to establish whether celiac disease can be passively transferred to mice by serum or immunoglobulins. Serum aliquots or purified immunoglobulins (Ig) were intraperitoneally injected into Hsd:Athymic Nude-Foxn1nu mice for 8 or 27 days. As mice do not have proper IgA transport from peritoneum to blood, sera with a high content of IgG-class anti-TG2 antibodies from untreated IgA-deficient celiac patients were used. Mouse sera were tested for celiac disease-specific autoantibodies and several tissues were analyzed for autoantibody deposits targeted to TG2. Morphological assessment was made of the murine small intestinal mucosa. Injection of celiac disease patient sera or total IgG led to a significant delay in weight gain and mild diarrhea in a subset of mice. The mice injected with celiac patient sera or IgG had significantly decreased villus height crypt depth (Vh/CrD) ratios and celiac disease –specific autoantibody deposits targeted to TG2 in several tissues, including the small intestine. None of these features was observed in control mice. We conclude that administration of IgA-deficient celiac disease patient serum or total IgG induce both deterioration of the intestinal mucosa and clinical features of celiac disease in mice. The experimentally induced condition in the mice injected with patient serum or IgG resembles early developing celiac disease in humans.

Keywords: autoantibodies; celiac disease; passive transfer; transglutaminase 2

INTRODUCTION

The development of celiac disease, an autoimmune-mediated enteropathy, is promoted in a subgroup of genetically susceptible HLA-DQ2 or -DQ8 positive individuals upon exposure to dietary gluten. The gluten-induced small-bowel mucosal damage in the patients develops gradually from a normal villus structure to mild mucosal inflammation and eventually to a totally flat mucosa with crypt hyperplasia. The clinical presentation of the disease is markedly variable, ranging from classical intestinal manifestations (diarrhea, abdominal pain, malabsorption and failure to thrive in childhood) to extraintestinal symptoms affecting liver, muscles and neurological functions [1, 2]. Interestingly, many of these have been reported to occur even before the development of villus atrophy [3, 4].

A key feature of celiac disease is a strong immunoglobulin (Ig) A-class antibody response mainly against the autoantigen transglutaminase 2 (TG2) [5]. This response is measurable in celiac disease patient serum using either anti-reticulin, endomysial or anti-TG2-antibodies, which nevertheless all target the same autoantigen [6]. In addition to being present in the circulation, the TG2-autoantibodies are sequestered locally in the small-bowel mucosa, where they are bound to extracellular TG2 below the epithelial layer and around blood vessels [2, 7-9]. Interestingly, such mucosal autoantibody deposits precede and even seem to predict the development of villus atrophy [3, 10, 11]. In addition to being present in small-intestinal mucosa, anti-TG2-autoantibodies have extraintestinal target sites, for example in the liver, muscles and brain, where they often coincide with local disease manifestations [2, 12].

Celiac disease has been associated with a number of conditions and an unusually high prevalence of the disease has been observed for instance in type 1 diabetes mellitus and Sjögren's syndrome [13, 14]. Besides these autoimmune disorders, celiac disease is also overrepresented among patients with selective IgA-deficiency. Nearly all IgA-deficient celiac disease patients mount an IgG-class instead of IgA-class humoral response against TG2 [15]. In addition to being present in circulation, the autoantibodies of IgA-deficient patients are bound to TG2 in various tissues [2, 16]. Importantly, the

clinical presentation of celiac disease in IgA-deficient patients is similar than in IgA-competent patients regardless of different class of TG2-targeted autoantibodies [15, 17].

Both A- and G-class immunoglobulins derived from celiac disease patients have been shown to have multiple biological impacts affecting for instance epithelial, endothelial and neuronal cell biology [18-22]. Despite these convincing results, it remains debatable whether celiac disease antibodies participate in the disease pathogenesis. A classical and widely used approach in demonstrating the role of autoantibodies in disease induction is to inject patient's serum or purified antibodies specific to culprit autoantigens into healthy recipients [23]. This approach was thus adopted, serum or immunoglobulins from celiac disease patients being injected into immune-compromised mice and the generation of clinical symptoms and alterations characteristic for celiac disease investigated.

MATERIALS AND METHODS

Ethical considerations

Ethical permission from the Heim Pál Children's Hospital Ethical Committee (licence number 20/1996, revised 19.10.2000) was obtained for using sera from Hungarian patients. The animal experiments were conducted under the approval by the National Ethics Committee for Animal Experiments in Finland (license numbers ESHL-2009-04108/Ym-23, ESLH-2009-08347/Ym-23 and ESAVI/2723/04.10.03/2011).

Patient sera and purification of human total IgG from serum

Serum specimens were obtained from IgA-deficient untreated biopsy-proven celiac disease patients. We chose to use IgA-deficient celiac disease patients' sera, since no murine equivalent of Fc-receptor for IgA (FcαR1) has been found and human IgA has been reported to be rapidly degraded in rodents [24, 25]. Four of the celiac disease patients were female and six male, mean age 6 years (range 2-13

years). According to the Marsh classification [26] four of the patients evinced IIIB and the rest IIIC lesion. All patients had total serum IgA-levels <0.05 g/l, regarded as classical selective humoral IgA deficiency, while having normal IgG and IgM levels in their sera. In all patients, serum IgG-class endomysial antibody (EmA) values were above 1:4000 (dilution of 1:10 or more considered positive) and TG2 antibodies above 100 U/ml (cut-off 7 U/ml). The patients had also been tested for IgA-class EmA and TG2 antibodies and were negative. The control sera used in the experiments were derived from healthy non-celiac subjects negative for both EmA and TG2 antibodies.

Total IgG fractions from a subset of patients and controls were purified using Protein G Sepharose 4B fast flow (Sigma-Aldrich, MO). The serum samples were passed through the column, whereafter the column was washed with 0.075 M Tris-HCl, pH 8.0, and 0.5 M NaCl prior to elution of the IgG with 0.1 M glycine-HCl in 0.5 M NaCl, pH 2.5. The collected IgG fractions were neutralized with 1 M Tris-HCl, pH 8.0, before removal of glycine by passing the samples through PD-10 columns (GE Healthcare, Buckinghamshire, UK). In purified serum IgG fractions the IgG-class TG2 antibody levels were measured with a commercial Celikey kit (Celikey IgG®; Phadia GmbH, Freiburg, Germany) according to manufacturer's instructions. The median value of TG2 antibodies in purified celiac serum samples was above 100 U/ml (min 88.9 U/ml, max >100 U/ml) and below cut-off value in control samples. The concentration of total IgG in the sera was determined by a human IgG enzyme-linked immunosorbent assay (ELISA) kit (Immunodiagnostik AG, Bensheim, Germany). Finally the IgG samples were lyophilized and resolubilized in phosphate-buffered saline (PBS) to a final concentration of 100 µg/ml.

Experimental animals, housing and husbandry

Female Hsd:Athymic Nude-Foxn1nu mice lacking T cells approximately 4-5 weeks of age supplied by Harlan (Venray, The Netherlands) were used in this study. All were kept in semi-barrier conditions in the facilities of the Central Animal Laboratory, in the University of Turku, Finland. Before

commencement of the studies, the mice were acclimatized for 2 weeks. The environment consisted in a temperature range of 20 to 23 °C, a relative humidity of 50 to 60%, and artificial illumination with a 12-h light/dark cycle. Throughout the study period, all mice were fed a standard gluten-containing mouse chow (Special Diet Services, Whitham, Essex, UK) ad libitum, and tap water was provided without restriction. The mice were determined to be healthy on the basis of gross clinical examinations, and were considered specific-pathogen-free based on the results of the microbiological screening routinely performed in the animal facility in accordance with European recommendations. The mice were cared for and used in accordance with the current Finnish legislation and the Council of Europe Convention ETS 123 on the care and use of vertebrate animals for scientific purposes.

Study design and experimental procedures

To investigate the effects of celiac patient sera or total IgG, the mice were injected with human sera and total IgG previously collected from untreated celiac disease patients and from healthy human controls. Sera were administered with a single daily intraperitoneal injection of 200 µl for either 8 (n=3 mice/group) or 27 days (n=5 mice/group). In another set of experiments, 200 µl of purified serum IgG from celiac disease patients or controls were injected for 8 days (n=5 mice/group). For celiac disease patient-derived IgG-injections the titers of TG2-specific IgG in total IgG fractions were adjusted to equal the levels found in injected celiac patient serum samples. For control IgG injections, the overall protein concentrations in the control samples were set to the level of those found in celiac IgG-samples. In addition, in the 27-day serum and the 8-day total IgG experiments also non-injected control mice were included (n=2 in each experiment).

During the experiments, the body weight of the animals was recorded daily and the results being given as percentage of starting weight. On day 0, mouse body weights ranged from 18.7 to 24.5 g (median 22 g). Fecal consistency was evaluated with a four-grade scale: normal feces (bullet-like, well-formed fecal pellets), mild diarrhea (well-formed pellets with clearly moist surface), moderate

diarrhea (very moist surface with loose consistency or viscous, clear diarrhea with some areas of better consistency) and severe diarrhea (clearly watery or pasty feces).

At the end of the experiments, the mice were anesthetized using Isoflurane, blood was collected by cardiac puncture, and sera were separated from the blood by centrifugation and stored at –70°C. After euthanasia tissue samples from the small bowel, brain, heart, kidney, liver, spleen and muscle were subsequently collected and harvested. Samples were either freshly embedded in optimal cutting temperature compound (OCT, Tissue-Tec, Miles Inc, Elkhart, IN), frozen in liquid nitrogen, and stored at –70°C, or fixed in 10 % phosphate buffered neutral formalin and embedded in paraffin.

Celiac serology and serum alanine aminotransferases from mouse sera

Human total IgG levels were determined in mouse sera by an Immunoglobulin G ELISA Kit specifically recognizing human immunoglobulins (ImmunoDiagnostik, Bensheim, Germany). The assay was performed according to the manufacturer's instructions and the levels of sera were given as mg/ml, based on the standards provided by the kit.

Serum IgG-class EmA values in the mouse sera were determined by an indirect immunofluorescence method, where human umbilical cord from a premature baby was used as substrate and a serum dilution of 1:10 or more was considered positive [15]. Positive sera were further diluted: 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000 and 1:4000. In addition, serum IgG class antibodies against TG2 were measured by ELISA using human recombinant TG2 as antigen (Celikey®, Phadia GmbH, cut-off 7 U/ml).

Serum alanine aminotransferase values were measured by standard kinetic methods on an Abbott Architect clinical chemistry analyser (Abbott Laboratories, Abbott Park, IL, USA).

Small-bowel mucosal morphology and immunohistochemistry

Paraffin-embedded samples from the proximal small intestine of mice were cut into 3-µm-thick well-oriented sections [27]. These were stained using monoclonal rabbit anti-Ki-67 IgG (clone SP6, Novus Biologicals, LLC, Littleton, CO, dilution 1:400) as primary antibody followed by biotinylated goat anti-rabbit IgG and avidin-biotin complex reagent (both Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA). Finally, the sections were incubated with peroxidase substrate solution and counterstained in hematoxylin. Stained sections were used to assess the small-intestinal morphology of mice. The mean of villus height, crypt depth and villus height/crypt depth ratio (Vh/CrD) were calculated from approximately 15 measurements per mouse. Further, the cellular infiltration in the lamina propria was determined by counting the total amount of all cells and Ki-67-positive cells per mm^2 of lamina propria.

Small-bowel mucosal TG2-specific IgG deposit stainings in mice

Unfixed 5-µm-thick sections from frozen proximal small-intestinal specimens were stained by direct immunofluorescence using DyLightTM594-conjugated goat antibody specifically against human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at a dilution of 1:800 in PBS, pH 7.4. To investigate whether human IgG and TG2 are co-localized in the small intestine of mice, the specimens were double-stained. First, the sections were stained for human IgG as described above and then for TG2 using polyclonal rabbit antibody to human tissue transglutaminase (Zedira GmbH, Darmstadt, Germany) diluted 1:50 in PBS followed by Alexa Fluor 488 conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR) at a dilution of 1:2000 in PBS. In other tissues deposit staining was established with fluorescein isothiocyanate-conjugated rabbit antibody against human IgG (Dako Denmark A/S, Glostrup, Denmark) at a dilution of 1:160 in PBS. The slides were mounted using a Vectashield mounting medium containing DAPI (Vector Laboratories, Inc.) and images were taken with an Olympus BX60 microscope (Olympus Europa GmbH, Hamburg, Germany).

Investigation of target specificity of small-bowel mucosal IgG deposits

Potassium thiocyanate (KSCN, E. Merck, Darmstadt, Germany), a substance which disrupts the binding of TG2 to fibronectin [28, 29], was used to elute TG2 from frozen mouse small-bowel sections. First unfixed 5-μm-thick cryosections were air-dried for 10 minutes and 2 M KSCN was applied to the sections for 30 minutes. PBS was used as control. The elution was followed by extensive washes with PBS and incubations with antibodies for IgG and TG2 similarly to IgG deposit staining as described in the previous paragraph. Finally the tissue sections were again washed with PBS and mounted with DAPI-containing Vectashield mounting medium (Vector Laboratories, Inc.). To check that fibronectin remained intact in the tissue sections, they were incubated with rabbit anti-human anti-fibronectin antibody (Sigma-Aldrich, St. Louis, MO) at a dilution of 1:500 in PBS for 30 minutes. After incubation the sections were washed with PBS and secondary goat anti-rabbit antibody was added at a dilution of 1:2000 in PBS for 30 minutes. Finally the tissue sections were again washed with PBS and mounted with DAPI-containing Vectashield mounting medium (Vector Laboratories, Inc.). After stainings images were taken with an Olympus BX60 microscope (Olympus Europa GmbH).

Statistics

Statistical comparisons were made using statistical analysis software (PASW Statistics 18, SPSS Inc., Chicago, IL). Data were compared by Kruskal-Wallis test and further tested by Mann-Whitney U test. Data are presented either as mean ± standard error of mean (SEM) or median and range, a p-value ≤ 0.05 being considered significant.

RESULTS

Celiac disease autoantibody and human IgG levels in the mouse sera

To ensure that injected human antibodies were transferred similarly to mice in all cases, mouse sera were tested for the presence of human IgG after completion of experiments. Human IgG was detected in the sera of all injected animals regardless of experimental group, proving that human IgG was successfully transported from the peritoneum to the circulation in all cases. The levels of human IgG were somewhat lower in the sera of mice injected with total human IgG. All the non-injected control mice were negative for human IgG (Table 1).

Next, we assayed the mouse sera for IgG-class celiac disease–specific EmA- and TG2-autoantibodies. Notably, sera from all mice injected with IgA-deficient celiac disease patients' sera or total IgG were positive for both EmA- and TG2-autoantibodies. In contrast, all non-injected control animals and those receiving control sera or IgG were negative for these autoantibodies. The medians of IgG-class EmA-titers in mice injected with celiac patient serum for 8 or 27 days or with total human IgG were 1:200, 1:2000 and 1:50, respectively. The corresponding TG2-IgG-titers were 96.6, >100 and >100 U/ml, respectively (Table 1).

Clinical observations

Mice were monitored for weight and diarrhea during the entire length of the experiments. Noteworthily, at the end of the 8-day serum experiment, weight gain in mice receiving celiac patient sera was virtually halted (increase 1.2 %), whereas in controls the weight increment was 8.7 %. The difference did not, however, reach statistical significance ($P = .121$) (Fig. 1A). In the experiment where human sera were injected into mice for 27 days, a statistically significant delay in weight gain was observed in mice receiving celiac sera up to day 10 when compared to mice treated with control serum ($P < .05$). After this time-point the differences between groups leveled off, although the weight gain in mice injected with celiac patients' sera still remained lower compared to controls (Fig. 1B). In the experiment where total IgG from celiac patients or controls were injected into mice, a statistically significant difference in weight gain between groups was noted on day 8 ($P < .05$) (Fig.

1C). Non-injected control mice, which were included in the latter two experiments, were compatible to mice treated with control sera or total IgG.

Strikingly, mild diarrhea was detected in a subset of animals receiving celiac sera or total IgG, whereas the stool consistency of the control animals was normal in all cases. In the 8-day serum experiment 2 out of 3, in the 27-day serum experiment 2 out of 5 and in the IgG experiment 3 out of 5 of mice presented with mild diarrhea (Table 1).

Mouse small-intestinal mucosal morphology and lamina propria cellular infiltration

The small-intestinal morphology of the mice was studied from Ki-67-stained paraffin sections of the proximal small intestine (Fig. 2). There was no significant difference in the villus height between groups receiving human sera for 8 days, whereas in mice injected with celiac patients' sera for 27 days or with celiac patient total IgG the villi were significantly shorter than in control groups ($P < .001$) (Table 2). Crypts were significantly elongated in mice receiving celiac sera for either 8 or 27 days compared to control serum mice ($P < .001$ and $P < .01$, respectively). In the total IgG injection experiment no such difference was observed (Table 2). Importantly, as seen in Table 2, Vh/CrD was significantly lower in animals injected with celiac sera for 8 or 27 days ($P < .05$ and $P < .001$, respectively) or celiac total IgG ($P < .001$) in comparison to their respective controls. Non-injected control animals matched well with control serum or control IgG injected animals except in the 27-day serum experiment considering crypt depth (Table 2).

In addition to determining the small bowel morphology we also quantified lamina propria cellular infiltration. Total number of infiltrative cells as well as number of infiltrative proliferating cells in mm^2 of lamina propria was significantly higher in the mice injected with celiac patients' sera or total IgG compared to mice injected with control sera or total IgG in all three experiments. Non-injected control mice differed significantly from celiac patient sera or IgG injected mice except when considering total amount of cells/mm2 of lamina propria in the 27-day serum experiment (Table 3).

Human IgG-deposits in mouse tissues

A characteristic feature for untreated celiac disease, regardless of mucosal morphology, is the presence of TG2-targeted autoantibody deposits in the small intestine [2, 7-9, 16]. Strikingly, all animals receiving celiac disease patients' sera or IgG evinced clear subepithelial human IgG positivity along the villus and crypt basement membranes (Fig. 3). In addition, human IgG deposits were also found around mucosal blood vessels. This pattern of human IgG positivity was identical to the normal tissue localization of the mouse *in situ* TG2, as demonstrated by the yellow color due to colocalisation (Fig. 3A). None of the control mice in any of the three experiments had such human IgG deposits in the small-intestinal mucosa (Fig. 3B).

To ascertain that human IgG is specifically bound to mouse TG2 in the small-intestinal mucosa, we disrupted the binding of TG2 to fibronectin with KSCN [28, 29]. Elution of TG2 from frozen sections led to the disappearance of human IgG antibody deposits along with the TG2 signal (Fig. 4A, B). In contrast, fibronectin staining in sections remained essentially unchanged (Fig. 4C).

Focusing on extraintestinal tissues, we detected human IgG deposits on reticulin fibers in liver, skeletal muscle, heart and kidney in all mice receiving celiac patients' sera or IgG, whereas all control animals were negative (Fig. 5).

DISCUSSION

This study demonstrates the passive transfer of celiac disease –like condition by anti-TG2 positive celiac disease patients' sera or IgG. More specifically, in this study we demonstrated that injection of IgA-deficient celiac disease patient sera or G-class immunoglobulins to immune-compromised mice leads to mild diarrhea in a subset of animals. In addition, the mice injected with celiac disease patient sera or total IgG gained less weight than the control animals, although there was slight variation between different experiments especially during the first days. Moreover, the mice injected with IgA-

deficient celiac disease patients' sera or IgG had significantly decreased Vh/CrD, increased density of lamina propria cells and TG2-targeted human autoantibody deposits in several tissues including the small intestine. None of these features were observed in control mice. Interestingly, there are publications showing that patients with early developing celiac disease with normal small-intestinal mucosal morphology also have autoantibody deposits bound to TG2 in their small bowel [2]. Moreover, these patients may suffer from various gastrointestinal symptoms, for example diarrhea [3, 4, 30]. Thus, the condition in the mice injected with sera or IgG from IgA-deficient celiac disease patients' is markedly similar to the early stage celiac disease in humans.

Perhaps the most significant finding in our study is the development of gastrointestinal symptoms coinciding with mild enteropathy in mice following the serum or immunoglobulin injections. Notably, our experimental setting differed from those previously reported [31-33] in that we utilized sera or immunoglobulins from IgA-deficient celiac disease patients containing high titers of IgG-class TG2-autoantibodies. Sera or total IgG from this patient group was used because it was anticipated that human IgA-class anti-TG2-antibodies from IgA-competent celiac disease patients might not be efficiently transported to the mouse circulation due to the absence of IgA-Fc-receptor expression [24]. It has also been reported that human IgA is rapidly degraded in the circulation of rodents [25]. Furthermore, since we wanted to specifically address the effects exerted by patient antibodies and to avoid the development of an anti-idiotypic response in mice [31], we used immunodeficient mice lacking T cells. Although to our knowledge the current article is the first to report celiac disease-specific TG2-targeted autoantibody deposits in the small-intestinal mucosa of mice, the causality between these deposits and the clinical observations remains to be established in further studies.

Previous studies addressing the effects of antibodies from celiac disease patients report the presence of autoantibody deposits not in the small intestine but for instance in liver, muscle fibers and brain [31-33]. Interestingly, these same tissues are target sites for extraintestinal manifestations [2, 12] and

it has been speculated that celiac autoantibodies may play a pathogenetic role. In the current article we addressed liver health by measuring alanine aminotransferase values from mouse sera, but no statistically significant differences were found between mice injected with celiac patient sera or total IgG and controls (data not shown).

There are several examples showing that passive transfer of plasma, serum or IgG-class antibodies from patients with a distinct autoimmune disorder characterized by specific IgG-class antibodies can induce features of the disease in animals [34-38]. The studies in question suggest that humoral autoimmunity can induce pathological changes consistent with the underlying disorders. Our present study is in line with these, since injection of IgA-deficient celiac disease patient sera or total IgG induced characteristics of celiac disease. Although the precise mode of action exerted by autoantibodies remains to be established, the mechanism might involve increased cellular infiltration in lamina propria. In addition, the development of overt small-bowel mucosal villus atrophy with crypt hyperplasia but also extraintestinal manifestations may require even longer exposure to celiac disease patient antibodies and/or additional factors.

We conclude that IgA-deficient celiac disease patient sera or total IgG with high titers of TG2-specific autoantibodies induces characteristics of early developing celiac disease in athymic mice after intraperitoneal injection. The results of this study therefore suggest that IgG-class antibodies from IgA-deficient celiac disease patients could participate in the disease pathogenesis and the generation of clinical symptoms.

DISCLOSURE OF POTENTIAL CONFLICT OF INTERESTS

The authors declare no conflict of interests related to this study.

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FIGURE LEGENDS

Figure 1. Increment of mouse body weight as percentage during the experiments. Athymic mice were injected daily (A) with serum for 8 days (n= 3 mice/group), (B) with serum for 27 days (n= 5 mice/group) or (C) with total IgG for 8 days (n= 5 mice/group). Grey circles represent non-injected control mice, black circles mice receiving control sera or total IgG and open circles mice receiving serum or total IgG from IgA-deficient celiac disease patients. Mean ± SEM, $*P < .05$

Figure 2. Representative pictures of Ki-67-stained proximal small-intestinal specimens from mice. Mice injected with healthy control (left panel) and celiac disease patient serum (right panel) (A) for 8 days or (B) for 27 days and (C) mice injected with healthy control (left panel) and celiac disease patient total IgG (right panel) for 8 days. Scale bar=100 μm

Figure 3. Representative pictures of small-bowel immunofluorescence stainings for human IgG (red) and transglutaminase 2 (TG2) (green) of a mouse injected with (A) celiac patient serum or (B) healthy control serum. Yellow color (arrows) in composite picture indicates colocalization of IgG deposits and TG2. Blue colour represent nuclear staining (DAPI). Three images on the right represent higher magnification of the area marked in the image on the left. The images are from mice injected with sera for 27 days. Scale bar=100 μm

Figure 4. Specificity of deposited human IgG for transglutaminase2 (TG2) in mice injected with celiac disease patient sera or total IgG. (A) In PBS-treated sections where the binding of TG2 (green) to fibronectin is intact, human IgG (red) is detectable and colocalized with TG2 (yellow). (B) After incubation with potassium thiocyanate (KSCN), which disrupts the binding of TG2 to fibronectin, deposited human IgG (red) and extracellular TG2 (green) disappear from the sections. (C) Fibronectin

staining was visible after KSCN treatment. The images are from mice injected with sera for 27 days. Scale bar=100 µm in the lower magnification images on the left and scale bar=50 µm in other images

Figure 5. Immunofluorescent stainings for human IgG (green) in kidney (A), liver (B) and skeletal muscle (C) and heart (D) of celiac disease patient serum-injected or control mice. Blue colour represent nuclear staining (DAPI). The images are from mice injected with sera for 27 days. Scale bar=100 µm

Table 1. Increment of body weight, severity of diarrhea and human antibody levels in individual mice in different experiments.

Experiment	Group	Increment of body weight (%)	Diarrhea evaluation	Human IgG (mg/ml) in mouse serum	Serum EMA-G titer in mouse serum	TG2-G (U/ml) in mouse serum
Serum injections, 8 days	**Celiac patient serum-injected mice (n=3)**	4.8	none	ND	1:500	>100
		1.8	mild	1.4	1:200	92.9
		0.5	mild	2.5	1:100	96.6
	Control serum-injected mice (n=3)	6.5	none	1.6	neg	neg
		10.8	none	2.0	neg	neg
		5.6	none	1.8	neg	neg
Serum injections, 27 days	**Celiac patient serum injected mice (n=5)**	2.2	mild	1.6	1:2000	>100
		5.6	none	2.2	1:1000	78.8
		10.9	mild	1.7	1:500	>100
		10.6	none	1.9	1:4000	>100
		8.3	none	1.1	1:2000	>100
	Control serum injected mice (n=5)	19.5	none	1.7	neg	neg
		7.2	none	1.9	neg	neg
		8.0	none	1.6	neg	neg
		11.6	none	1.6	neg	neg
		5.1	none	1.4	neg	neg
	Non-injected control mice (n=2)	16.6	none	0.0	neg	neg
		6.0	none	0.0	neg	neg
Total IgG injections, 8 days	**Celiac patient total IgG-injected mice (n=5)**	5.2	mild	0.5	1:50	>100
		6.5	none	0.5	1:10	>100
		6.8	mild	0.5	1:50	>100
		5.8	mild	0.5	1:50	>100
		4.6	none	0.4	1:50	>100
	Control total IgG-injected mice (n=5)	10.6	none	0.4	neg	neg
		6.9	none	0.6	neg	neg
		10.4	none	0.7	neg	neg
		6.4	none	0.6	neg	neg
		6.9	none	0.6	neg	neg
	Non-injected control mice (n=2)	10.0	none	0.0	neg	neg
		8.8	none	0.0	neg	neg

For the serum EMA-G results, a serum dilution of 1:≥10 was considered positive; for TG2-G results, ≥7 U/ml was considered positive; EMA-G, IgG-class endomysium antibody; TG2-G, IgG-class transglutaminase2 antibody; ND, not determined; neg, negative.

Table 2. Small-bowel mucosal morphology of mice. The mean values were calculated from approximately 15 measurements per mouse.

Experiment	Group	Villus height, mean (range), µm	Crypt depth, mean (range), µm	Villus height crypt depth ratio (Vh/CrD), mean (range)
Serum injections, 8 days	**Celiac patient serum-injected mice (n=3)**	454 (313-579)	112 (75-161)	4.13 (3.05-5.87)
	Control serum-injected mice (n=3)	440 (249-569)	101 (74-138)***	4.42 (2.65-6.32)*
Serum injections, 27 days	**Celiac patient serum injected mice (n=5)**	497 (354-641)	130 (71-185)	3.92 (2.60-5.96)
	Control serum-injected mice (n=5)	549 (364-732)***	123 (82-182)**	4.57 (2.72-6.88)***
	Non-injected control mice (n=2)	550 (367-735)***	129 (85-200)	4.29 (2.44-5.58)***
Total IgG injections, 8 days	**Celiac patient total IgG-injected mice (n=5)**	499 (376-668)	129 (82-180)	3.94 (2.32-5.69)
	Control total IgG-injected mice (n=5)	558 (356-781)***	124 (64-176)	4.63 (3.15-7.71)***
	Non-injected control mice (n=2)	559 (482-647)***	129 (79-174)	4.46 (2.85-6.21)**

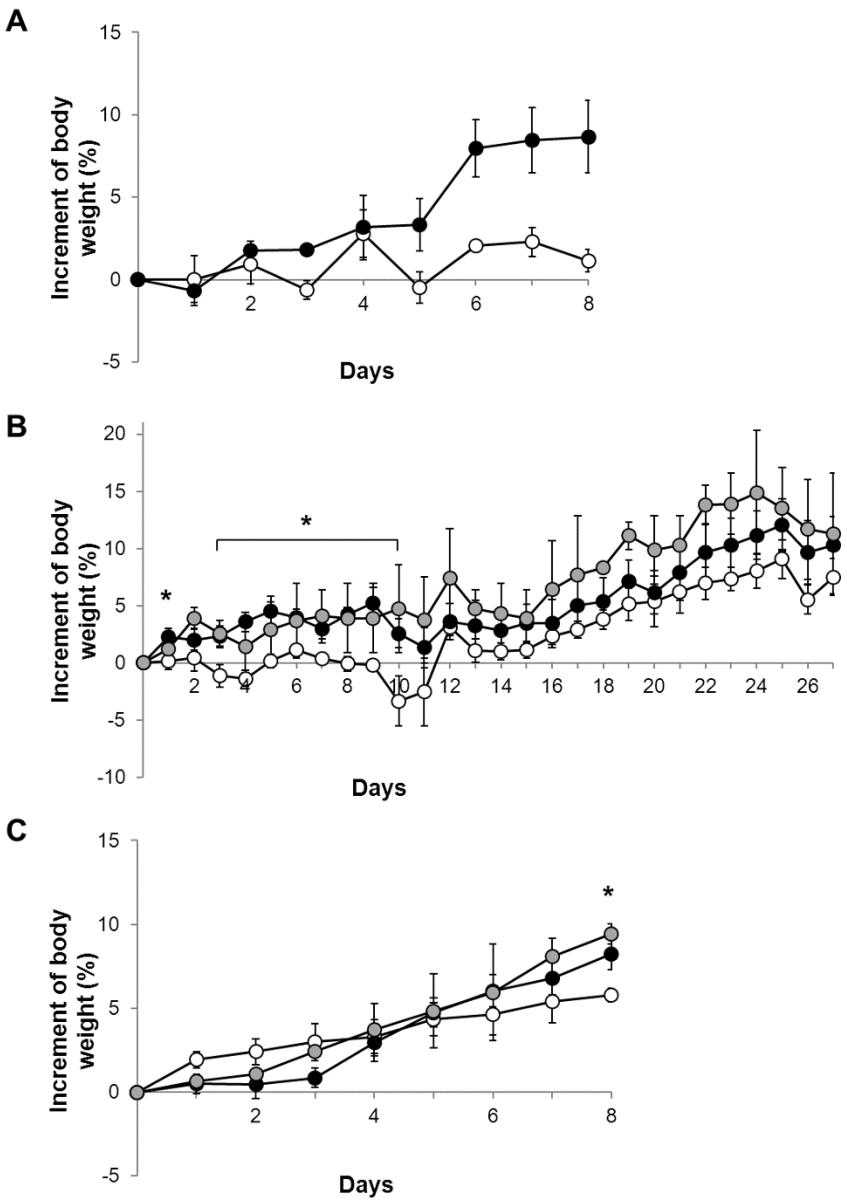
*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ when compared to the celiac disease patient derived serum or total IgG treated group within the experiment.

Table 3. Lamina propria infiltration of mice. The mean values were calculated from approximately 30 measurements per mouse.

Experiment	Group	Total amount of cells/mm^2 in lamina propria	Ki-67-positive cells/mm^2 in lamina propria
Serum injections, 8 days	**Celiac patient serum-injected mice (n=3)**	10854 (3750-21875)	1201 (0-4375)
	Control serum-injected mice (n=3)	7139 (1875-11250)***	694 (0-3125)***
Serum injections, 27 days	**Celiac patient serum injected mice (n=5)**	9867 (3125-25000)	1017 (0-3750)
	Control serum-injected mice (n=5)	7933 (2500-16250)***	767 (0-3750)**
	Non-injected control mice (n=2)	8729 (3750-15625)	729 (0-2500)*
Total IgG injections, 8 days	**Celiac patient total IgG-injected mice (n=5)**	13242 (0-26250)	2267 (0-11250)
	Control total IgG-injected mice (n=5)	8117 (2500-17500)***	883 (0-4375)***
	Non-injected control mice (n=2)	7729 (3125-13125)***	656 (0-2500)***

*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ when compared to the celiac patient derived serum or total IgG treated group within the experiment.

Figure 1.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Increment of body weight (%)
Days
B
Increment of body weight (%)
Days
*
*
C
Increment of body weight (%)
Days
*

Figure 2.

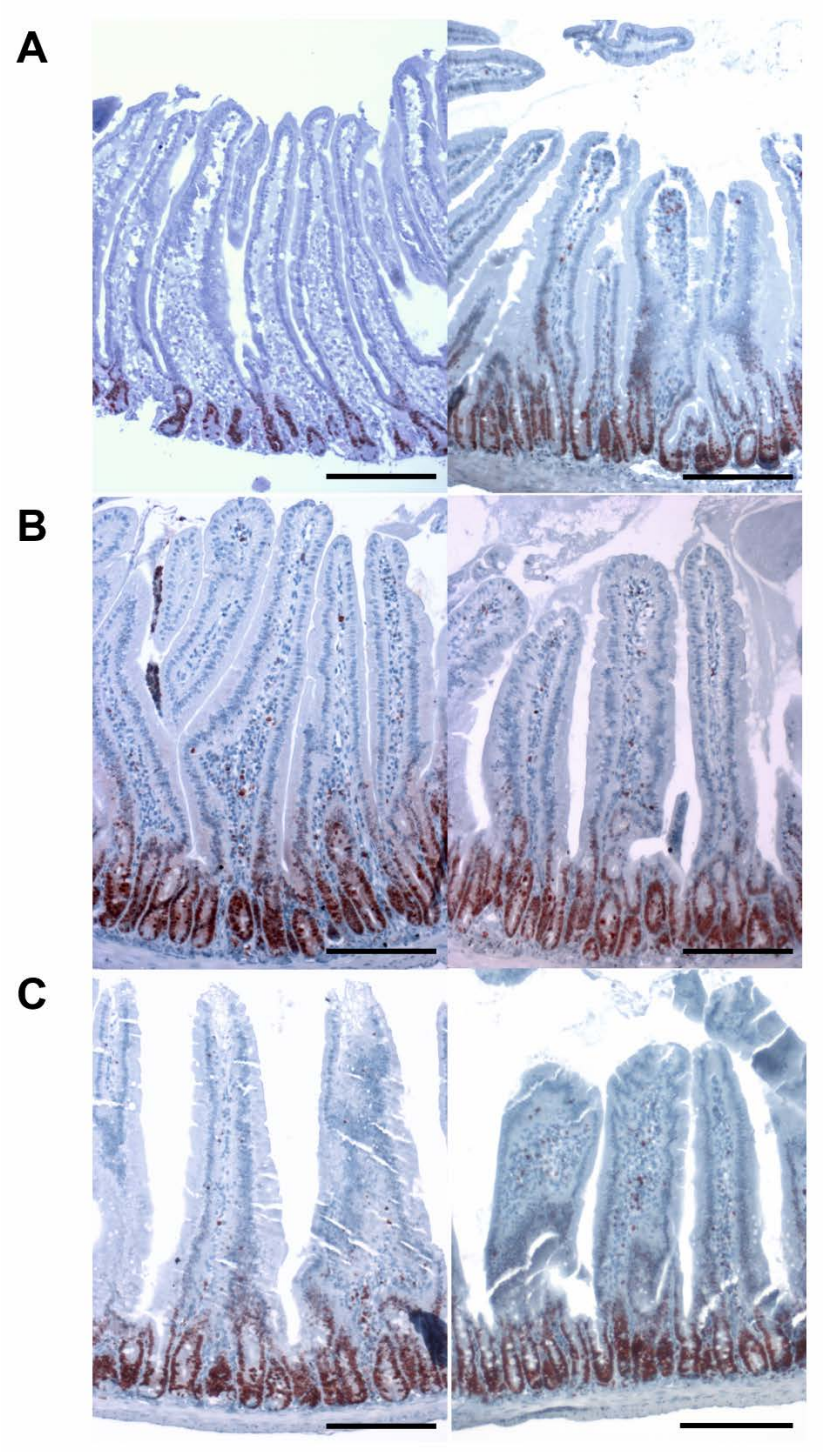


Figure 3.

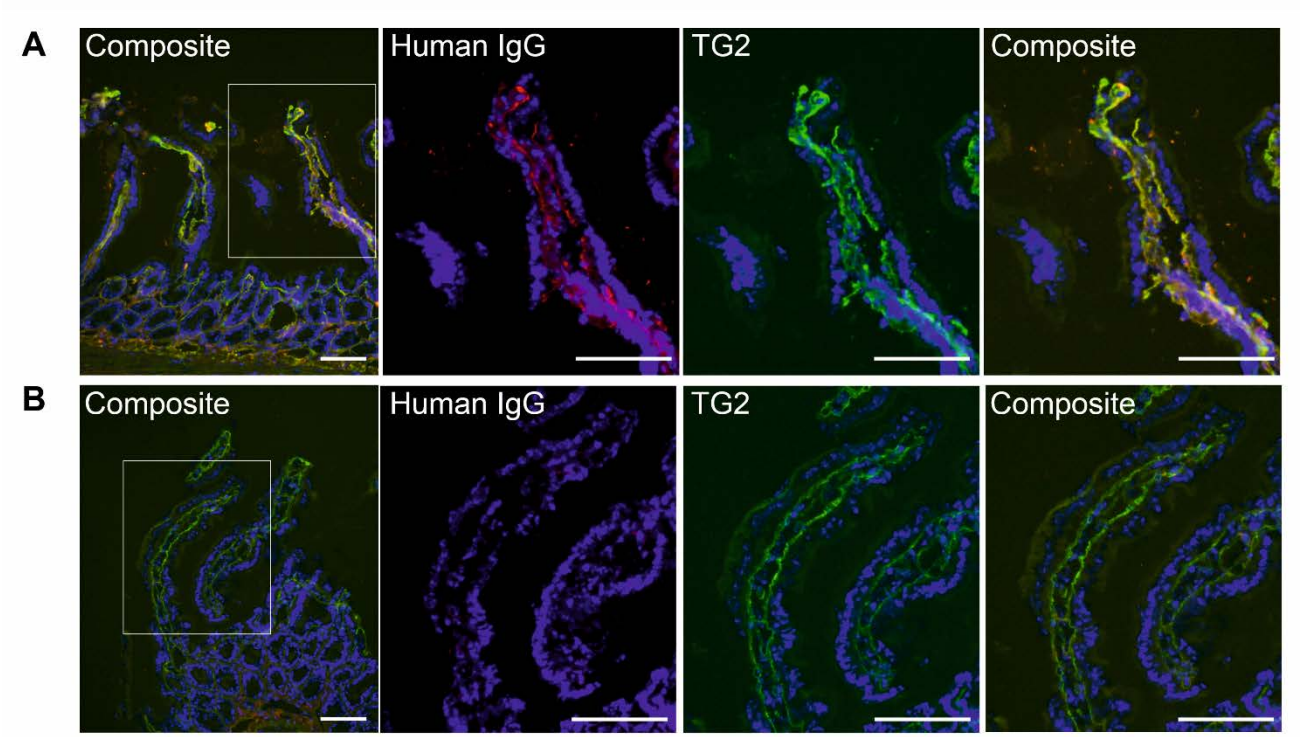

A
Composite
Human IgG
TG2
Composite
B
Composite
Human IgG
TG2
Composite

Figure 4.

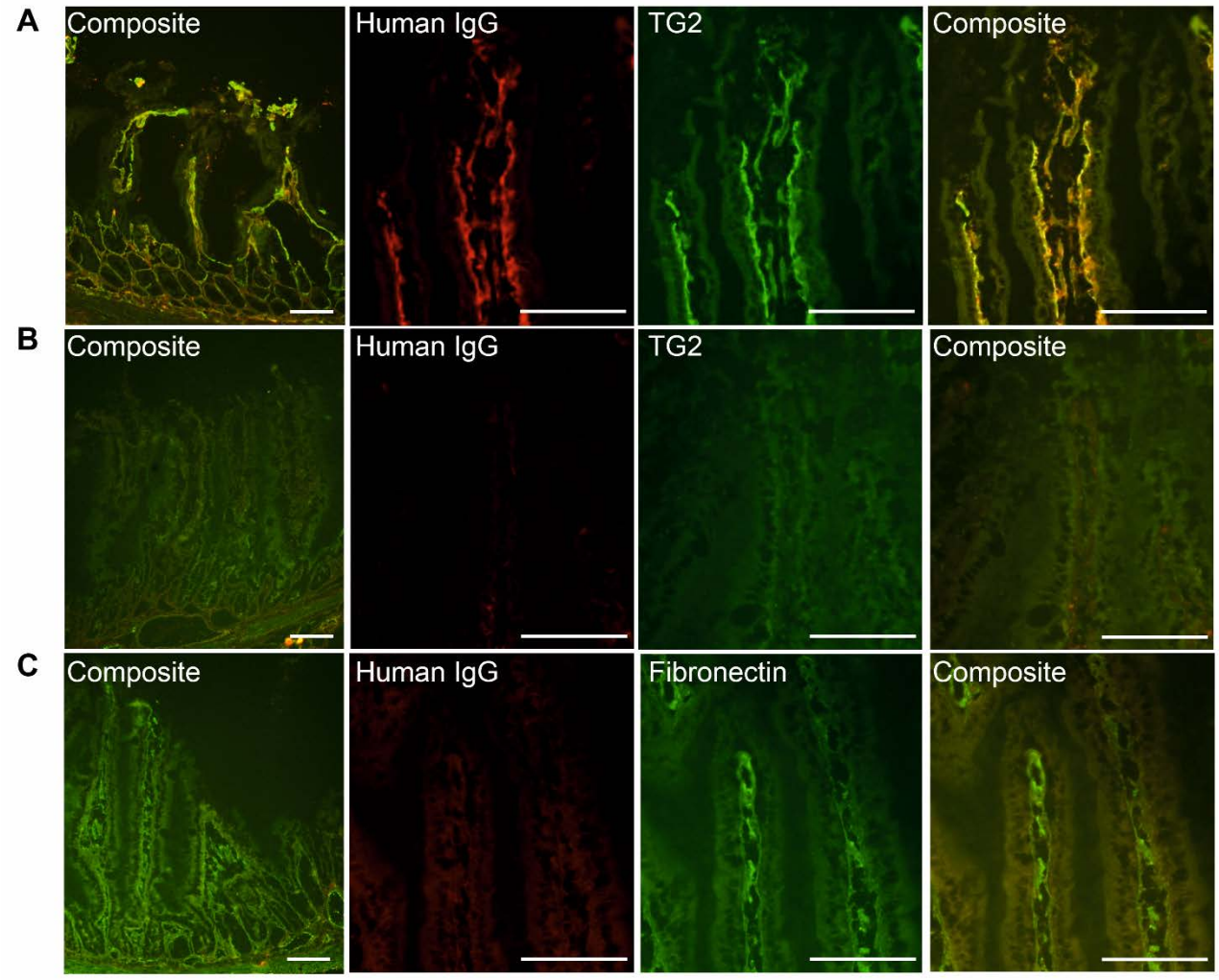
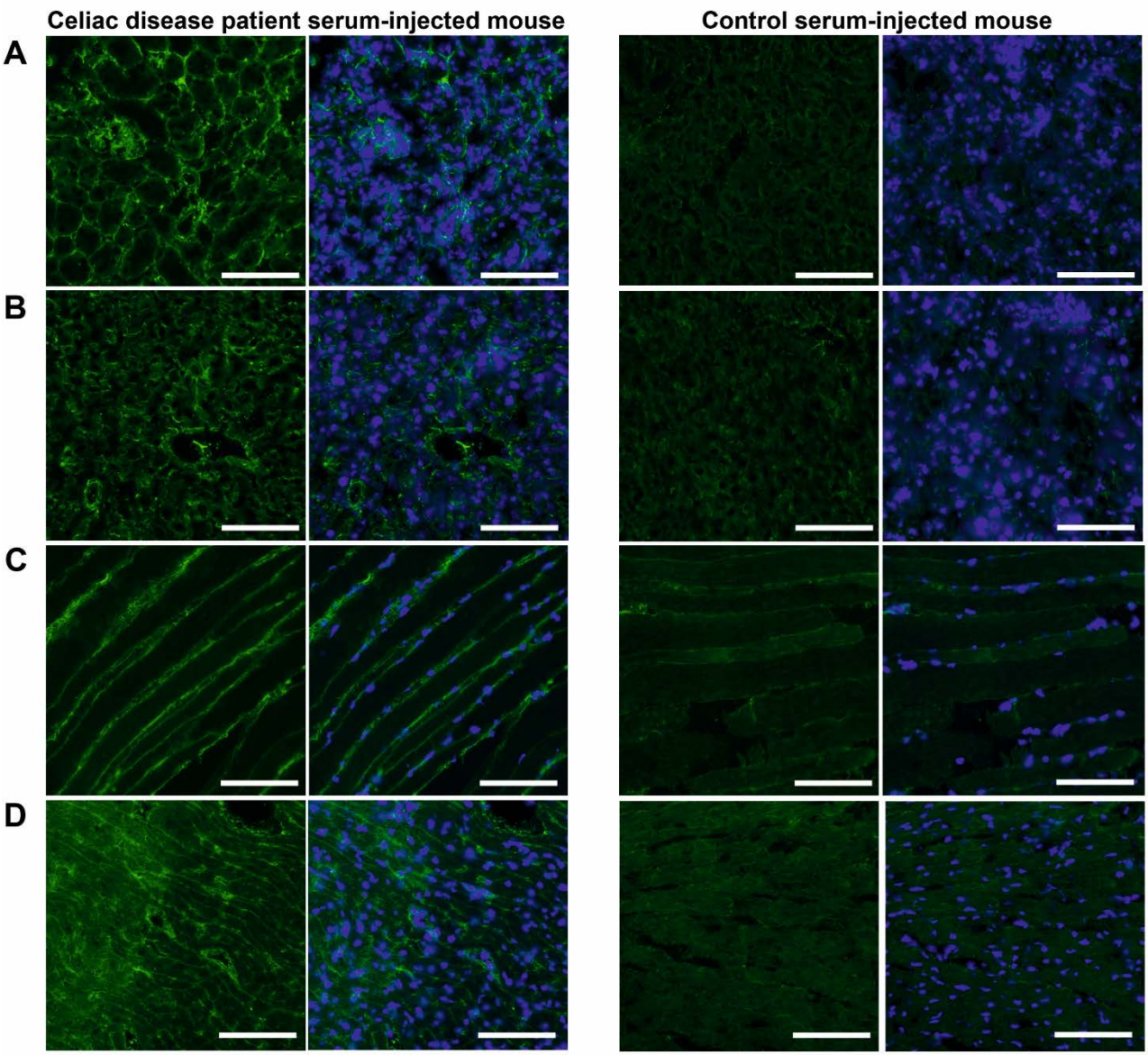

A
Composite
Human IgG
TG2
Composite
B
Composite
Human IgG
TG2
Composite
C
Composite
Human IgG
Fibronectin
Composite

Figure 5.


Celiac disease patient serum-injected mouse
Control serum-injected mouse
A
B
C
D

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Celiac Disease–Specific TG2-Targeted Autoantibodies Inhibit Angiogenesis *Ex Vivo* and *In Vivo* in Mice by Interfering with Endothelial Cell Dynamics

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Abstract

A characteristic feature of celiac disease is the presence of circulating autoantibodies targeted against transglutaminase 2 (TG2), reputed to have a function in angiogenesis. In this study we investigated whether TG2-specific autoantibodies derived from celiac patients inhibit angiogenesis in both *ex vivo* and *in vivo* models and sought to clarify the mechanism behind this phenomenon. We used the *ex vivo* murine aorta-ring and the *in vivo* mouse matrigel-plug assays to address aforementioned issues. We found angiogenesis to be impaired as a result of celiac disease antibody supplementation in both systems. Our results also showed the dynamics of endothelial cells was affected in the presence of celiac antibodies. In the *in vivo* angiogenesis assays, the vessels formed were able to transport blood despite impairment of functionality after treatment with celiac autoantibodies, as revealed by positron emission tomography. We conclude that celiac autoantibodies inhibit angiogenesis *ex vivo* and *in vivo* and impair vascular functionality. Our data suggest that the anti-angiogenic mechanism of the celiac disease-specific autoantibodies involves extracellular TG2 and inhibited endothelial cell mobility.

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Introduction

Angiogenesis, the formation of blood vessels, has emerged as an essential phenomenon involved in various disorders. Also intestine-related diseases, such as inflammatory bowel disease, ascites and peritoneal adhesions, are characterized or contributed by dysregulated blood vessel growth or formation [1]. In inflammatory bowel disease, for instance, it has been demonstrated that increased vascularization is present in the inflamed colonic mucosa of the patients and the expression of several angiogenic factors is markedly increased [2,3]. Similarly, untreated celiac disease patients have been reported to evince abnormalities in their small-intestinal mucosal vasculature [4,5]. In addition to these vascular aberrations, untreated celiac patients have disease-specific circulating autoantibodies targeted against transglutaminase 2 (TG2) in their sera and as deposits in their small-intestinal mucosa. In the mucosa autoantibodies are bound to TG2 below the epithelium on the basement membrane and interestingly also around blood vessels [6,7].

The target of the celiac autoantibodies, TG2, is a ubiquitously expressed enzyme involved in a wide range of cellular processes including angiogenesis. TG2, expressed highly by endothelial cells, contributes to angiogenesis by cross-linking a variety of extracellular matrix (ECM) proteins through the formation of Ca^{2+}-dependent covalent linkages [8,9]. Celiac disease-specific TG2-targeted autoantibodies have been proposed to disturb endothelial cell biology *in vitro* [10,11], but information about their capability to interfere with vessel formation and function in more complex *ex*

vivo and *in vivo* systems is not available. This study was designed specifically to address the question what kind of effects the celiac disease-specific autoantibodies have on vascular formation and functionality *ex vivo* and *in vivo* and to discover the mechanism behind.

Materials and Methods

Ethics statement

The protocol for mouse studies was approved by the Finnish and Hungarian authorities, the Turku Central Animal Laboratory (University of Turku, Finland) and the Debrecen University animal facility (Debrecen, Hungary). The study protocol for using human serum samples was approved by the Ethics Committee of Tampere University Hospital, Tampere, Finland, and written informed consent was received from all subjects.

Animals

For *ex vivo* and *in vivo* studies, 4–6-week-old female Balb/c mice (Harlan Laboratories Inc. Horst, the Netherlands) or C57BL/6 wild type or TG2 knockout mice [12], were housed at 22°C in a 12-hour light/dark cycle with water and food freely available. The animals were cared for and used in accordance with the regulations in Finland, Hungary and the European Union (86/609/EC).

Purification of serum IgA and production of monoclonal antibodies

Serum samples from three biopsy-proven celiac disease patients on a gluten-containing diet and positive for both anti-TG2 (>100 U/ml; Celikey, Phadia GmbH, Freiburg, Germany) and endomysial antibodies (1:>2,000) were employed in the study. As controls we used serum samples from three non-celiac controls, which all were negative for the above-mentioned antibodies. Total IgA fractions from serum samples were purified as previously described [10], using cyanogen bromide-activated Sepharose 4B (Pharmacia Upjohn, Uppsala, Sweden) coupled with 7 mg/ml rabbit anti-human IgA antibodies (Sigma Aldrich, St Louis, MO, USA). Thereafter, the IgA samples were lyophilized and resolubilized in Hank's balanced salt solution to a final concentration of 100 μg/ml. Purified antibodies were used in the experiments at a concentration of 1 μg/ml.

The following IgG-class recombinant monoclonal autoantibodies prepared from celiac patients were used: celiac patients' anti-TG2 specific monoclonal antibodies targeting the major celiac epitope; clone 4.1 (CD Mab) and irrelevant control antibodies (clones 5.1 and 6.2, non-CD Mab) targeted against Escherichia coli proteins M5 and M6 [13,14]. Recombinant technology was essentially applied in Chinese hamster ovary cells to produce the monoclonal antibodies as previously described [15], which were used in the experiments at a concentration of 1 μg/ml.

Ex vivo aorta ring and *in vivo* matrigel plug angiogenesis assays

Mouse aortas were cut into 0.5 mm-thick rings and embedded in matrigel (BD Biosciences, Bedford, MA, USA) containing CD Mabs or their respective controls, non-CD Mabs. The aorta rings were cultured in EGM-2 plus endothelial cell growth factors provided in the EGM-2 Bulletkit (Clonetics, San Diego, CA, USA) for ten days. Thereafter, images of endothelial sprouts and interconnected capillary tubes were randomly taken using a Zeiss inverted microscope and Axiovision 3.0 program (Carl Zeiss Vision GmbH, München-Hallbergmoos, Germany). The pictures were analyzed using ImageJ software (http://rsb.info.nih.gov/ij) [16].

In vivo mouse angiogenesis assays were performed using matrigel (BD Biosciences) containing 10 μg/kg of erythropoietin (EPO; Sigma Aldrich, St. Louis, MO, USA), which was injected subcutaneously into the backs of mice. This system is based on the EPO-induced self-production of vessels by cells migrating into the matrigel plugs from the host [17–20]. CD Mabs or their respective controls were mixed with matrigel and injected to recipient mice. After eight days, some of the mice were examined by positron emission tomography (PET). Finally, the matrigel plugs were removed from all animals, snap-frozen and stored at −80°C for further analysis.

In vitro angiogenesis assays

In vitro assays were performed using human umbilical vein endothelial cells (HUVECs) purchased from Lonza (Cambrex Bio Science, Walkersville, MD, USA). HUVECS were cultured at 37°C and 5% CO^2 in endothelial growth medium-1 (EGM-I; Clonetics). EGM-I consists of endothelial cell basal medium (EBM-I; Clonetics) and endothelial cell growth factors provided in the EGM-I Bulletkit (Clonetics).

HUVECs (2.5×10^5cells/well) were mixed with matrigel (BD Biosciences; diluted 1:3 in EGM-1) and celiac patient-derived IgA (CD IgA) or CD Mab or their respective controls (non-CD IgA and non-CD Mab). In a subset of experiments we used an active site-directed irreversible extracellular TG2 inhibitor R281 at a concentration of 200 μM [21]. In this case, the inhibitor was administered one hour prior to addition of antibodies. After 48 hours images of different fields were randomly taken using a Zeiss inverted microscope and Axiovision 3.0 program (Carl Zeiss Vision GmbH, München-Hallbergmoos, Germany). Length, branch, area and number of endothelial tubules were measured using ImageJ analysis software.

In addition, HUVECs embedded in matrigel as described above were cultured for ten days in Cell-IQ (Chip-man Technologies LTD, Tampere, Finland). Briefly, the system consists of a cell incubator containing an integrated system designed to take and analyze images. During the assays images were taken every five minutes. The Cell IQ software was used for further enumeration of apoptotic and immobile cells and video editing. For the analysis of cell movements cells were tracked using MTrack tool in ImageJ software.

In vivo vascular functionality assay

Balb/c mice, injected subcutaneously with matrigel plugs as described above, received an intravenous injection of Hoechst 33342 (Hoechst; Invitrogen, Carlsbad, CA, USA) five minutes before euthanasia. Thereafter, the plugs were snap-frozen after excision and stored at −80°C until further analysis.

Positron emission tomography

In this experiment one mouse received three matrigel implants, each treated with PBS (basal group), non-CD Mab or CD Mab and injected subcutaneously into separate limbs. Mice were anesthetised with isoflurane and intravenously injected with 2-[^{18}F]-fluoro-2-deoxy-D-glucose ([^{18}F]FDG). PET imaging for 20 minutes was performed using an Inveon Multimodality scanner (Siemens Medical Solutions, Knoxville, TN) at 60 minutes post injection and reconstructed with the ordered-subsets expectation maximization 2D algorithm (OSEM2D). After PET imaging the animals were euthanised and the matrigel plugs excised, weighed and measured for radioactivity using a gamma counter (Triathler 3″, Hidex, Turku, Finland), which was cross-calibrated with a dose

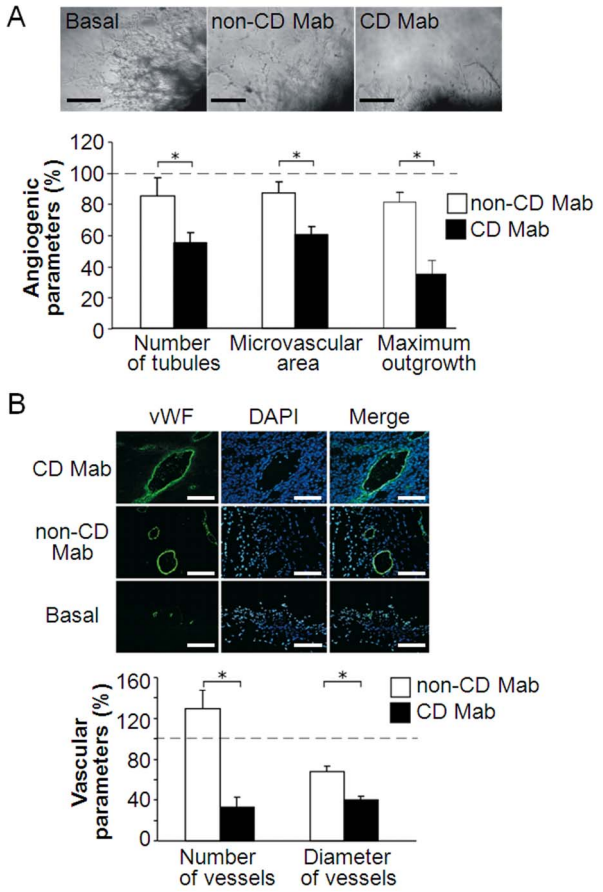

Figure 1. Angiogenesis is impaired *ex vivo* and *in vivo* after treatment with celiac disease patient monoclonal autoantibodies. (A) Representative figures taken from mouse aorta rings cultured inside matrigel for ten days in the presence of monoclonal celiac disease-specific transglutaminase 2 antibody (CD Mab) or its respective control (non-CD Mab). Scale bars represent 100 μm. Bars represent different angiogenic parameters. (n = 4 aortas per group). (B) Representative pictures of mouse matrigel implants treated with CD Mab or non-CD Mab. After eight days the implants were removed and stained for blood vessels with von Willebrand factor (vWF)-antibody (green) and blue color indicates nuclei (DAPI). Scale bar represents 200 μm. Bars show vascular parameters measured from matrigel implants. (n = 8 animals per group). Bars in both charts, A and B, represent the average value as percentage + SEM. All data was normalized to the basal group (dotted line). * represents P≤0.001 statistical difference.
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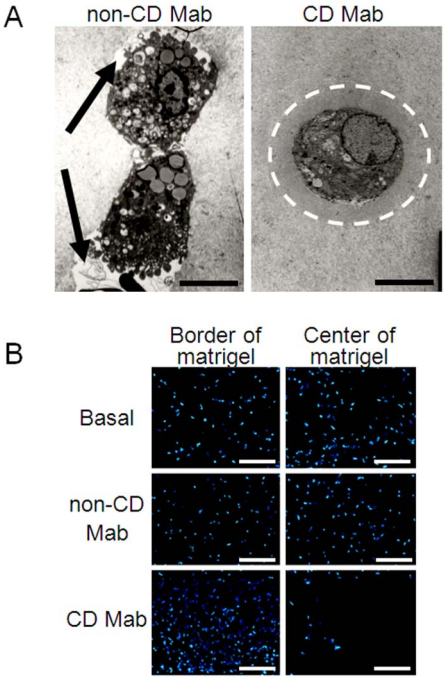

Figure 2. Cellular behavior is altered in the presence of celiac autoantibodies. (A) Transmission electron micrographs of mouse aorta ring experiments in the presence of monoclonal celiac patient-derived transglutaminase 2-targeted antibody (CD Mab) or its respective control (non-CD Mab). Arrows indicate the extracellular matrix (ECM)-free area in the leading edges of the cells treated with non-CD Mab and the white dotted line shows an area of densely organized ECM around a cell treated with CD Mab. Scale bar represents 6 μm. (B) Representative images taken from mouse matrigel implants treated with CD Mab or non-CD Mab. Nuclei are shown as blue (DAPI) from the border and center of the implants. Scale bar represents 300 μm.
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calibrator (VDC-202, Veenstra Instruments, Joure, the Netherlands). Quantitative analysis was performed by drawing regions of interest in the matrigel plug areas (Vinci software, version 2.54; Max Planck Institute for Neurological Research, Cologne, Germany). The average radioactivity concentration (kBq/ml) in the regions of interest was used to verify *in vivo* results.

Electron microscopy

Aortas collected from the mice and treated as described above were prepared for electron-microscopy analysis. Briefly, the specimens were cut to 1 mm thickness, fixed in 5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA) in 0.16 M s-collidin buffer (pH 7.4) and post-fixed with potassium ferrocyanide-osmiun tetroxide as previously described [22]. Samples were embedded in epoxy resin (Glycidether 100, Merck, Darmstadt, Germany) and cut into thin sections (70 nm) [22]. Sections were stained with 5% uranyl acetate and 5% lead citrate in an Ultrostainer (Leica, Wien, Austria) and examined under a JEM-100SX transmission electron microscope (JEOL, Tokyo, Japan).

Immunofluorescence studies

Frozen sections from mouse matrigel plugs were fixed in 4% paraformaldehyde and processed to 5–7-μm-thick sections. The presence of vessels in the matrigel implants was studied by immunofluorescent labeling with an anti-von Willebrand factor (vWF) antibody (1:200, Dako, Glostrup, Denmark) and counterstained with a secondary antibody labeled with Alexa-488 (1:1000, Molecular Probes, Eugene, OR, USA) at room temperature. Nuclei were stained with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA, USA). Images were taken with an Olympus BX60 microscope (Olympus Europa GmbH, Hamburg, Germany) and Cell^{D} imaging software (Olympus Europa GmbH, Hamburg, Germany). Quantitative data were obtained by counting the number and diameter of vWF-positive blood vessels as well as the total number of cells per matrigel area in digitalized images using ImageJ software.

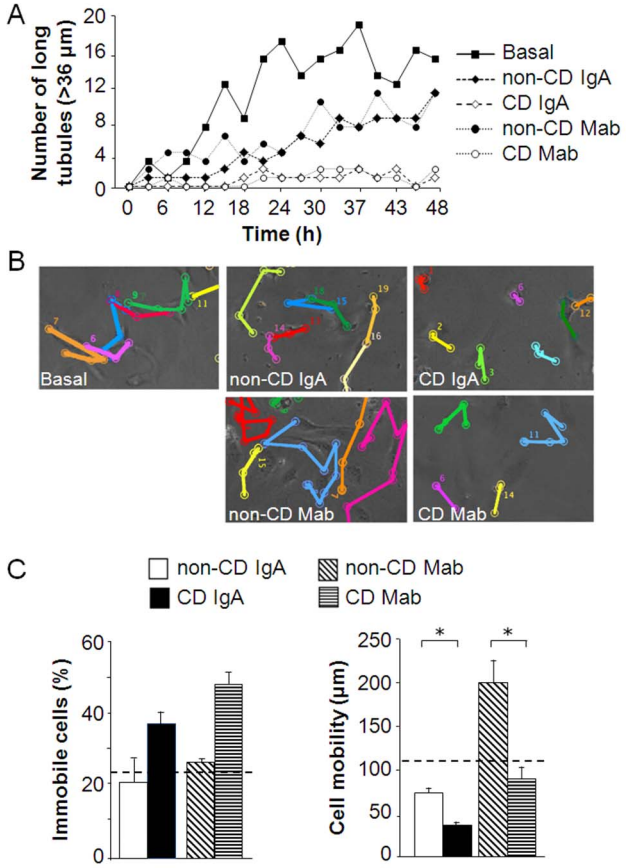

Figure 3. Endothelial cell dynamics in the presence of celiac patient-derived total IgA (CD IgA) or monoclonal antibodies (CD Mab), or their relevant controls (non-CD IgA or non-CD Mab). (A) The number of long tubules (>36 µm) was counted from videos taken from human umbilical vein endothelial cell (HUVEC) cultures during a 48 hour time course in the presence of CD IgA or CD Mab, or their relevant controls non-CD IgA or non-CD Mab. (B) HUVECs were tracked for 48 hours and cellular mobility during that time course is indicated in representative pictures. Each color represents an individual cell. (C) The percentage of immobile cells (left panel) and the cell mobility in micrometers (right panel) were determined in the cell cultures treated with CD IgA or CD Mab or their relevant controls. Bars represent the average value as percentage + SEM. Basal group is indicated as a dotted line. * represents P≤0.001 statistical difference.
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Demonstration of TG2 in the matrigel

First, 100 µl of matrigel (BD Biosciences) was jellified and mixed with 100 µl of Laemmli-buffer. After ten minutes the samples were sonicated and centrifuged for ten minutes at 12 000 rpm at 4°C. Subsequently, the samples were concentrated with acetone. The protein concentrations were measured by the Bradford method and 20 µg of total protein was loaded on 10% polyacryl amide gels (1.5 M Tris–HCl, pH 8.8; sodium dodecyl sulfate, 12%; acrylamide/bis-acrylamide, 30%; APS, 10%; and TEMED), followed by transfer to a Hybond-P membrane (Amersham Biosciences, Little Chalfont, Bucks, UK). After blocking in 5% milk, the membrane was incubated with mouse monoclonal antibody against TG2, CUB7402 (1:200, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), followed by a secondary rabbit antibody conjugated with horseradish peroxidase. Detection was performed with the ECL PlusWestern Blotting Detection System (ECL, GE Healthcare Biosciences, Pittsburgh, PA, USA).

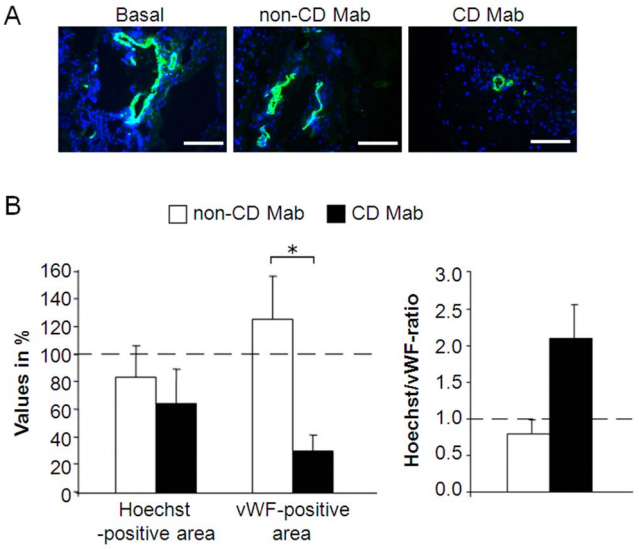

Figure 4. Functionality of vessels in mice in the presence of celiac patient-derived monoclonal autoantibodies (CD Mab) or their relevant controls (non-CD Mab). (A) Representative images of mouse matrigel implants were treated with CD Mab or non-CD Mab. Hoechst 33342 (Hoechst) was injected intravenously into a tail vein five minutes prior to euthanasia. The removed implants were stained for blood vessels with anti-von Willebrand factor (vWF)-antibody (green). Blue color indicates Hoechst-stained nuclei (DAPI) and scale bar represents 200 µm. (B) Vascular functionality parameters were determined from matrigel implants (left panel) and the ratio of Hoechst- and vWF-positive area was calculated (right panel). (n = 4 animals per group). Bars represent the average value as percentage + SEM. All data was normalized to the basal group (dotted line). * represents P≤0.05 statistical difference.
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Statistical analysis

Statistical comparisons were made using statistical analysis software (PASW Statistics 18, SPSS Inc., Chicago, IL, USA). The data were first tested for homogeneity. When the data fulfilled this criterion and there were three or more groups involved in an experiment, one-way ANOVA analysis was used. A two-way ANOVA within subjects was used to compare the effect of TG2 inhibitor with respect to each treated group. In both cases a Student Newman Keuls (SNK) test was performed as *post hoc* analysis. Non-homogeneous data were compared with Kruskal-Wallis test and further tested by Mann-Whitney U test. The data are presented as mean ± standard error of mean (SEM), a p-value≤0.05 being considered significant.

Results

Celiac disease antibodies impair angiogenesis *ex vivo* and *in vivo*

To investigate the effects of celiac disease antibodies on angiogenesis occurring in an assay, which recapitulates all the key steps in the process including matrix degradation, migration, proliferation and reorganization, we performed e*x vivo* aorta ring experiments. Using this system, we observed that the number of the tubules, the microvascular area and the maximum microvessel outgrowth were significantly reduced in the presence of CD Mab as compared to non-CD Mab (P≤0.001; Figure 1A). As definitive tests for angiogenesis require *in vivo* experiments, we next performed mouse matrigel experiments. In this assay our results were parallel to those obtained from aorta ring experiments as both the number and diameter of the vessels inside the matrigel

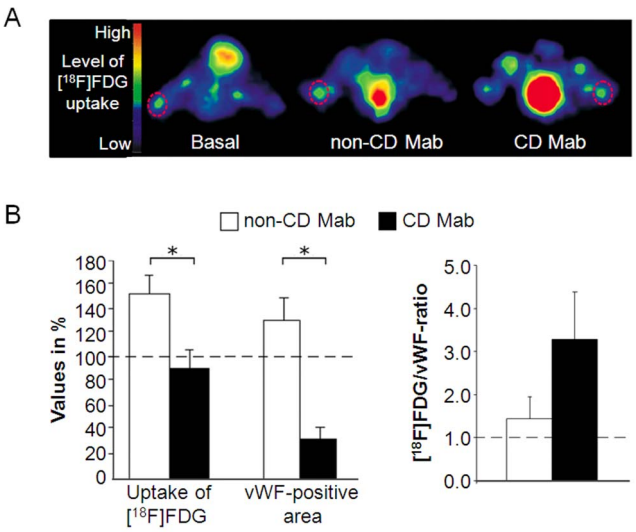

Figure 5. Vessel functionality studied by positron emission tomography (PET). (A) 2-[^{18}F]-fluoro-2-deoxy-D-glucose ([^{18}F]FDG) PET scans of mice implanted with matrigel (basal) or supplemented with celiac disease-specific monoclonal autoantibodies (CD Mab) or control antibodies (non-CD Mab). Each mouse received three matrigel implants (basal, CD Mab or non-CD Mab), each injected subcutaneously into separate limb as highlighted by circles in the scan. The magnitude of [^{18}F]FDG uptake is defined in different colors. (n = 3) (B) The uptake of [^{18}F]FDG in the implants was analyzed from the PET images and the area occupied by vessels was determined by von Willebrand factor (vWF)-antibody staining (left panel). The ratio of [^{18}F]FDG uptake and vascular area was calculated (right panel). Bars represent average value as percentage + SEM. All data was normalized to the basal group (dotted line). * represents P≤0.05 statistical difference.
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were significantly decreased in the presence of CD Mab (P≤0.001; Figure 1B).

To gain further insight into the effects of celiac antibodies, we took transmission electron microscope images from *ex vivo* mouse aorta ring assays, and observed that in control cultures there were pseudopodia in the protruding leading edges of cells and pericellular areas free of ECM (Figure 2A). In contrast, in the presence of celiac patient TG2-targeted autoantibodies cells outgrowing from mouse aortas were round and did not exhibit cellular processes characteristic for the leading edge during migration. Furthermore, the mouse matrigel implants were used to study the localization of cells migrating from the mice to the plug. We found that in the basal group and in the group treated with non-CD Mab, cells had migrated from the animal even to the center of the matrigel and were evenly distributed inside the matrigel implant. In contrast, in the matrigels treated with CD Mab, cells were mainly located in the border of the matrigel and only few were found in the central area (Figure 2B).

Dynamics of endothelial cell behavior is altered in the presence of celiac disease antibodies

The data obtained from aorta ring and mouse matrigel experiments suggested that the anti-angiogenic effects exerted by celiac antibodies could be contributed by defective migration. To study this further we took videos of HUVECs growing inside matrigel. In cultures supplemented with control antibodies, the formation of tubule-like structures was evident after 20 hours of culture. However, in the presence of celiac antibodies (both total CD IgA and CD Mab) the formation of tubule-like structures, especially long ones (>36 μm), was inhibited when compared to controls during the 48 hour time course (Figure 3A, Video S1). Concurrently, the number of branches and tubules after 48 hours of culture were significantly reduced in the cultures treated with IgA and CD Mab compared to controls (Figure S1).

Tracking of cells grown inside matrigel revealed that in the presence of celiac antibodies the number of immobile cells was increased. The cells that did move regardless of the presence of celiac antibodies, moved half of the distance of those cultured in the presence of their relevant control antibodies (P≤0.001). However, cellular mobility showed extensive variability among experimental groups (Figure 3B, C). To exclude the possibility that the anti-angiogenic effects of celiac disease antibodies are due to increased apoptosis, we quantified the number of apoptotic cells and found that their number was not increased in the presence of celiac antibodies (Figure S2).

Celiac disease TG2-specific antibodies affect *in vivo* vascular functionality

We next studied capillary perfusion in *in vivo* matrigel implants using an intravenous tail vein injection of Hoechst [23]. In all treatment groups the vessels inside the matrigel were functional, since in each case the injected dye was located around blood vessels (Figure 4A). The percentage of the Hoechst-positive area was similar in all groups, whereas the area occupied by vWF-staining was significantly reduced in the group treated with CD Mab (P≤0.05). The ratio between the Hoechst- and vWF-positive areas was 2.6 times higher in the group treated with CD Mab than in the relevant control (Figure 4B).

The functionality of the vessels formed inside *in vivo* matrigel implants was also studied by PET (Video S2). We found that [^{18}F]FDG uptake was decreased in the matrigel treated with CD Mab in comparison with non-CD Mab (P≤0.05) but not with basal group. However, the matrigel implants treated with CD Mab contained significantly fewer vessels than controls (P≤0.05). The ratio of [^{18}F]FDG uptake and vessel area was increased more than two fold in CD Mab-treated matrigel implants than in the controls (Figure 5A, B).

The contribution of extracellular TG2 in the anti-angiogenic response

As extra- and intracellular TG2 have been demonstrated to be involved in distinct endothelial cell processes [24], we next studied whether the anti-angiogenic response by celiac disease antibodies is mediated by cellular or extracellular TG2. To this end, we first tested the role of extracellular TG2 by pretreating HUVEC cultures with the cell-impermeable TG2 inhibitor R281 and found that the inhibitor was able to restore the tubule length of CD IgA- or CD Mab-treated cells to control level (P≤0.001; Figure 6A).

To confirm the contribution of extracellular TG2, we performed experiments using TG2 knockout mice. By definition, TG2 knockout mice do not express cellular TG2 [12], whereas Figure 6B clearly demonstrates that matrigel itself, used in the following experiments as extracellular growth environment, contains significant amounts of extracellular TG2. *Ex vivo* aorta ring assay with TG2 knockout mouse tissue showed that in the presence of CD Mab the microvessel and maximum outgrowth areas were decreased slightly but not significantly (Figure 6C). On the other hand, the *in vivo* matrigel plug assays performed in the knockout animals demonstrated that the vascular area in the implants was significantly reduced after CD Mab treatment (P≤0.01; Figure 6D). It is of note that the overall angiogenic

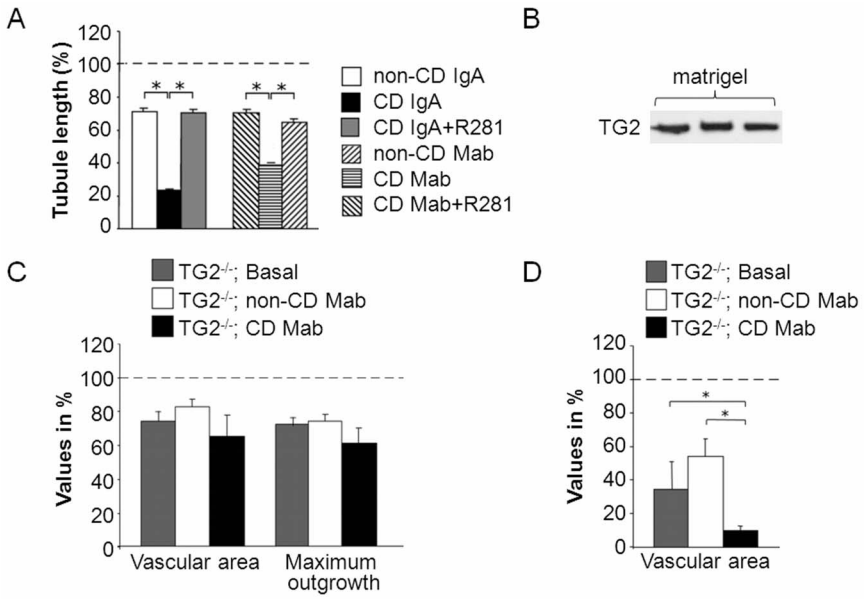

Figure 6. The contribution of extracellular transglutaminase 2 (TG2) in the anti-angiogenic response exerted by celiac antibodies. (A) Endothelial tubule length in three-dimensional human umbilical vein endothelial cell (HUVEC) cultures in the presence of celiac patient-derived total IgA (CD IgA) or monoclonal antibodies (CD Mab), or their relevant controls (non-CD IgA or non-CD Mab) and with/without TG2-inhibitor R281. All data was normalized to the basal group (dotted line). Bars represent the average value as percentage + SEM. * represents $P \leq 0.001$ statistical difference (n = 9). (B) Western blot using anti-TG2 antibody CUB7402 showing the presence of TG2 in the matrigel. (C) Mouse aorta rings derived from TG2 knockout mice ($TG2^{-/-}$) were cultured inside TG2-containing matrigel for ten days without supplementation (basal) or in the presence of CD Mab or non-CD Mab. After the culture period the vascular area and the maximum outgrowth were measured (n = 5 aortas per group). (D) *In vivo* angiogenesis assays were performed in $TG2^{-/-}$ mice. Matrigel containing endogenous TG2 was supplemented with CD Mab or non-CD Mab and injected to mice. After eight days the implants were removed and the vascular area determined by anti-von Willebrand factor (vWF)-antibody staining. (n = 8 animals per group). Bars in charts C and D represent the average value as percentage + SEM. All data was normalized to the wild type mouse basal group (dotted line). * represents $P \leq 0.01$ statistical difference.
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response in TG2 knockout mice was reduced when compared to wild type animals of the same mouse strain (Figure 6C–D).

Discussion

Previous *in vitro* findings suggest that celiac disease-specific TG2-targeted autoantibodies disturb angiogenesis in two-dimensional cell cultures [10,14,25]. The data presented in this article clearly demonstrate that celiac disease autoantibodies are anti-angiogenic also in more complex *ex vivo* systems using mouse aorta rings grown in three-dimensional conditions. Moreover, our data show for the first time that angiogenesis is hindered by celiac antibodies also *in vivo* in mice. In addition, the present results demonstrate that celiac disease-specific TG2 autoantibodies affect the functionality of vessels *in vivo*, which would confirm the previously suggested effects of celiac autoantibodies on endothelial permeability [11].

The data presented in Figure 3 strongly suggest that the anti-angiogenic effects of celiac antibodies are attributable to affected endothelial cell mobility, but also their reduced capacity to form sprouts and to establish cell-cell contacts. During the angiogenic process, the breakdown of the matrix in the leading edge of a migrating cell is a prerequisite for proper movement [26,27]. In cultures supplemented with CD Mab, such areas of proteolytic degradation were not present and instead the cells were surrounded by electron-dense areas of ECM. These observations would indicate that in the presence of celiac autoantibodies ECM degradation/remodeling is disturbed, which could contribute to defective mobility discussed above. Interestingly, it has been shown that the administration of catalytically active TG2 causes an accumulation of ECM and defective angiogenesis *in vitro* [9]. Together this and our current finding, that cell-impermeable TG2-inhibitor R281 prevented the anti-angiogenic effects of celiac antibodies, would suggest that extracellular TG2 activity is involved in impaired angiogenic response in the presence of celiac antibodies. Another plausible explanation how R281 could rescue impaired angiogenesis is by preventing the binding of the celiac antibodies to TG2 but according to previous data [11] this did not seem to be the case.

Results obtained from experiments performed with TG2 knockout mice showed that in the *in vivo* matrigel plug *de novo* angiogenesis assay, where only the extracellular TG2 was present supplied by the matrigel itself, the angiogenic response was significantly decreased after CD Mab treatment. This observation underlines the importance of extracellular TG2 in the anti-angiogenic effect exerted by celiac disease antibodies. In the aorta ring experiments there was only a trend towards reduced microvessel and maximum outgrowth areas in the presence of CD Mab, the change was not however statistically significant. These somewhat discrepant results could be partly explained by the fact that in the TG2 knockout mice the overall angiogenic response in matrigel was greatly reduced compared to wild type animals while in the aorta ring experiment the reduction was not that extensive. This is probably due to differences in experimental settings, because in the matrigel plug assay the cells first have to migrate to plug to form vessels, whereas in the aorta ring assay cells can initiate the angiogenic process immediately. It is possible that also other factors, such as the proportion endogenous ECM secreted by the aortic tissue or individual migrating cells, might contribute to discrepancy.

The data derived from *in vivo* Hoechst injections and PET scans suggest that the vessels formed in matrigel in the presence of celiac autoantibodies are functional, as they transported the marker molecules injected into the circulation. However, the vascular area was smaller in the groups treated with CD Mab in both experiments. This would suggest that the functionality of the vessels was impaired either due to increased permeability or immaturity of the vessels. Because the number of vessels and their diameter were diminished in the presence of celiac autoantibodies, it is reasonable to assume that the latter alternative, the immaturity of the vessels, could explain our findings of impaired functionality. A similar state of immaturity, lacking a sufficient smooth muscle supportive layer, has previously been demonstrated in the small-intestinal mucosa of celiac disease patients [4].

Our present findings show that the celiac disease-specific autoantibodies interfere with angiogenesis *in vivo*. Thus, TG2-targeted autoantibody deposits around blood vessels in celiac patient small bowel mucosa [7] could be anti-angiogenic and lead to altered small bowel mucosal vasculature. This abnormal vascular network would no longer be able to provide mechanical support to the villi and thereby contribute to the development of villus atrophy. Interestingly, the association between morphological changes in intestinal mucosa and angiogenesis is supported by a recent paper, which describes that olmesartan, one of several angiotensin II receptor antagonists used for management of hypertension, was found to be associated with unexplained chronic diarrhea and small intestinal villus atrophy in patients taking the drug [28]. The same drug has been published to reduce angiogenesis in mice [28,29].

The changes in the small-bowel mucosal vasculature in celiac disease might not only affect the mucosal architecture; they may also contribute to the pathogenesis in other ways. The celiac autoantibodies acting in concert with other factors including inflammatory mediators [30] could also potentiate the small-bowel mucosal inflammatory response by increasing the permeability of the vessels.

We conclude that celiac autoantibodies inhibit angiogenesis in three-dimensional *ex vivo* experiments, but also importantly *in vivo* in mice. The data presented in this article suggest that the anti-angiogenic mechanism of the disease-specific autoantibodies involves extracellular TG2 activity contributing to disruption of ECM remodeling. These could lead to inhibited endothelial cell mobility and finally to impaired angiogenesis. Defective angiogenesis could be coupled with compromised vascular function induced by celiac patient TG2-targeted autoantibodies and explain the findings of abnormal small-bowel vasculature in untreated celiac disease.

Supporting Information

Figure S1 Three-dimensional endothelial cell tubule formation assay. Several angiogenic parameters quantified from human umbilical vein endothelial cells cultured inside matrigel for 48 h without any supplementation (basal) or in the presence of celiac patient-derived total IgA (CD IgA) or monoclonal antibodies (CD Mab), or their relevant controls (non-CD IgA or non-CD Mab; n = 9). Bars represent the average value as percentage + SEM. All data was normalized to the basal group (dotted line). * represents P≤0.001 statistical difference.
(TIF)

Figure S2 The percentage of apoptotic cells in the presence of celiac antibodies Apoptotic human umbilical vein endothelial cells inside matrigel cultures without any supplementation (basal) or in the presence of celiac patient-derived total IgA (CD IgA) or its respective control (non-CD IgA), or monoclonal celiac or control antibodies (CD Mab or non-CD Mab, respectively) were enumerated after 1, 15, 30 and 48 hours of culture with Cell-IQ from the videos. Results are given as percentages of total cell number.
(TIF)

Video S1 Tubule dynamics of endothelial cells supplemented with celiac or control antibodies. Human umbilical vein endothelial cells were grown inside matrigel in the presence of celiac patient-derived total IgA (CD IgA) or monoclonal antibodies (CD Mab), or their relevant controls (non-CD IgA or non-CD Mab) for ten days in a Cell-IQ system. During the assay pictures were taken every five minutes.
(MPEG)

Video S2 Positron emission tomography (PET) and positron emission tomography/computed tomography (PET/CT) scanning 3D video from a mouse with matrigel implants. PET and PET/CT scanning video of a mouse with matrigel implants without any supplementation (basal) or supplemented with celiac disease-specific transglutaminase 2-targeted monoclonal autoantibodies (CD Mab) or its relevant control (non-CD Mab). One mouse received three implants (basal, CD Mab or non-CD Mab), each injected subcutaneously into separate limbs as highlighted by circles in the video.
(MPEG)

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Author Contributions

Conceived and designed the experiments: SC KL. Performed the experiments: SK SC. Analyzed the data: SK SC. Contributed reagents/materials/analysis tools: AMS DS MG KK. Wrote the paper: SK SC KL. Gave technical support: IRKS MAP ZS RF SM AR LJP. Critically reviewed the manuscript content: AMS IRKS RF AR LJP CE MM KK. Obtained funding and supervised the study: SC KL. Interpreted the data: SK SC KL.

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Correction: Celiac Disease–Specific TG2-Targeted Autoantibodies Inhibit Angiogenesis Ex Vivo and In Vivo in Mice by Interfering with Endothelial Cell Dynamics

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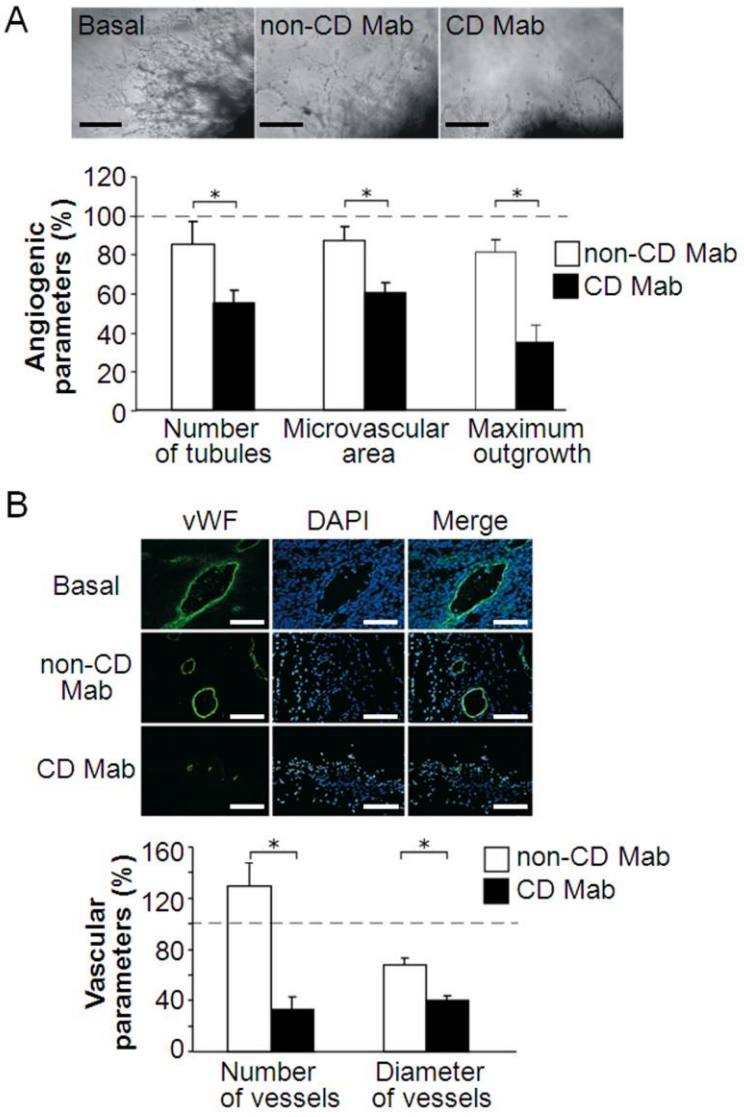

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Competing interests: No competing interests declared.

SUPPLEMENTARY MATERIAL

Celiac Disease–Specific TG2-Targeted Autoantibodies Inhibit Angiogenesis Ex Vivo and In Vivo in Mice by Interfering with Endothelial Cell Dynamics

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Figure S1.

Three-dimensional endothelial cell tubule formation assay. Several angiogenic parameters quantified from human umbilical vein endothelial cells cultured inside matrigel for 48 h without any supplementation (basal) or in the presence of celiac patient-derived total IgA (CD IgA) or monoclonal antibodies (CD Mab), or their relevant controls (non-CD IgA or non-CD Mab; n = 9). Bars represent the average value as percentage + SEM. All data was normalized to the basal group (dotted line). * represents $P \leq 0.001$ statistical difference.

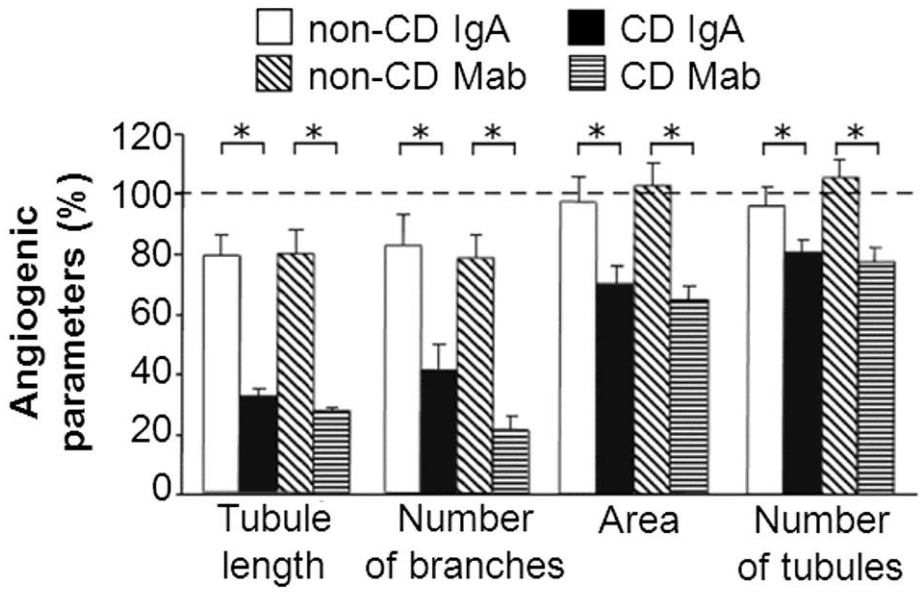
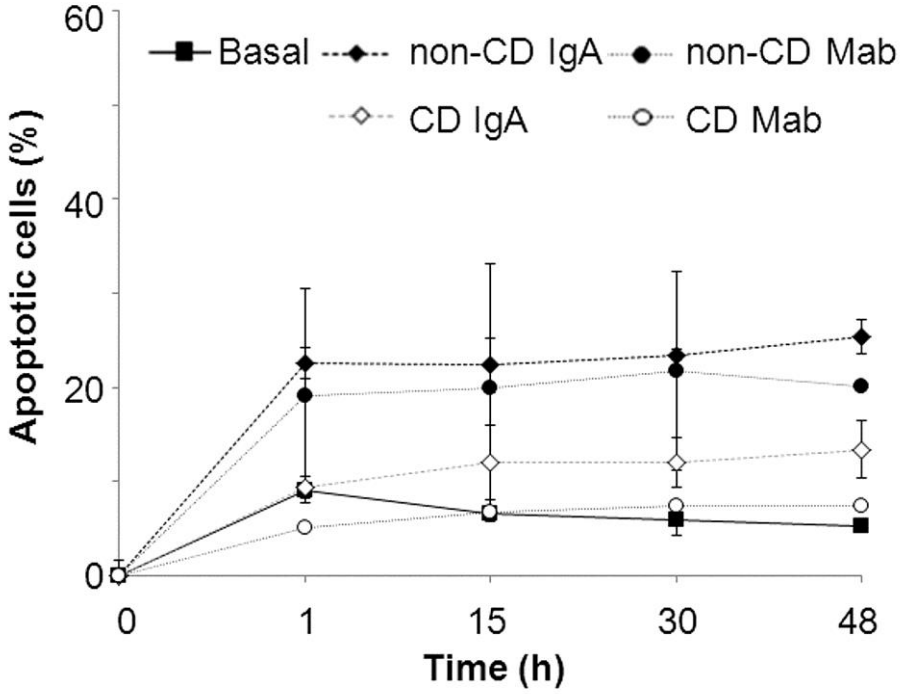


Figure S2.

The percentage of apoptotic cells in the presence of celiac antibodies. Apoptotic human umbilical vein endothelial cells inside matrigel cultures without any supplementation (basal) or in the presence of celiac patient-derived total IgA (CD IgA) or its respective control (non-CD IgA), or monoclonal celiac or control antibodies (CD Mab or non-CD Mab, respectively) were enumerated after 1, 15, 30 and 48 hours of culture with Cell-IQ from the videos. Results are given as percentages of total cell number.



Video S1.

Tubule dynamics of endothelial cells supplemented with celiac or control antibodies. Human umbilical vein endothelial cells were grown inside matrigel in the presence of celiac patient-derived total IgA (CD IgA) or monoclonal antibodies (CD Mab), or their relevant controls (non-CD IgA or non-CD Mab) for ten days in a Cell-IQ system. During the assay pictures were taken every five minutes.

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Video S2.

Positron emission tomography (PET) and positron emission tomography/computed tomography (PET/CT) scanning 3D video from a mouse with matrigel implants. PET and PET/CT scanning video of a mouse with matrigel implants without any supplementation (basal) or supplemented with celiac disease-specific transglutaminase 2-targeted monoclonal autoantibodies (CD Mab) or its relevant control (non-CD Mab). One mouse received three implants (basal, CD Mab or non-CD Mab), each injected subcutaneously into separate limbs as highlighted by circles in the video.

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