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Transglutaminase 2-specific coeliac disease autoantibodies induce morphological changes and signs of inflammation in the small-bowel mucosa of mice

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ABSTRACT

Coeliac disease is hallmarked by an abnormal immune reaction against ingested wheat-, rye- and barley-derived gluten and the presence of transglutaminase 2 (TG2)-targeted autoantibodies. The small-bowel mucosal damage characteristic of the disorder develops gradually from normal villus morphology to inflammation and finally to villus atrophy with crypt hyperplasia. Patients with early-stage coeliac disease have TG2-autoantibodies present in serum and small-intestinal mucosa and they may already suffer from abdominal symptoms before the development of villus atrophy. Previously we have shown that intraperitoneal injections of coeliac patient-derived sera or purified immunoglobulin fraction into mice induce a condition mimicking early-stage coeliac disease. In the current study, we sought to establish whether recombinantly produced patient-derived TG2-targeted autoantibodies are by themselves sufficient for the development of such an experimentally induced condition in immune-compromised mice. Interestingly, mice injected with coeliac patient TG2-antibodies had altered small-intestinal mucosal morphology, increased lamina propria cellular infiltration and disease-specific autoantibodies deposited in the small bowel, but did not evince clinical features of the disease. Thus, coeliac patient-derived TG2-specific autoantibodies seem to be sufficient for the induction of subtle small-bowel mucosal alterations in mice, but the development of clinical features probably requires additional factors such as other antibody populations relevant in coeliac disease.

Keywords: Cellular infiltration, cytokine, intestinal permeability, small-intestinal morphology, intestinal autoantibody deposits

INTRODUCTION

Coeliac disease is a common autoimmune disorder which occurs in a subset of genetically predisposed individuals in response to ingestion of gluten-containing wheat, rye and barley. Patients present with a wide constellation of symptoms and clinical manifestations, of which the most typical are chronic diarrhoea, malabsorption and failure to thrive in childhood (Kaukinen et al. 2010). A characteristic feature of the disease is the presence of disease-specific antibodies in patient serum. These circulating antibodies target both gluten-derived deamidated gliadin peptides (DGP) (Kaukinen et al. 2007) and a self-antigen, transglutaminase 2 (TG2) (Dieterich et al. 1997). Evidence shows that the TG2-autoantibodies are produced in the small-intestinal mucosa (Marzari et al. 2001; Picarelli et al. 1996), where they can bind TG2 present in the basement membrane and around blood vessels and form deposits characteristic of the disease (Di Niro et al. 2012; Korponay-Szabó et al. 2004; Koskinen et al. 2008; Marzari et al. 2001). The TG2-targeted autoantibodies have been demonstrated to mediate a wide spectrum of effects at *in vitro, ex vivo* and *in vivo* levels (Caja et al. 2011; Caputo et al. 2013; Kalliokoski et al. 2013; Martucciello et al. 2012; Paolella et al. 2013), but their contribution to the disease pathogenesis remains as yet incompletely understood.

In untreated coeliac disease, the damaged small-bowel mucosa is hallmarked by villous atrophy and crypt hyperplasia as well as the activation of both adaptive and innate immune responses. The adaptive immune component encompasses a pro-inflammatory response characterized by secretion of cytokines such as interferon (IFN)-γ, interleukin (IL)-1β and IL-6 (Lahat et al. 1999; Nilsen et al. 1995; Przemioslo et al. 1994). In contrast, a key player in the innate immune response is cytokine IL-15, which contributes to disease development for instance by promoting small-bowel mucosal epithelial damage (Kim et al. 2015). As a result, the impaired epithelium is no longer viable as a proper barrier, which leads to increased intestinal permeability in coeliac disease (van Elburg et al. 1993; Reims et al. 2002; Schulzke et al. 1998).

The small-bowel mucosal damage in coeliac disease evolves gradually and may take years or even decades to develop (Lähdeaho et al. 2005; Mäki et al. 1990; Marsh 1992). However, it is known that many signs of the disease are already present in the early phases of the disease, prior to the appearance of villous atrophy. Patients with early developing coeliac disease still have a morphologically normal small-intestinal mucosal villus structure, but they may already have autoantibodies in their circulation and TG2-autoantibody deposits and an increased number of inflammatory cells in their small-bowel mucosa (Korponay-Szabó et al. 2004; Salmi et

al. 2006). Moreover, these patients may suffer from various gastrointestinal symptoms, for example diarrhoea and abdominal pain, before development of villous atrophy (Kaukinen et al. 2001; Kurppa et al. 2009).

Recently, we demonstrated that injection of coeliac patient sera or purified serum immunoglobulins into immune-compromised mice leads to a condition with characteristics of early developing coeliac disease (Kalliokoski et al. 2015). The mice had intestinal inflammation and TG2-autoantibodies in their sera and small-bowel mucosa. Moreover, a subset of the animals failed to gain weight and evinced signs of mild diarrhoea. In the current study, we further investigated the effects of the coeliac disease patient antibodies, and addressed the question whether patient-derived TG2-targeted autoantibodies are by themselves able to induce the early developing coeliac disease–like condition in mice and factors associated with the alterations.

MATERIALS AND METHODS

Production and purification of monoclonal miniantibodies

The following IgG-class recombinant monoclonal miniantibodies were used in the study: coeliac patient anti-TG2-specific monoclonal antibody clone 4.1 (referred to in the article as CD Mab) and an irrelevant control antibody against a Helicobacter pylori antigen (referred to as non-CD Mab). They were produced in scFv-Fc format in Chinese hamster ovary cells (CHO-S) transfected with pUCOE vector as previously described (Boscolo et al. 2012).

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 dialysed against phosphate-buffered saline (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.

An indirect immunofluorescence method was used to determine IgG-class coeliac disease-specific endomysium antibody (EmA) titres in the miniantibody preparations. Human umbilical cord from a premature baby was used as substrate, and a serum dilution of 1:10 or more was considered positive (Korponay-Szabó et al. 2003). Positive antibody preparations were further diluted 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000, 1:4000, 1:8000, 1:12000 and 1:16000. An enzyme-linked immunosorbent assay (ELISA) method using human recombinant TG2 as antigen was used to measure IgG-class antibodies against TG2 (cut-off 3 U/ml, Celikey®, Phadia GmbH, Freiburg, Germany) in the miniantibody preparations.

Experimental animals, housing and husbandry

Twenty-five female *Hsd:Athymic Nude-Foxn1nu* mice (Envigo, Gannat, France) 6 weeks of age were used in the study. All animals were kept in semi-barrier conditions in the facilities of the Central Animal Laboratory, University of Turku, Finland. All mice were acclimatized at least two weeks prior to the commencement of the study. After the acclimatization period, all animals were randomly assigned to single cages. At the beginning of the experiments, each animal was 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 19-22 ℃, the relative humidity 42 to 60%, and artificial illumination followed a 12-h light/dark cycle (lights on at 6 am). 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. The animal experiments were conducted as appoved by the National Ethics Committee for Animal Experiments in Finland (license numbers ESAVI-2010-06223/Ym-23, ESAVI/4279/04.10.07/2013 and ESAVI/781/04.10.07/2015).

Study design and experimental procedures

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-antibody 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 mice were injected with CD Mab (n=10 mice), irrelevant non-CD Mab (n=10 mice) or sterile PBS (n=5 mice). Single daily intraperitoneal injections of antibodies or PBS (10 ml/kg mouse) were performed for 8 days. The body weights of the animals were recorded daily during the experiment. At the beginning of the daily injections, the median weight of CD Mab-receiving mice was 24.7 g (range 21.1-30.2 g), non-CD Mab-receiving mice 25.4 g (range 22.0-30.3 g) and PBS-receiving mice 27.1 g (range 23.1-28.1 g). Faecal consistency was evaluated daily and graded as normal faeces, mild diarrhoea, moderate diarrhoea and severe diarrhoea as previously described (Kalliokoski et al. 2015).

To measure intestinal permeability, an iohexol test was performed on five mice per group essentially as described elsewhere (Frias et al. 2009; Frias et al. 2014). The test was carried out two weeks before initiation of antibody or PBS injections and again after completion of the 8-day injections. For iohexol permeability testing, 10ml/kg of Omnipaque 300 (647 mg iohexol mg/ml Amersham Health, the Netherlands) was administered to the mice by oral gavage, whereafter they were placed in metabolic cages for 24 h for urine collection. After recording of total urine volume, the urine samples were collected and stored at –20 °C for iohexol measurements.

At the end of the experiment the mice were anaesthetized using isoflurane (Vetflurane 1000 mg/g, Virbac, Suffolk, UK), blood collected by cardiac puncture, and sera separated by centrifugation at 1500 x g for 10 minutes and stored at –70°C. After euthanasia tissue samples from the small bowel, heart, kidney, liver and skeletal muscle were collected. The samples were either freshly embedded in optimal cutting temperature compound (Tissue-Tek, 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 experiment, one mouse receiving PBS died on day six after starting the antibody and PBS injections and thus the serum and tissue samples from this animal were collected at this time point.

Human antibody measurements from mouse sera

Total human 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 IgGs were given as mg/ml, based on the standards provided in the kit. IgG-class EmA titres and TG2-antibody levels in mouse sera were determined as described above.

Laboratory analysis of iohexol in mouse urine

Iohexol levels in mouse urine were determined using the FIT-GFR Iohexol Kit (BioPAL, Inc., Worcester, MA, USA). Urine samples were disregarded in the case of incomplete collection or faecal contamination. Iohexol standards from the kit were prepared using BioPAL diluent (0.01 0.03, 0.1, 0.3, 1.0, 3.0, 10.0 µg/ml). The urine samples were diluted 1:5000 and the test then carried out according manufacturer's instructions. The absorbance at 450 nm for each sample was recorded by a plate reader (EnSight™ multimote plate reader, Kaleido™ Data Acquisition and Analysis software), and data on the standards were fitted to a four-parameter logistic function. Finally, the amount of iohexol in mouse urine was calculated as described by Frias and colleagues (Frias et al. 2014) as follows:

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

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

The results are presented as percentage of iohexol in urine.

Immunohistochemistry and small-bowel mucosal morphology

For immunohistochemical stainings, 3-µm-thick well-oriented sections were cut from paraffin blocks of formalin-fixed proximal parts of mouse small intestines. Thereafter, the sections were deparaffinized in xylene and rehydrated in descending ethanol series. Antigens were exposed by heat-induced antigen retrieval, 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. Rabbit anti-mouse antibodies against Ki-67 (1:400, Novus Biologicals, LLC, Littleton, CO, USA), and active caspase-3 (1:250, Cell Signalling Technology,

Danvers, MA, USA), neutrophil elastase (1:1000, Abcam, Cambridge, UK) and F4/80 (1:100, Novus Biologicals) were used as primary antibodies. Subsequently, the sections were incubated with biotinylated goat anti-rabbit antibody (1:200, Vector Laboratiories Inc, Burlingame, CA, USA), HRP-conjugated swine anti-rabbit antibody (1:200, Dako Denmark A/S, Glostrup, Denmark), Rabbit-on-Rodent HRP-polymer (Biocare Medical, LLC., Concord, CA, USA) or ImmPRESS Reagent (ImmPRESS HRP Anti-Rabbit IgG (Peroxidase) Polymer Detection Kit, Vector Laboratories), respectively. Immunodetection was done using ABC solution (Vectastain Elite ABC kit, Vector Laboratories) followed by 3-amino-9-ethylcarbazole (AEC) or 3,3'-diaminobenzidine (DAB). The sections were counterstained with Harris haematoxylin.

Ki-67-stained small-intestinal sections of mice were used for morphological assessments as previously described (Kalliokoski et al. 2015). Villous height, crypt depth and villous height crypt depth ratio (Vh/CrD) were determined approximately from 15 measurements per mouse. In addition, total amounts of cells and Ki-67-positive proliferative cells per mm^2 of lamina propria were enumerated by approximately 30 measurements per mouse. Active caspase-3-stained sections were analysed 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.

Quantification of epithelial cell subsets by quantitative real-time PCR

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 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 manufacturer's instructions. Primer

sequences are described in supplementary Table 1. 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 relative to those of the housekeeping gene in the results.

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

IgG deposits were stained essentially as previously described (Kalliokoski et al. 2015). In brief, frozen proximal small-intestinal sections were double-stained for human IgG and TG2 using DyLightTM594-conjugated goat anti-human IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and polyclonal rabbit antibody to tissue transglutaminase (Zedira GmbH, Darmstadt, Germany), followed by Alexa Fluor 488-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR, USA). Fluorescein isothiocyanate-conjugated rabbit antibody against human IgG (Dako Denmark A/S) was used for deposit staining in other tissues. The slides were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Inc.), and images taken with an Olympus BX60 microscope (Olympus Europa GmbH, Hamburg, Germany).

Cytokine analysis

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. Tissues were homogenized with Ultra Turrax T8 tissuemizer (IKA Labortechnik, Staufen, Germany), supernatants collected after centrifugation at 4°C and total protein concentration measured using Bio-Rad CD Protein Assay (Bio-Rad). Supernatants were diluted to 10 mg protein/ml with PBS and used in the assay. Protein expression levels of 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), tumour necrosis factor (TNF)-α, epithelial neutrophil activating peptide (ENA-78) and macrophage inflammatory protein (MIP)-2) were measured with the Bio-Plex 200 system (Bio-Rad) using a ProcartaPlex™ Multiplex Immunoassay (eBioscience, San Diego, CA, USA) in accordance with the manufacturer's instructions. The

amounts of the cytokines were calculated based on the standard curves. Values below the detection limit were set to zero for statistical analysis.

Statistics

Statistical analysis was made using statistical analysis software (IBM SPSS Statistics, SPSS Inc., Chicago, IL, USA). Data are presented as medians and ranges. Kruskal-Wallis test followed by Mann-Whitney U test were used to compare differences between the groups and Wilcoxon test to compare changes within the groups. Correlations were studied using Spearman's correlation test. In the correlation analysis, data on Vh/CrD and lamina propria cell infiltration from a previously published study (Kalliokoski et al. 2015) were also included. These data include Vh/CrD and lamina propria cell infiltration values from mice injected with coeliac disease patient (n=8 mice) or control individual (n=8 mice) sera as well as total serum IgG from coeliac patients (n=5 mice) or control individuals (n=5 mice), and non-injected mice (n=4). A p-value ≤ 0.05 being considered significant.

RESULTS

Determination of human antibodies and small-bowel morphology

To confirm that the intraperitoneal injections of recombinantly produced human CD Mab and non-CD Mab into mice were successful, human IgG levels in mouse sera were measured at the end of the experiment. All the mice injected with antibodies had human IgG in their circulation. Despite the comparable total protein concentrations of the injected antibodies, the mice injected with CD Mab had lower levels of human IgG in their sera (median 0.08 mg/ml) than the non-CD Mab-injected mice (median 0.35 mg/ml). All PBS-injected mice were negative for human IgG (Table 1).

Mouse sera were also tested for IgG-class coeliac disease-specific EmA- and TG2-autoantibodies. Notably, all mice receiving CD Mab were positive for both autoantibodies, the median levels being 1:1000 and 87 U/ml for EmA- and TG2-antibodies, respectively. None of the non-CD Mab- and PBS-injected mice had detectable levels of either of these antibodies (Table 1).

Interestingly, mice receiving CD Mab had significantly lower small-intestinal mucosal villi (median 434 µm, range 355-541 µm) than mice injected with non-CD Mab (median 470 µm, range 357-585 µm) or PBS (median

474 µm, range 382-519 µm) (Fig. 1a). There was no significant difference in crypt depth between CD Mab and non-CD Mab injected mice (medians 103 µm and 94 µm, respectively). However, the control mice injected with PBS had significantly shorter crypts (median 80 µm) than the mice injected with either CD Mab or non-CD Mab. (Fig. 1b). Of note, Vh/CrD was significantly lower in mice receiving CD Mab (median 4.41) in comparison to those receiving non-CD Mab (median 4.86) or PBS (median 5.33). The difference in Vh/CrD between non-CD Mab-treated mice and PBS-treated mice was also statistically significant. (Fig. 1c).

All mice receiving CD Mab evinced clear human IgG positivity in the small-intestinal mucosa at the basement membrane and around blood vessels. Notably, the coeliac patient TG2-targeted miniantibodies localized with mouse TG2 at these sites (Table 1). Human IgG-class miniantibody deposits were also detected in heart, kidney, liver and skeletal muscle (data not shown). The presence of CD Mab in several mouse tissues could account for the lower levels of human IgG observed in the circulation of the mice injected with CD Mab. None of the non-CD Mab- or PBS-injected mice exhibited such deposits.

Clinical observations and intestinal permeability

Weight and faecal consistency were monitored throughout the experiment. At the end of the experiment, median weights of CD Mab-, non-CD Mab- and PBS-injected mice were 25.0 g (range 20.4-29.5 g), 25.2 g (range 22.6-29.6 g) and 26.0 g (range 21.9-28.0 g), respectively, and thus no significant differences in the increment of body weight were found between groups. In addition, within each treatment group, the body weights remained fairly constant during the procedure.

During the entire length of the experiment, 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 (data not shown).

Intestinal permeability in the mice was studied by determining the percentage of orally administered iohexol in mouse urine. Before antibody injections, the median percentage of iohexol in the CD Mab-group was 1.00% (range 0.15-2.22%), non-CD Mab-group 0.15% (range 0.14-8.64%) and PBS-group 0.31% (range 0.04-1.39%). After the 8-day antibody- or PBS-injection 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.

Epithelial cell subsets and apoptosis

To assess whether the observed changes in the small-intestinal mucosal morphology of CD Mab-injected mice could be due to altered differentiation of epithelial cells, we evaluated the mRNA expression of distinct intestinal epithelial cell differentiation markers by qPCR. The mRNA expression level of the enterocyte marker ALP1, the enteroendocrine marker CHGA, the Paneth cell marker LYZ1, the goblet cell marker MUC2 and stem cell marker LGR5 were similar in CD Mab and non-CD Mab injected mice (Supplementary Fig. 1).

In the case of apoptosis, it was observed that all mice evinced active caspase-3 positivity in single epithelial cells located mostly in the villous tips. In the lamina propria only rare caspase-3 positive cells were detected. However, no differences in the level of apoptosis were observed between CD Mab and the control groups (Supplementary Fig. 2).

Lamina propria cellular infiltration

In order to quantify mucosal lamina propria cellular infiltration, we enumerated the total number of cells and Ki-67-expressing proliferative cells per mm^2 of lamina propria in mouse small intestines. The total number of lamina propria cells was significantly higher in mice injected with CD Mab (median 9553, range 7083-12145 cells) when compared to non-CD Mab- (median 7344 cells, range 5841-8229 cells) or PBS-injected mice (median 7042 cells, range 6473-7917 cells) (Fig. 2a). A parallel finding was observed in the number of Ki-67 positive proliferating lamina propria cells, where the amounts were 990 (range 130-1583 cells), 505 (range 208-833 cells) and 229 cells (range 167-458 cells) in CD Mab-, non-CD Mab- and PBS-injected animals, respectively (Fig. 2b). The values in the CD Mab group were statistically different from those in both controls groups. The difference between the non-CD Mab and PBS groups was also statistically significant.

Notably, a significant negative correlation was found between Vh/CrD and number of proliferative cells in the lamina propria (P=0.007) (Fig. 3a). As we had the Vh/CrD data and the number of lamina propria cells available from our previous experiments, where we injected either coeliac or control serum or total serum IgG from coeliac patients or controls to mice (Kalliokoski et al. 2015), we performed the correlation analysis also

taking this data set into consideration. In this larger data set, a negative correlation was found between Vh/CrD and number of proliferative cells in the lamina propria ($P \leq 0.001$) (Fig. 3b) and also between Vh/CrD and total number of cells in the lamina propria ($P \leq 0.001$) (Fig. 3c).

Cytokine expression levels in small intestines of mice

The expressions of 11 cytokines were determined in small-intestinal tissue homogenates from mice receiving CD Mab or non-CD Mab. Protein levels of IL-3, IFN-γ, IL-17A and GM-CSF were below the detection limit range in 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 mice (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 addition, TNF-α levels correlated with both the Ki-67-positive cells in the lamina propria and the total amount 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).

DISCUSSION

In this study, we demonstrated that the administration of coeliac disease-specific TG2-targeted recombinantly produced miniantibodies into athymic mice induces an alteration in the small-intestinal mucosal morphology coupled with increased cellular infiltration in the lamina propria. CD Mab-injected mice also evinced coeliac patient autoantibodies in the serum and small-bowel mucosa, where they co-localized with TG2. Thus, these

results are parallel to those in our previous study with coeliac patients' sera and immunoglobulin injections (Kalliokoski et al. 2015). However, the injection of CD Mab did not affect the occurrence of diarrhoea, intestinal permeability or increment of body weight.

According to our results epithelial cell apoptosis was not increased in the presence of CD Mab, thus paralleling earlier findings (Barone et al. 2007). In addition, CD Mab injections 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 in the current study. Interestingly, decreased expression of distinct junctional proteins (Rauhavirta et al. 2014) and increased permeability (Smecuol et al. 2005) have been reported in gluten-sensitive individuals with normal small-intestinal mucosal morphology. Therefore, other factors along with TG2-targeted autoantibodies are likely needed for the induction of epithelial permeability at the early phases of coeliac disease development. On the other hand, the injections of CD Mab to mice induced elongation of crypts suggesting that crypt epithelial cell proliferation could be increased. Indeed, a previous study shows that antibodies against TG2 are able to induce the entry of epithelial cells into S-phase (Barone et al. 2007). However, in the CD Mab-injected mice, changes in both crypt depth and also villous height in mice were milder than observed in untreated overt coeliac patients present with considerable villous atrophy and large-scale crypt hyperplasia, and there might be several reasons for that. For instance, the mice used in the present and previous (Kalliokoski et al. 2015) study lack T-cells and also the correct major histocompatibility complex (MHC) molecules, which both 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 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.

In contrast to our previous study, the administration of TG2-targeted autoantibody into mice did not led to a significant delay in weight gain. Surprisingly, the mouse body weight in the current study actually 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. Furthermore, in the current study there was no difference in the

occurrence of mild transient diarrhoea between groups, in contrast to the previous study (Kalliokoski et al. 2015). Several factors might account for this difference. Firstly, the current study was performed by injecting TG2-specific autoantibodies targeting a single previously identified classical coeliac epitope, while autoantibodies against an additional three major TG2-epitopes also exist in coeliac patients (Iversen et al. 2013; Simon-Vecsei et al. 2012). Secondly, even though not reported, DGP-antibodies were found in the majority of the mice injected with coeliac patient-derived sera or immunoglobulins in the previous study, and also other antibody populations in addition to the TG2-antibodies were very likely present in the injections. Thus, we propose that a combined action of TG2-autoantibodies targeting different epitopes and/or other antibody populations such as DGP-antibodies might be required for 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 (Salmi et al. 2006; Kurppa et al. 2009).

To our knowledge, only two previous studies have assessed the effects of autoimmunity against TG2 using murine models, and neither reports any signs of diarrhoea or effects on weight gain (Di Niro et al. 2008; Freitag et al. 2004). The first study by Freitag and co-workers included the induction of a humoral response against TG2 by immunizing mice with exogenous TG2 (Freitag et al. 2004). Although high levels of anti-TG2 antibodies in mouse sera were detected, no histological changes in the small intestine were observed, in contrast to our study. Even though not reported in the article, it is very likely that the epitopes of the murine TG2-autoantibodies deviate considerably from those of the coeliac patients, thus providing a possible explanation for the differences in the findings. In the second study, Di Niro and colleagues expressed TG2-miniantibodies in immune competent mice by means of adeno-associated virus vectors (Di Niro et al. 2008). Regardless of using a coeliac patient-derived single-chain TG2-miniantibody similarly to the one used in our study, they observed no morphological alterations in small-bowel mucosa despite the presence of the antibodies in serum. On the other hand, in their study, a strong anti-idiotypic response was apparent in the mice, which may explain the lack of small-bowel mucosal morphological changes. Interestingly, the idiotypic antibodies observed in Di Niro's study competed with TG2 for miniantibody binding, and might thus have prevented the formation of deposits in most tissues, including the small intestine. It is thus conceivable that autoantibodies deposited in the small intestine might be one factor inducing decreased Vh/CrD.

Although as discussed above, intestinal epithelial cell alterations likely do not contribute to the alteration in mucosal morphology in CD Mab-injected mice, our results suggest the autoantibodies deposited to the small bowel mucosa might play a role. One plausible mechanism is the activation of the complement system, although our data does not support this conception (data not shown). In addition, an increased density of both all infiltrative cells and the proliferating infiltrative cells was present in CD Mab-injected mice compared to controls. This increased density of infiltrative cells in the lamina propria of the mice injected with CD Mab, however, might not be attributable to neutrophils or macrophages. The number of neutrophil elastase-positive cells was very low in all animals and no differences between groups were observed (data not shown). Macrophages were abundantly present in the lamina propria, but their amount did not differ between study groups either (data not shown). Nonetheless, there was a significant negative correlation between Vh/CrD and number of Ki-67-positive proliferating cells in the lamina propria. In the larger data set combining mice from our previous serum and total IgG experiments, both infiltration parameters, Ki-67-positive cells and total number of cells in the lamina propria, correlated negatively with Vh/CrD. Our results would thus indicate that the infiltrative cells in the lamina propria could be involved in the process of mucosal deterioration. Further research is needed to determine which cell types are responsible for the increased infiltration in these mice.

We showed that lamina propria infiltration parameters also correlated with the levels of IL-27 and TNF-α. Although the expression levels of these cytokines in CD Mab-injected mice did not differ significantly from those in control mice, the observed trend towards increased levels of IL-27 and TNF-α in mice receiving CD Mab could have reached statistical significance by increasing the number of animals. Our finding of IL-27 protein in the small intestine of the CD Mab-injected mice is intriguing, as in active coeliac disease patients' biopsy specimens the expression of IL-27 mRNA has been reported to be increased (Garrote et al. 2008). With regard to TNF-α, numerous studies report elevated levels of TNF-α in coeliac disease patients (Cataldo et al. 2003; Kontakou et al. 1995; Manavalan et al. 2010; Przemioslo et al. 1994; Westerholm-Ormio et al. 2002). However, the entire small-intestinal cytokine profile of the mice did not resemble that observed in coeliac disease patients (Garrote et al. 2008; Lahat et al. 1999; Iacomino et al. 2016). For instance, no differences in the levels of IL-15, the innate immunity cytokine overexpressed in coeliac patients' small-intestinal epithelia and lamina propria (Di Sabatino et al. 2006; Iacomino et al. 2016; Maiuri et al. 2000; Mention et al. 2003),

were observed between CD Mab- and control group mice. In addition, the levels of other pro-inflammatory cytokines such as IFN-γ, IL-1β and IL-6 have been increased in active coeliac patients in several studies (Fornari et al. 1998; Kontakou et al. 1994; Manavalan et al. 2010; Nilsen et al. 1998), but no significant increase was found in our CD Mab-treated mice compared to controls. This difference in the cytokine profile between coeliac patients and CD Mab-injected mice is not surprising, since the mice we used in the study were immune-compromised T-cell deficient mice and thus unable to mount the cytokine response characteristic of T-cell-mediated adaptive immune activation.

In summary, we observed that injections of coeliac disease-specific miniantibodies targeting major TG2 epitope into mice induced a condition resembling early phase disease in coeliac patients, encompassing slight albeit significant morphological alterations, increased cellular infiltration and TG2-antibody deposits along the basement membrane and blood vessels in the small intestines of the mice. The small-bowel histological changes occurred in conjunction with autoantibody deposits, lamina propria cellular infiltration and IL-27 and TNF- α, rendering those possible contributors to the process. However, the results suggest that the development of clinical features such as diarrhoea requires TG2-antibodies targeting other epitopes, entirely other antibody populations and/or longer exposure to the antibodies.

COMPLIANCE WITH ETHICAL STANDARDS

Disclosure of potential conflicts of interest: The authors declare no conflict of interests related to this study.

Research involving human participants and animals: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. This article does not contain any data from studies involving human participants performed by any of the authors.

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TABLES

Table 1 Human IgG-class antibody levels in mouse sera and the presence of miniantibody deposits in the small bowel of mice.

	CD Mab-injected mice (n=10)	**Non-CD Mab-injected mice (n=10)**	**PBS-injected mice (n=5)**
Human total IgG (mg/ml), median (range)	0.08 (0.03-0.28)	0.35 (0.11-0.62)	neg
EmA-G titre, median (range)	1:1000 (1:200, 1:8000)	neg	neg
TG2-G (U/ml), median (range)	87 (60.4, >100)	neg	neg
Human anti-TG2-antibody deposits in small bowel (% of mice)	100	0	0

For the serum EmA-G results, a serum dilution of 1:≥10 was considered positive; for TG2-G results, ≥3 U/ml was considered positive; CD Mab, coeliac patient-derived anti-TG2 miniantibody; non-CD Mab, control antibody; EMA-G, IgG-class endomysium antibody; TG2-G, IgG-class transglutaminase2 antibody; neg, negative; TG2, transglutaminase 2

Table 2 Correlations between cytokine levels and lamina propria infiltration in the mice treated with TG2-targeted miniantibodies (CD Mab, n=5) or control miniantibodies (non-CD Mab, n=5). Correlation coefficients (R) were defined by Spearman's correlation and *P* values lower than 0.05 were considered statistically significant.

	IL-27		**TNF- α**	
	R	***P* value**	**R**	***P* value**
Total amount of cells/mm^2 in lamina propria	0.619	0.056	0.666	0.036
Ki-67-positive cells/ mm^2 in lamina propria	0.625	0.042	0.653	0.041
IL-27	-	-	0.724	0.018
IL-1β	0.515	0.128	0.653	0.041

IL-27, interleukin-27; IL-1β; interleukin-1β; TNF-α, tumour necrosis factor-α

FIGURE CAPTIONS

Fig. 1 Small-bowel mucosal morphology of mice. Villous heights (**a**) and crypt depths (**b**) were measured and villous height crypt depth ratio (Vh/CrD) calculated (**c**). Each dot represents the median value for one mouse calculated from approximately 15 measurements, and black lines indicate the median values of each group. Panel **d** shows representative images of small intestines from coeliac disease-specific anti-TG2-antibody (CD Mab)-, control antibody (non-CD Mab)- and PBS-injected mice, respectively. *P* values lower than 0.05 were considered statistically significant and only significant *P* values are reported in the figure. Scale bar = 50µm

Fig. 2 Lamina propria infiltration in the mice. Total amount of cells per mm^2 of lamina propria (**a**) and Ki-67-positive proliferating cells per mm^2 of lamina propria (**b**) were counted in coeliac disease-specific anti-TG2-antibody (CD Mab)-, control antibody (non-CD Mab)- and PBS-injected mice. 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 lower than 0.05 were considered statistically significant and only significant *P* values are reported in the figure

Fig. 3 Correlations between villous height crypt depth ratio (Vh/Crd) and lamina propria infiltration. Panel **a** shows the correlation (R, correlation coefficient) between Vh/Crd and number of proliferative Ki-67-positive cells per mm^2 of lamina propria in mice injected with coeliac (CD Mab) or control antibody (non-CD Mab) or PBS. Panels **b** and **c** present correlations between Vh/Crd and number of proliferative Ki-67-positive cells or total amount of cells per mm^2 of lamina propria, respectively. In **b** and **c** data from our previous study (Kalliokoski et al. 2015) are included. These data represent results from 8- and 27-day experiments with coeliac or control serum (CD or non-CD serum, respectively) injections and the 8-day experiment with coeliac or control total IgG (CD or non-CD IgG, respectively) injections. Results also include non-injected mice from serum and total IgG experiments. *P* values lower than 0.05 were considered statistically significant

Fig. 1

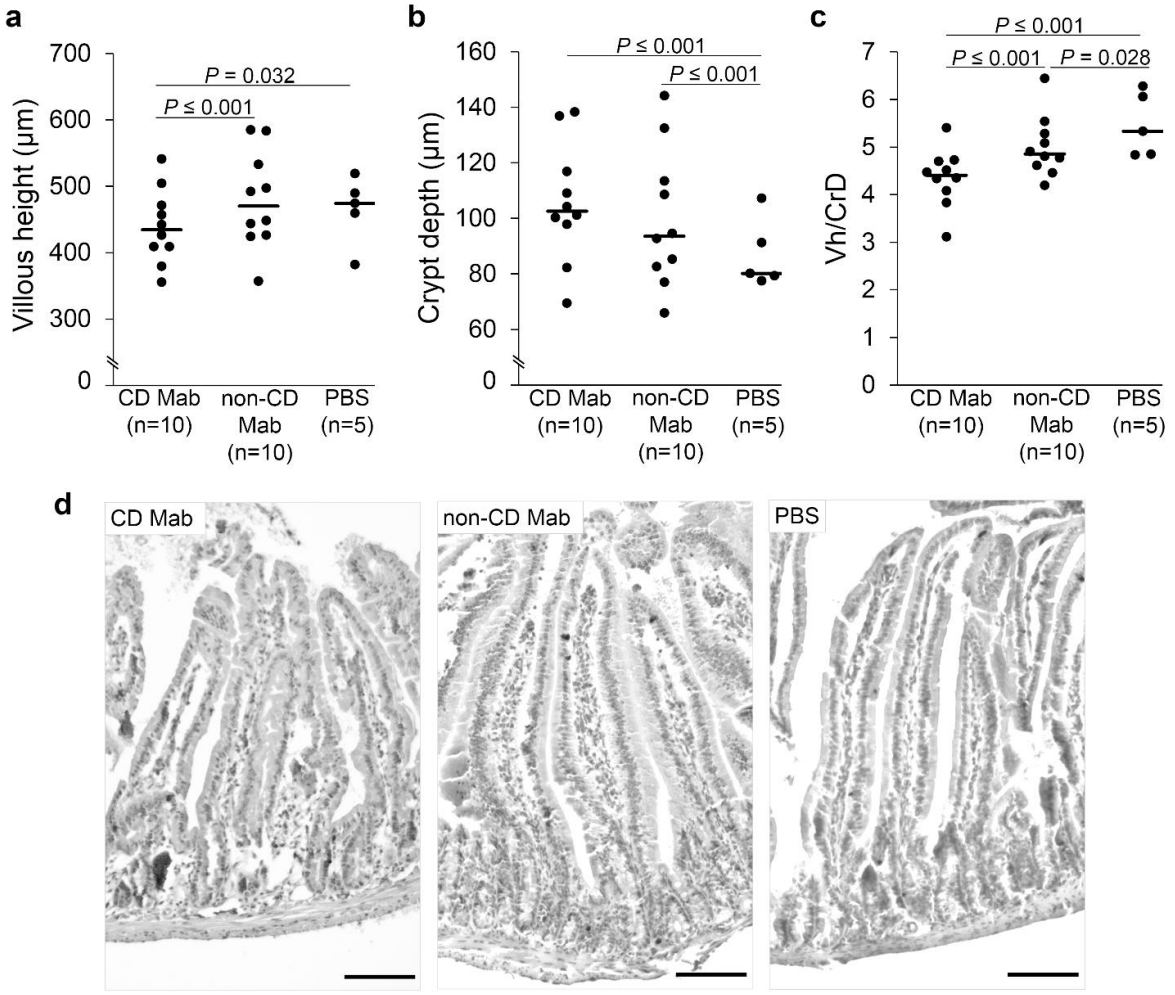

a
Villous height (µm)
700
600
500
400
300
0
P = 0.032
P ≤ 0.001
CD Mab (n=10)
non-CD Mab (n=10)
PBS (n=5)
b
Crypt depth (µm)
160
140
120
100
80
60
0
P ≤ 0.001
P ≤ 0.001
CD Mab (n=10)
non-CD Mab (n=10)
PBS (n=5)
c
Vh/CrD
7
6
5
4
3
2
1
0
P ≤ 0.001
P ≤ 0.001
P = 0.028
CD Mab (n=10)
non-CD Mab (n=10)
PBS (n=5)
d
CD Mab
non-CD Mab
PBS

Fig. 2

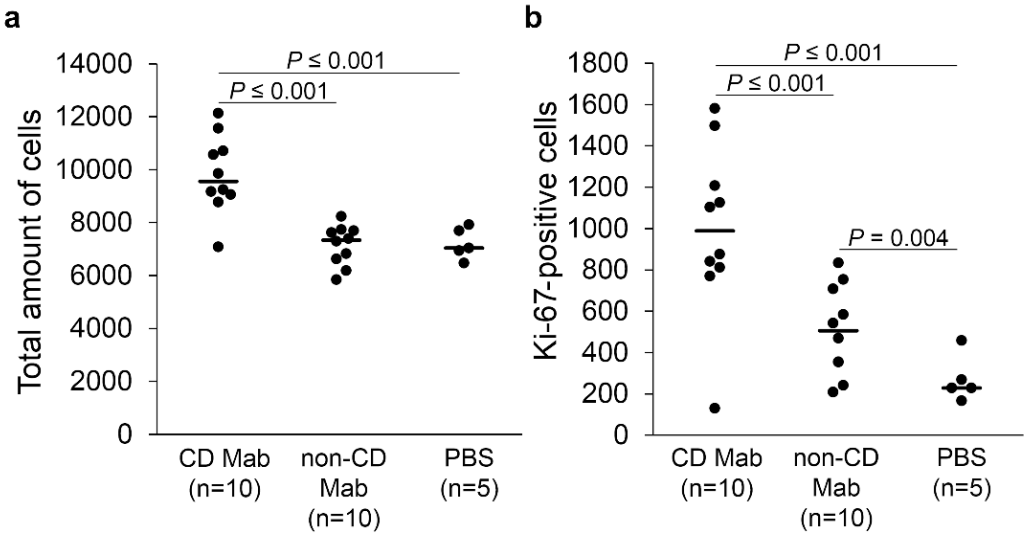
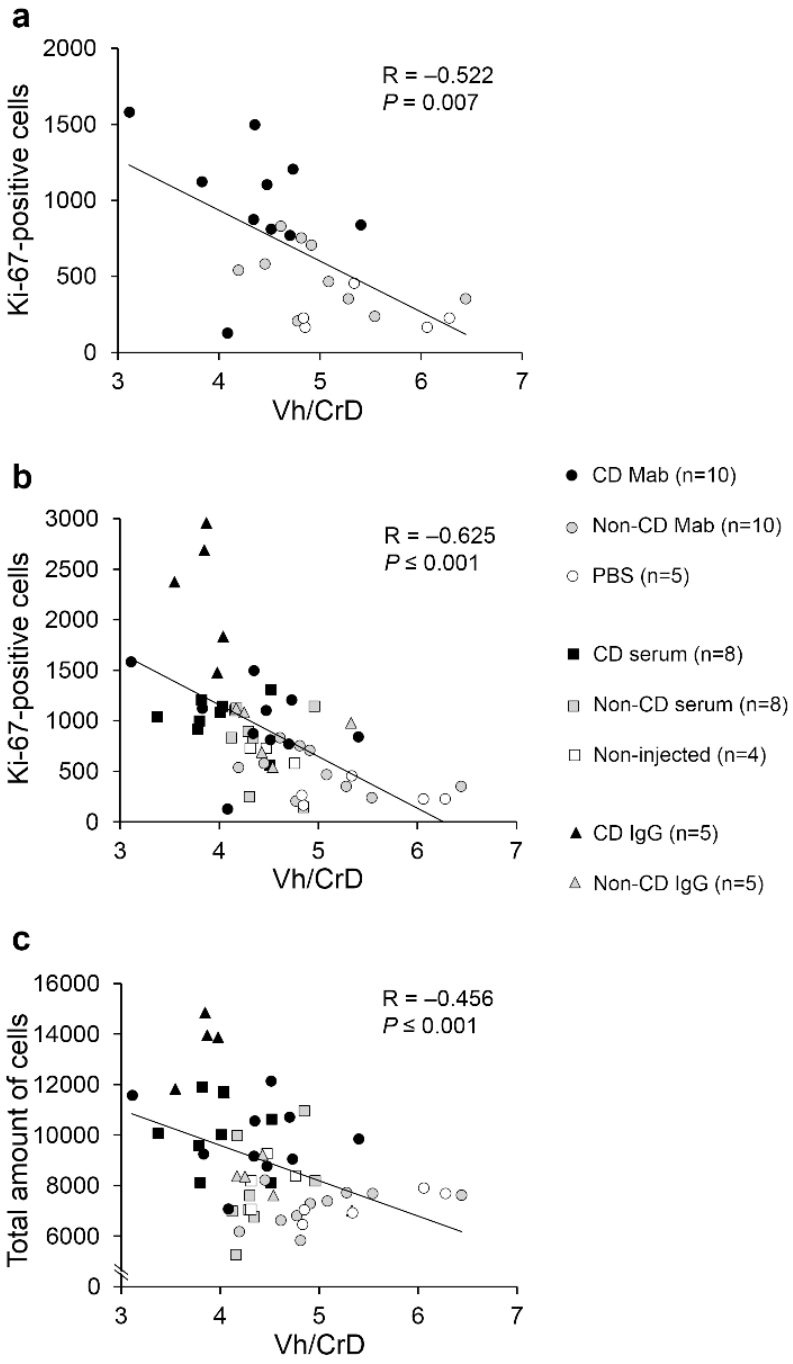

a
Total amount of cells
14000
12000
10000
8000
6000
4000
2000
0
P ≤ 0.001
P ≤ 0.001
CD Mab (n=10)
non-CD Mab (n=10)
PBS (n=5)
b
Ki-67-positive cells
1800
1600
1400
1200
1000
800
600
400
200
0
P ≤ 0.001
P ≤ 0.001
P = 0.004
CD Mab (n=10)
non-CD Mab (n=10)
PBS (n=5)

Fig. 3


a
Ki-67-positive cells
2000
1500
1000
500
0
3
4
5
6
7
Vh/CrD
R = –0.522
P = 0.007
b
Ki-67-positive cells
3000
2500
2000
1500
1000
500
0
3
4
5
6
7
Vh/CrD
R = –0.625
P ≤ 0.001
CD Mab (n=10)
Non-CD Mab (n=10)
PBS (n=5)
CD serum (n=8)
Non-CD serum (n=8)
Non-injected (n=4)
CD IgG (n=5)
Non-CD IgG (n=5)
c
Total amount of cells
16000
14000
12000
10000
8000
6000
0
3
4
5
6
7
Vh/CrD
R = –0.456
P ≤ 0.001

SUPPLEMENTARY MATERIAL

Article title: Transglutaminase 2-specific coeliac disease autoantibodies induce morphological changes and signs of inflammation in the small-bowel mucosa of mice

Journal: Amino Acids

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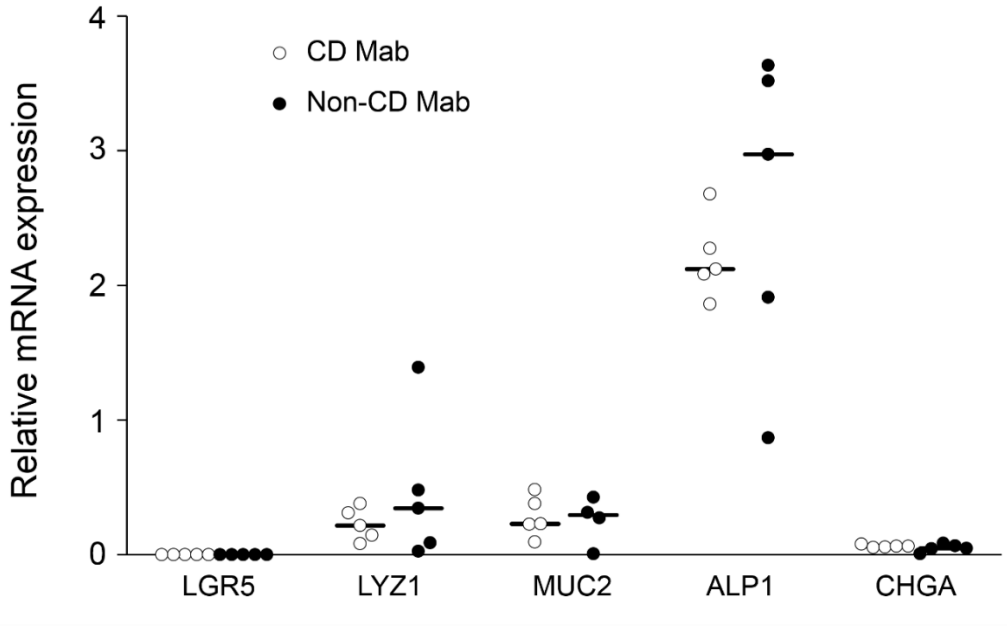
Supplementary table 1. List of quantitative real-time PCR oligos used in this study

Oligonucleotide	Species	Sequence (5' to 3')
Gapdh_fwd	*Mm*	TGTGTCCGTCGTGGATCTGA
Gapdh_rev	*Mm*	CCTGCTTCACCACCTTCTTGA
Lgr5_fwd	*Mm*	GACAATGCTCTCACAGACGTCC
Lgr5_rev	*Mm*	CAGGGAGTGGATTCTATTATTATGGAG
Alpi_fwd	*Mm*	CCACAAGGCTTCTACCTCTTTGTAG
Alpi_rev	*Mm*	CGGGTGTAGGATTTGTCATCTAGG
Lyz_fwd	*Mm*	GGAATGGATGGCTACCGTGG
Lyz_rev	*Mm*	CACAGGCATTCTTAGATCTTGGG
Chga_fwd	*Mm*	GTGCGTCCTGGAAGTCATCTCC
Chga_rev	*Mm*	GAGAGCCAGGTCTTGAAGTTCC
Muc2_fwd	*Mm*	GGAACCGGGAAGATGCACTC
Muc2_rev	*Mm*	GTCAGCAGCCTCTCACATTCG

Supplementary Fig. 1 Messenger RNA (mRNA) expression of distinct intestinal epithelial cell differentiation markers evaluated by quantitative real-time PCR. The levels of the enterocyte marker ALP1, the enteroendocrine marker CHGA, the Paneth cell marker LYZ1, the goblet cell marker MUC2 and stem cell marker LGR5 are shown. Each dot represents the median value for one mouse calculated from three replicate samples and black lines show median values per group. *P* values lower than 0.05 were considered statistically significant, but no significant differences were found between coeliac (CD Mab)- and control antibody (non-CD Mab)-injected mice



Supplementary Fig. 2 Epithelial cell apoptosis was determined by active caspase-3 staining. Representative pictures of **a)** coeliac antibody (CD Mab)- and **b)** control antibody (non-CD Mab)-injected mice show single cell positivity mainly at the tips of villi. Higher magnification images (scale bar=50µm) show the area marked in the lower magnification images (scale bar=100 µm)