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Abstract: Celiac disease is an enteropathy resulting from an abnormal immune response to gluten-derived peptides in genetically susceptible individuals. This immune response is initiated by intestinal transport of intact p31-49 and 33-mer gliadin peptides through an unknown mechanism. Here we show that the transferrin receptor, CD71, is responsible for apical to basal retrotranscytosis of gliadin peptides, a process during which p31-49 and 33-mer peptides are protected from degradation. In patients with active celiac disease, CD71 is overexpressed in the intestinal epithelium and colocalizes with IgA. Intestinal transport of intact p31-49 and 33-mer peptides was blocked by polymeric and secretory IgA and by soluble CD71 receptors, pointing to a role of secretory IgA-gliadin complexes in this abnormal intestinal transport. This retrotranscytosis of secretory IgA-gliadin complexes may promote the entry of harmful gliadin peptides into the intestinal mucosa, thereby triggering an immune response and perpetuating intestinal inflammation. Our findings strongly implicate CD71 in the pathogenesis of celiac disease.

Celiac disease (CD) is an inflammatory enteropathy induced by gluten-derived prolamines in genetically susceptible individuals. CD affects about one in 100 individuals in Europe and the United States[1;2]. The associated intestinal inflammation results from synergism between innate and adaptive immune responses to gliadin peptides. The adaptive immune response is orchestrated by $CD4^+$ T cells recognizing various deamidated gliadin peptides[3], including a 33-mer (peptide 56-88)[4], bound to HLA-DQ2/8 molecules[5]. Peptide 56-88 is a powerful immunodominant gliadin peptide extremely resistant to gastrointestinal digestion, and has far higher T cell stimulatory potency than its 12-mer counterparts[6]. The innate immune response in CD is triggered by a distinct set of gliadin peptides: one prototype innate peptide is peptide 31-49 (p31-49), common to the N terminus of A-gliadins and shown to be toxic for CD patients both *in vitro* and *in vivo* [7-9]. This peptide was recently shown to stimulate the synthesis of IL-15[10;11], a proinflammatory cytokine that can promote the $CD4^+$ adaptive immune response[11] and can activate cytotoxic activity and IFNγ production in intraepithelial lymphocytes[12-14].

This activation of the local immune system implies that undigested gliadin fragments present in the intestinal lumen somehow cross the intestinal epithelium. Indeed, apical to basal transport and processing of gliadin peptides, and particularly of p31-49 and 33-mer, are severely altered in active CD, leading to the release of intact peptides on the basal side, while the same peptides are almost entirely degraded during their intestinal transport in control individuals and treated CD patients[15;16]. Several lines of evidence argue against simple non specific paracellular leakage of gliadin peptides across the celiac mucosa. In particular, a 12-mer gliadin peptide, peptide 57-68, is completely degraded during intestinal transport in patients with active CD, suggesting no paracellular leakage of molecules of this size or larger. In addition, only 0.3% of the apical peptide crosses the epithelium during a 3-hour incubation period, arguing against free diffusion across a damaged mucosa. The “protected” transport of p31-49 thus involves a transcellular pathway[15;16], that enables the peptide to escape lysosomal degradation. As active CD is associated with high

concentrations of anti-gliadin IgA antibodies in the intestinal lumen[17;18], we postulated that the transport of intact gliadin peptides might result from abnormal retrotranscytosis of IgA-gliadin complexes. Indeed, although anti-gliadin IgA antibodies are a hallmark of CD, they have no known pathogenic role. We obtained evidence that polymeric/secretory IgA can mediate protected transport of p31-49 and 33-mer gliadin peptides through their binding to CD71, the transferrin receptor. Importantly, we found that this receptor was abnormally expressed at the apical pole of enterocytes in patients with active CD. Although initially implicated in endocytosis of iron-loaded transferrin, CD71 was recently recognized as an IgA receptor mediating mesangial deposition of IgA1 complexes in IgA nephropathy[19].

RESULTS

High-molecular-weight immune complexes containing gliadin-specific IgA and transglutaminase are present in patients with active CD

Active CD is associated with enhanced intestinal secretory immunity[17;20] and with higher antigliadin antibody titers in serum and jejunal secretions than in controls and treated CD patients[17;20;21]. In addition, gliadin co-localizes with transglutaminase (the autoantigen in CD) in the epithelium and subepithelium of patients with active CD[22]. We postulated that, as in IgA nephropathy, high-molecular-weight immune complexes[23] might be present in CD patients. As shown in Fig. 1A, anti-gliadin IgA antibodies and transglutaminase were detected within high-molecular-weight immune complexes, which were found in larger amounts in the serum and duodenal secretions of patients with active CD than in those of treated CD patients and controls. The presence of transglutaminase in these immune complexes may be related to the cross-linking capacity of this enzyme, contributing to the formation of IgA/gliadin/transglutaminase complexes. Consistent with a possible role of gliadin-specific IgA in the luminal uptake of gliadin peptides in active CD, immunofluorescence studies of duodenal biopsies revealed large amounts of IgA at the apical pole of the surface epithelium in patients with active CD, whereas in control subjects and treated CD patients, IgA was restricted to the crypts and was not observed in villous epithelium (Fig. 1 B and Fig. S1). In patients with active CD, co-localization of IgA with cytokeratin or alkaline phosphatase (a brush-border enzyme) confirmed the presence of IgA at the apex of surface epithelial cells (Fig. 1 C).

CD71 is overexpressed in the apical pole of enterocytes in active CD

IgA involvement in the intestinal transport of gliadin peptides would imply the presence of IgA receptors on the apical pole of enterocytes. Besides the polymeric Ig receptor (pIgR, also called membrane secretory component or SC), which permits transcytosis of dimeric IgA (dIgA) from

the *lamina propria* and its delivery into the intestinal lumen, complexed to the cleaved extracellular part of pIgR (bound SC), in the form of secretory IgA (SIgA), enterocytes express a second receptor able to bind IgA, namely CD71[19;24]. In normal intestinal epithelium, CD71 ~~is mainly expressed on the basolateral membranes predominantly in the crypt compartment, and~~ mediates the rapid endocytosis/recycling of transferrin necessary to deliver iron to rapidly proliferating epithelial cells[25]. The level of CD71 expression is also linked to body iron stores and correlates negatively with the serum iron concentration. CD71 can be upregulated in CD patients with iron-deficiency anemia[26]. The recent demonstration that CD71 can bind IgA1 at the surface of mesangial cells in IgA nephropathy[19], together with a previous report indicating upregulation of intestinal CD71 by IL-15 in CD[27], led us to investigate whether CD71 mediates abnormal IgA retrotransport in CD.

The distribution of CD71 in active CD was assessed in duodenal biopsies by both immunoperoxidase and immunofluorescence detection (Fig. 2A, B). In control biopsies, CD71 expression was confined to the basolateral pole of villous epithelial cells and was observed at both the basal and apical poles of crypt epithelial cells. In contrast, in active CD, CD71 was strongly expressed all over the epithelial layer, including the flat surface epithelium. Staining was not restricted to the basolateral compartment but was also detected at the apical pole of epithelial cells, in a sub-apical rather than strictly apical location, possibly corresponding to the apical recycling compartment from which this receptor is rapidly recycled to the cell surface[28]. A similar abnormal distribution of CD71 expression was observed in patients with refractory celiac sprue, who have a flattened over-proliferating mucosa. In contrast, CD71 expression was normal in treated CD patients who had recovered a normal or subnormal epithelial architecture. The strong expression of CD71 contrasted with the lack of significant expression of CD89, the myeloid IgA Fc receptor[29], on either enterocytes or immune cells in the *lamina propria* of controls and CD patients

([30] and data not shown). Taken together, these results were compatible with a role of CD71 in IgA binding at the apical surface of enterocytes in active CD.

IgA co-localizes with CD71 and p31-49 at the apical pole of enterocytes in active CD

The possible role of apically expressed CD71 in the binding of IgA/gliadin peptide complexes in patients with active CD was first addressed by performing co-localization immunofluorescence studies on frozen sections. Co-localization (white) of IgA (green) and CD71 (blue) was observed at the apical pole (and the basal pole) of epithelial cells (Fig. 2C) in fresh biopsies from patients with active CD but not from treated CD patients or controls. The presence of CD71 at the brush border membrane of enterocytes in patients with active CD, and its co-localization with IgA, was confirmed by immunogold electron microscopy (Fig. 2 D and Fig. S2).

As secretory IgA (SIgA) is the main IgA subtype present in the intestinal lumen, we examined whether it can bind CD71. The capacity of polymeric IgA (pIgA) and SIgA to bind to Daudi cells, which express CD71 as the only IgA receptor[19], was studied by flow cytometry. Both pIgA and SIgA were able to bind Daudi cells, and this binding was inhibited by prior incubation of IgA with soluble CD71 receptors (Fig. 2E), acting as a competitive inhibitor.

Finally, co-localization of IgA (green) and p31-49-TAMRA (red) was studied after applying the peptide for 15 minutes at 37°C on the apical side of biopsies mounted in Ussing chambers. As shown in Fig. 2F, co-localization of p31-49 and IgA was detected in biopsies from patients with active CD but not in those from treated CD patients (or controls, not shown). The lack of IgA co-localization with p31-49 in treated patients was compatible with total degradation of the peptide during intestinal transport (Fig. 3A). The red fluorescence observed in the *lamina propria* of treated CD patients (and, to a lesser extent, in patients with active CD) is likely related to the release of the TAMRA fluorescent label after endocytosis and hydrolysis of p31-49 by epithelial

cells. Together, these results support the hypothesis that CD71 may bind SIgA/gliadin peptide complexes at the apical surface in active CD.

IgA mediates intestinal transport of intact p31-49 through CD71

To directly address the role of IgA in the protected apical to basal transport of gliadin peptides, duodenal biopsies were mounted in Ussing chambers and ^{3}H-p31-49 was applied to the apical side. Intestinal transport was evaluated by analysing ^{3}H-labeled fragments in the basal compartment three hours later. Confirming our published results[15], a large fraction of ^{3}H-p31-49 was transported intact across the duodenal mucosa of patients with active CD, who have both a flat mucosa and IgA lining the surface epithelium (Fig. 3A). In contrast, this peptide was almost totally degraded during transport through samples from control subjects and treated CD patients, who had a normal intestinal epithelium with much less CD71 and IgA than patients with active CD. Furthermore, the peptide was similarly degraded during transport across duodenal biopsies from three patients with refractory celiac sprue, a complicated form of CD associated with severe villous atrophy, CD71 apical overexpression but no detectable IgA on the epithelial cell surface (Fig. 1B and Fig. 3A), indicating that non-specific leakage due to epithelial flattening is not sufficient to explain the increased transport of the intact peptide observed in active CD. The percentage of intact peptide 31-49 and active fragments found in the basal compartment in active CD is significantly higher than that observed in controls and treated CD patients (Fig. 3B). Adding an excess of dIgA, pIgA or SIgA to the apical compartment of duodenal biopsies from patients with active CD, as competitive inhibitors of endogenous gliadin-specific IgA, strongly inhibited the transport of intact p31-49 (Fig. 3C and D), indicating that large IgA molecules are involved in this transport. In contrast, monomeric IgA (mIgA) had no inhibitory effect, as expected in view of its low affinity for CD71[31]. ~~Immunoperoxidase staining with anti-secretory component antibody revealed, in addition to a strong epithelial labeling, diffuse lamina propria staining and labeling of~~

~~mononuclear cells, more intense in active CD patients than in controls (Fig. 3E). This observation is compatible with the retro-transport of SIgA immune complexes from the intestinal lumen into lamina propria in active CD patients, although diffusion of free SC back to the lamina propria cannot be excluded, as previously discussed [32].~~

To better understand the mechanism underlying intestinal transport of p31-49, we tested potentially inhibitory molecules. IgG and human transferrin (Tf) were unable to inhibit the "protected" transport pathway (Fig. 3E), indicating that gliadin-specific IgG does not play a major role in the intestinal absorption of gliadin peptides in patients with active CD. The failure of Tf to inhibit p31-49 transport is in line with its inability to inhibit IgA binding to CD71 on Daudi cells [31] and suggests that IgA and Tf do not share the same binding site on CD71. In addition, the anti-CD71 monoclonal antibody A24 did not inhibit the transport of intact p31-49 in active CD, suggesting that A24, like transferrin, does not bind CD71 at the same site as IgA[33] (Fig. S3 A). Also, an mAb directed to SC was not able to block the transport of IgA/p31-49 complexes (Fig. S3 B). Finally, we used a monoclonal antibody to transglutaminase II (anti-Tgase II, 6B9) or dansyl cadaverin, an inhibitor of Tgase activity, to test the involvement of Tgase II: no inhibitory effect was observed.

To confirm the role of CD71 in the transport of intact p31-49, we tested the capacity of a soluble CD71 receptor to competitively inhibit this transport in duodenal biopsies from six patients with active CD, using Ussing chambers. When soluble CD71 receptor was added to the apical compartment, RP-HPLC analysis of the radioactive material recovered in the basal compartment after intestinal transport of ^{3}H-p31-49 showed that transport of the intact peptide was significantly inhibited (Fig. 4A and B). In contrast, soluble CD89 receptor had no inhibitory effect, in keeping with the absence of CD89 expression in epithelial cells.

IgA mediates the intestinal transport of intact 33-mer via CD71

Intestinal transport of 33-mer in the form of the intact peptide and large immunogenic 12-mer[16] fragments (35% and 28%, respectively) was significantly higher in active CD than in controls (4% and 9%) and in treated CD (12% and 8%) (Fig. 5A and 5B left panel), confirming our previous results[15;16]. In active CD, competitive inhibition of 33-mer transport was attempted with dIgA, pIgA and soluble CD71 (Fig. 5B right panel). The percentage of 33-mer crossing the intestinal mucosa in intact form was significantly reduced by dIgA and pIgA (7% and 14%, respectively) and by soluble CD71 (19%) compared to the peptide alone (46%, $p<0.01$), further supporting CD71 mediation of protected IgA/33-mer complex retrotransport. ~~Yet, 33-mer large 12-mer fragments were still found in the basal compartment after intestinal transport.~~

DISCUSSION

This study reveals that "protected" transport of gliadin peptides in celiac disease is driven by retrotranscytosis of SIgA through the transferrin receptor (CD71), which is abnormally expressed on the apical side of the intestinal epithelium. This process may sustain anti-gliadin immune responses and aggravate intestinal inflammation in CD patients.

Immunoglobulin-mediated transport of luminal antigens across the epithelium has already been reported in various situations. Protected apical to basal intestinal transport of IgE/allergen complexes through CD23, the low-affinity IgE receptor, has been shown in allergic patients and might elicit rapid activation of intestinal mast cells[34-36]. Comparable protected transport of IgG/antigen complexes via the neonatal Fc receptor (FcRn) has also been demonstrated. This receptor, initially described at the apical surface of enterocytes in newborn rodents[37] and human fetal intestine[38;39], could transport IgG into the intestinal lumen and recycle IgG/antigen complexes back into the *lamina propria*, thereby promoting a specific immune response[39].

Here, we tested the hypothesis that, in active CD, SIgA-gliadin immune complex retrotransport could be mediated by CD71 and thus explain the protected transport of gliadin peptides in celiac patients. In healthy individuals, vectorial intestinal transport of IgA consists mainly of basal to apical transcytosis of dIgA via the pIgR, leading to the release of SIgA in the intestinal lumen[40] where it retains microbial and food antigens and confers protective mucosal immunity. While basal to apical transfer of IgA is the norm in epithelia, apical to basal retrotransport of SIgA through M cells overlying Peyer's patches has been documented in mice[41;42], although specific IgA receptors are not identified. ~~This process was, however, not described in columnar epithelial cells~~. Several lines of evidence support retrotransport of SIgA-gliadin complexes in active CD. First, the intestinal lumen of patients with active CD contains elevated levels of anti-gliadin IgA antibodies[17], which can bind a set of gliadin peptides[43;44] including the 33-mer[45] and peptide 31-55[46]. Here we show the presence of high-molecular-weight gliadin-specific IgA immune

complexes containing transglutaminase. This is an important finding, as IgA in form of high-MW complexes binds CD71 with high affinity[31]. Secondly, ~~secretory component (SC) seems to be present in the *lamina propria* of active CD patients, a result reminiscent of past study suggesting the abnormal presence of SIgA in lamina propria and serum of patients with active CD~~. ~~It is not excluded however, that SC originates from the retro-diffusion of free SC abundantly produced by epithelial cells in active CD~~ we show that IgA is more strongly concentrated at the apical pole of surface epithelial cells in active CD than in treated CD patients, refractory celiac sprue patients and control subjects, and that this pattern correlates with substantial transport of intact p31-49 and 33-mer in active CD, whereas this transport is negligible in controls, treated CD patients and patients with refractory celiac sprue. Finally, IgA involvement in the protected transport of these gliadin peptides is indicated by the competitive inhibition of their transport by polymeric IgA observed in this study. Although inhibition of 33-mer transport by IgA concerns the intact peptide only and not its large 12-mer fragments, these latter fragments have been shown to stimulate T cells much less potently than the intact peptide[6]. ~~The persistence of these fragments could be due to the fluid-phase transcytosis of 33-mer, in parallel to the receptor-mediated process, leading to incomplete degradation of this peptide by lysosomal enzymes.~~ One possible explanation is partial resistance of 33-mer to lysosomal degradation during fluid-phase transcytosis occuring in parallel to receptor-mediated transcytosis.

A role of IgA in protected transcytosis of gliadin peptides would imply the presence of a receptor able to bind polymeric/secretory IgA at the apical surface of enterocytes. Our findings highlight the role of the transferrin receptor CD71, a newly identified IgA receptor. Previous studies have shown that CD71 binds pIgA but not mIgA[31]. Here we show that CD71 can also bind SIgA, the main form of IgA present in the intestinal lumen. In the normal intestine, CD71 is mainly expressed in crypts (restricted to the basolateral membrane of epithelial cells)[47] with little expression in the villous epithelium ~~where its expression is low~~. In active CD, villous flattening,

increased epithelial renewal and iron-deficiency anemia are all associated with substantial CD71 upregulation all over the surface epithelium, and this may be responsible for its mis-sorting toward the apical pole of enterocytes. This is compatible with a role of CD71 in the endocytosis of IgA complexes from the apical cell surface. Previous studies have shown in non-polarized cells that pIgA binding to CD71 induces the internalization and rapid addressing of IgA-loaded CD71 into recycling vesicles[31]. The co-localization that we observed between IgA and CD71 and between IgA and p31-49 in active CD but not in controls suggests that IgA/gliadin complexes might bind CD71. These findings, combined with the demonstration that soluble CD71 receptors, as well as dIgA, pIgA and SIgA, can block the protected transcellular transport of intact p31-49 and 33-mer, provide strong evidence that CD71 is the receptor that allows gliadin peptides bound to SIgA to translocate from the intestinal lumen into the *lamina propria* in active CD. Interestingly, the capacity of CD71 to mediate polymeric IgA binding to mesangial cells of patients with IgA nephropathy also underlines the pathogenic role of CD71/IgA interactions in IgA nephropathy[19;31]. The presence of glomerular IgA deposits in a significant proportion of newly diagnosed CD patients[48] and the abnormally elevated incidence of CD in patients with IgA nephropathy[49] provides a link between these diseases.

The specific retrotranscytosis of SIgA/gliadin complexes in CD may appear puzzling given the presence of SIgA with diverse specificities in the intestinal lumen. Firstly, large IgA complexes bind CD71 with higher affinity than smaller IgA species such as SIgA[31]. Secondly, SIgA/gliadin complexes could be selectively retrotranscytosed with the help of tissue transglutaminase, which we detected in the high-molecular-weight IgA immune complexes found in duodenal secretions. Indeed, transglutaminase can cross-link gliadin peptides and promote receptor-mediated endocytosis[50], particularly the internalization step of CD71[51] and was recently detected at the surface of enterocytes in active CD[52]. However, neither transglutaminase antibodies nor an inhibitor of Tgase activity could inhibit the protected transport pathway in biopsy specimens from

patients with active CD, suggesting that transglutaminase is not directly involved in the transport process or that transglutaminase effect is irreversible in biopsies from patients with active celiac disease mounted in Ussing chambers. Finally, the pIgA and SIgA concentrations used in our Ussing chamber experiments were high enough -- in view of the small exposed surface area (0.025 cm^2) -- for competitive inhibition of IgA-gliadin transport to occur.

Gliadin-specific SIgA is present at high titers in the intestinal lumen of patients with active CD but is also found in healthy individuals[53;54]. In contrast, apical to basal delivery of intact gliadin peptides is only observed in active CD. Our data indicate that CD71 overexpression and mis-addressing to the apical pole of enterocytes is a key event in the intestinal retrotransport of SIgA/gliadin complexes (Fig. 6). They also indicate that the normal function of SIgA, namely the containment of harmful antigens in the intestinal lumen, is deficient in CD. This could account for the abnormal immune response to gluten in genetically susceptible (HLA-DQ2/8) individuals.

It is unclear whether this abnormal transport is the triggering event in CD or whether it becomes operational secondarily, perpetuating inflammation once the mucosa has flattened. Several environmental factors may serve as initial triggers for SIgA/gliadin complex entry into intestinal tissue. Among these factors, iron deficiency anemia, (~~frequently observed during pregnancy or infancy)~~by inducing CD71 upregulation, could promote the delivery of SIgA/gliadin complex, triggering abnormal intestinal responses in susceptible individuals.~~ Notably, celiac sprue can sometimes be related to pregnancy[55] and celiac disease is known to affect females more than males[56]. With this respect, it can be suggested that strict treatment of iron deficiency might be helpful to prevent the development of CD in at risk individuals and might help, in treated patients, to prevent relapse induced by inadvertent ingestion of small amount of gluten~~. In addition, strong intestinal epithelial cell proliferation secondary to epithelial damage by intestinal infection might also stimulate CD71 overexpression and thereby favor the onset of CD in susceptible individuals.

It is noteworthy that frequent rotavirus infection is associated with a higher risk of autoimmunity, defined as positivity for tissue transglutaminase, in early childhood[57].

Finally, if CD71 is necessary for CD to develop, why do patients with treated CD react so rapidly to the ingestion of gluten in the absence of CD71 expression? Some CD patients on a gluten-free diet still have minor small bowel abnormalities ~~probably due the high reactivity of the immune system to deliberate or inadvertent micro-challenge with gluten.~~, and there is evidence that the celiac epithelium may be persistently activated even after successful treatment and mucosal healing[58]. It is possible that residual expression of CD71 at the apical membrane of enterocytes may drive the entry of small amounts of gliadin peptides that are nonetheless sufficient to reactivate gluten-sensitive memory T cells and participate in rapid relapse.

In conclusion, we describe a novel mechanism of CD71-mediated IgA transcytosis that enables gliadin peptides to enter the *lamina propria* of patients with active CD. Inhibition of this protected transport pathway ~~by means of molecules such as soluble CD71 or by down-regulating apical CD71 expression~~ might provide a new therapeutic option, blocking the cascade that perpetuates innate and adaptive immune responses to gluten in patients with active CD.

MATERIALS AND METHODS

Patients

We studied 26 patients with active CD, 13 treated CD patients who had been on a gluten-free diet for at least one year, four patients with refractory celiac sprue, and 10 non-coeliac control subjects. Diagnosis of active CD was based on subtotal or total villous atrophy and positivity for anti-gliadin, anti-transglutaminase and anti-endomysium IgA antibodies. Refractory celiac sprue was defined as CD resistant to a gluten-free diet, characterized by the persistence of villous atrophy in the absence of anti-gliadin IgA antibodies. The control subjects underwent upper endoscopy for routine diagnostic purposes (dyspepsia, chronic diarrhea) and had a normal intestinal mucosa. All the patients underwent duodenal endoscopy, during which 4-6 additional biopsy samples were taken from the distal duodenum for research purposes. In some cases, duodenal secretions were obtained by infusion-aspiration of 50 ml of phosphate buffered saline. Local ethics committee approval was obtained and all the patients signed an informed consent to participate in the study.

Purification of IgA and synthesis of soluble IgA receptors

Competing IgA used in the Ussing chamber studies was human myeloma IgA1 protein prepared according to Chevailler et al[59]. Monomeric, dimeric and polymeric fractions of IgA1 (mIgA, dIgA and pIgA) were separated by gel filtration on Sephacryl S300 columns (Amersham Pharmacia Biotech, >98% pure)[60]. SIgA consisting of pooled human colostrum was from Fitzgerald or Biotrend. Soluble IgA receptors, sCD71 (TfR) and sCD89, were used. Both soluble receptors were expressed in a lytic baculovirus/insect cell expression system as described previously[19].

Measurement of IgA and IgA-containing complexes in serum and duodenal secretions

IgA-immune complexes (IC) in serum samples were analyzed by precipitation with polyethylene glycol 6000 (PEG 6000) as described elsewhere[61]. PEG precipitates were dissolved in 500 µl of 0.01 M phosphate buffer, pH 7.4, containing 0.5 M NaCl and 0.05% Tween 20. IgA-IC in duodenal secretions was measured after precipitation with saturated ammonium sulfate. Total IC

content was then estimated by measuring OD at 280 nm. The specificity of the IC present in serum and duodenal secretions and the presence of transglutaminase in these complexes was checked by enzyme-linked immunosorbent assay (ELISA). Ninety-six-well plates were coated with either pepsin/trypsin gliadin hydrolysate (PT-gliadin, 5 µg/ml) or rabbit polyclonal anti-transglutaminase antibody (US-biological) (5 µg/ml) respectively, blocked with 5% gelatin in borate buffer, and incubated overnight at 4°C with serum PEG IC diluted 1:10 and 1:100 in PBS containing 0.05% Tween 20 or with undiluted IC from duodenal secretions. After washing, alkaline phosphatase (AP)-anti-IgA (Southern Biotechnology Associates Inc) 1/2 000, was added for 2 h at 37°C. The reaction was developed by adding AP substrate and absorbance was read at 405 nm. Results are expressed as OD.

Flow cytometry analysis of secretory IgA binding to CD71

SIgA binding was examined with an indirect immunofluorescence assay in which Daudi cells (0.5 x 10^6), that express CD71 as the only IgA receptor[19], were incubated with 10 µl of SIgA (Fitzgerald, 0.5 mg/ml) for 1 hour on ice before washing and incubation for 20 min at 4°C with a biotinylated anti-IgA mAb clone CH-EB6-8 that recognizes both IgA1 and IgA2[62]. After washes, allophycocyanin (APC)-streptavidin (Southern Biotechnology Associates) was used as a developing reagent. For inhibition studies, SIgA was pre-incubated with soluble CD71 receptor at 0.5 mg/ml for 1 hour before adding the cells. Immunofluorescence was analyzed by flow cytometry (FACScalibur, Becton Dickinson, NJ).

Immunohistochemical analysis of duodenal biopsies

Immunoperoxidase labeling

CD71, CD89 and SC were detected on cryosections (6 µm) of duodenal biopsies fixed in cold acetone for 15 min at -20°C and rehydrated with PBS/BSA 0.1%. Endogenous peroxidase and biotin were blocked with 3% H_2O_2 and the Blocking Biotin system (Dako), respectively, and other non-specific binding sites were blocked with horse serum. Sections were incubated for 60 min

with anti-CD71 mAb A24[60] (22 µg/ml), biotinylated anti-CD89 mAb, A77 (10 µg/ml)[60], or anti-hSC mAb (10 µg/ml, Monosan), respectively. After rinsing in PBS/BSA 0.1%, primary antibodies were detected with an indirect biotin/streptavidin-peroxidase labeling kit (ChemMate, Dako), with DAB as substrate. Sections were counterstained with Mayer's hematoxylin.

Immunofluorescence labeling:

Immunofluorescence studies comprised two parts: 1) a study of fresh-frozen duodenal biopsy sections, and 2) a study of duodenal biopsy specimens incubated for 15 min at 37°C in Ussing chambers in the presence of fluorescent TAMRA-peptide 31-49 in the apical compartment, before freezing and cryosectioning.

In the first part, fresh duodenal biopsies were frozen in Tissue-Tek® (-80°C), cut into 6-µm-thick sections and kept at -20°C until staining. Biopsy sections were thawed, fixed in cold acetone and rehydrated in PBS/BSA 0.1%, and non-specific sites were blocked for 30 min with antibody diluent (ChemMate ™, DakoCytomation). Primary antibodies -- polyclonal goat anti-hIgA-FITC, 24 µg/ml (Abcam), anti-CD71(A24) mAb[60], 22 µg/ml, anti-human cytokeratin (epithelial) mAb (US-Biological), 50 µg/ml, or rabbit polyclonal anti-alkaline phosphatase, 25 µg/ml, (Abcam) -- were incubated for 60 to 90 min. After rinsing, secondary antibodies to A24 [Cy5-conjugated goat anti mouse IgG (H+L) Ab, 15 µg/ml], anti-cytokeratin mAb (Texas red-conjugated goat anti-mouse IgG (H+L), 30 µg/ml) or anti-alkaline phosphatase Ab (Texas red-conjugated sheep polyclonal anti-rabbit IgG(H+L), 20 µg/ml, Abcam) were added for 30 min. Tissue sections were mounted with anti-fade mounting medium (Vectashield®) and stored at 4°C until analysis.

All immunolabelling experiments included negative controls in which the primary Ab was replaced by concentration-matched isotype controls (Becton-Dickinson, St Quentin en Yvelines, France). The isotype control used for the anti-IgA-FITC primary conjugate was a goat IgG-FITC (Abcam). In some experiments, nuclei were labelled with TOPRO-3 (Molecular probes) or

propidium iodide (blue or red fluorescence, respectively). Slides were read with a LSM 510 Zeiss (Germany) laser scanning confocal microscope.

In the second part, peptide 31-49-TAMRA (200 µg/ml) was added to the apical compartment of duodenal biopsies from patients with active CD, patients with treated CD and control patients, placed in Ussing chambers. After 15 min at 37°C, the biopsies were removed, embedded in Tissue-Tek®, cryo-sectioned and submitted to immunofluorescent staining with a goat anti-hIgA-FITC, 24 µg/ml (co-localization peptide/IgA).

Immunogold electron microscopy

Biopsies were fixed with 2% paraformaldehyde/0.2% glutaraldehyde (Electron Microscopy Sciences) in 0.2 M phosphate buffer pH 7.4. Free aldehyde groups were quenched with 50 mM glycine in PBS and cells were embedded in 10% gelatin, infused in 2.3 M sucrose and frozen in liquid nitrogen. Ultrathin cryosections were prepared with a Leica FCS ultracryomicrotome (Leica) and double immunogold-labeled with anti-CD71 mAb (H68.4, Zymed laboratories), followed by rabbit anti-mouse Ig (Dako) and protein A conjugated to 15-nm gold particles and a goat polyclonal anti-human IgA antibody (Abcam) followed by rabbit anti-goat Ig (Dako) protein A conjugated to 10-nm gold particles. Protein A conjugated to gold particles was purchased from Cell Microscopy Centre (AZU, Utrecht, The Netherlands). To avoid cross-over of protein A binding, double labelling was performed sequentially and sections were treated with glutaraldehyde between each label to prevent interference between the different antibody-gold complexes[63].

Synthesis and radiolabeling of gliadin peptides

Peptides 31-49 (19-mer, LGQQQPFPPQQPYPQPQPF, MW: 2221) and 56-88 (33mer: LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF, MW: 3903) were synthesized (Covalab,

Lyon, France) and radiolabeled (tritiated) on selected proline residues as previously described[15]. The specific activity of the radiolabeled peptide batches used in this study was between 2 and 3 Ci/mmole. Radiolabeling allowed us to follow peptide degradation during intestinal transport, using RP-HPLC chromatography with online detection of radioactivity.

Immunofluorescence studies were performed with p31-49 coupled to 5-TAMRA (5-carboxytetramethylrhodamine) via a spacer (Ahx), at the N-terminal part of the peptide (Covalab, France).

Transport of peptides 31-49 and 33-mer across duodenal biopsies in Ussing chambers

Four to six duodenal biopsies from each patient were mounted in adapted Ussing chambers, exposing a surface area of 0.025 cm² as previously described[15]. Each biopsy was used to quantify apical to basal flux of radiolabeled p31-49 or 33-mer and to analyze tritiated-peptide fragments in the basal compartment. Peptide 31-49 or 33-mer was placed on the apical side at 0.2 mg/ml, together with 1850 kBq (50 µCi) of ^{3}H-peptide.

All inhibitors tested, namely non-specific IgA1 (mIgA, dIgA, pIgA final concentration 50 µg/ml, prepared as described in the section entitled Purification of IgA), SIgA (50 µg/ml, Biotrend, Köln, Germany), soluble IgA receptors (sCD71 or sCD89, 30 µg/ml), IgG (50 µg/ml, Biotrend, Köln, Germany), human transferrin (10 µg/ml, Sigma-Aldrich T3309), anti-CD71 mAb A24[60] (20 µg/ml), anti-human SC monoclonal antibody (30 µg/ml SC-05, Monosan, Tebu-bio, France), anti-Tgase II monoclonal antibody 6B9 (10µg/ml, kind gift from Prof. Issekutz T, Dalhousie University, Canada), and dansyl cadaverin, a Tgase II inhibitor (Sigma-Aldrich, 500 µM) were added to the apical compartment 30 min before adding the peptide.

Finally, preliminary experiments indicated no non-specific binding of ^{3}H-p31-49 to myeloma pIgA1, SIgA or sCD71 (not shown).

RP-HPLC analysis

P31-49 or p56-88 (33-mer) and their fragments present in the apical and basal compartments bathing the duodenal biopsies in the Ussing chamber were analyzed after 3 hours of incubation by radio-RP-HPLC, as previously described[15]. Acquisition, integration and calculation of the percentage of radioactivity eluted in each peak were performed with Radiostar software. In this setting, free ^{3}H-proline (the radiolabeled amino acid in the peptide sequence) is eluted with a retention time (Rt) of 4 min, while intact p31-49 is eluted at 24.5 min and intact 33-mer at 30 min. As already reported[15], there was no degradation of p31-49 or 33-mer in the apical compartment after 3 hours of incubation. Radio-RP-HPLC analysis of the basal compartments allowed us to evaluate p31-49 and 33-mer degradation during their intestinal transport by calculating the relative percentage of intact peptides and their degradation fragments. Our previous mass-spectrometry studies[16] showed that p31-49 fragments eluting between 17 and 21 min (named "active fragments"), correspond mainly to peptide 31-43, a toxic sequence of p31-49[11], and that large 33-mer fragments eluting between 23 and 28 min correspond to immunostimulant 12-mer peptides (named "large fragments").

Statistical analyses

The results are reported as means ± standard deviation (SD) or as scatter plots and medians. Multiple comparisons (analysis of variance) followed by group to group comparisons were performed with the general linear model procedure or the non parametric Wilcoxon test of the SAS package (SAS Institute, Cary, NC). Differences were considered to be statistically significant at p values <0.05.

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FIGURE LEGENDS

Figure 1: IgA and IgA/gliadin complexes are over-expressed in CD

A. Analysis of IgA immune complexes (IC) isolated from the serum and duodenal secretions of patients with active or treated CD and controls. Polyethylene glycol (PEG) precipitates containing high-molecular-weight IC were analysed by ELISA. Plates were coated with Frazer's fraction (pepsin/trypsin gliadin hydrolysate) or anti-transglutaminase antibody, followed by anti-human IgA-HRP. Results are presented as optical density values obtained with all sera or duodenal secretions tested. A significant increase in IC recognizing gliadin peptides and containing transglutaminase was observed in the active CD group compared to the treated CD and control groups, in both serum and duodenal secretion. *: significantly different from control ($p<0.0005$) and treated CD ($p<0.01$), #: significantly different from control ($p<0.01$) and treated CD ($p<0.02$).

B. IgA is concentrated at the apical pole of surface enterocytes in active CD. Cryosections of duodenal mucosa from control subjects, patients with active or treated CD and patients with refractory celiac sprue were labeled with anti-IgA-FITC Ab and TOPRO-3 (blue nuclei). In controls, treated CD patients and patients with refractory sprue, IgA staining of epithelial cells was located at the basal pole of villous cells and at the apical and basal poles of crypt cells. In contrast, in patients with active CD, IgA staining was concentrated at the apical pole of the surface epithelium (and of crypt cells, Fig. S1). Results are representative of three controls, seven patients with treated CD and six patients with active CD.

C. IgA overexpression observed at the apical pole of epithelial cells of patients with active CD was located inside the cell, including the brush border membrane, as shown by its co-localization with cytokeratin and alkaline phosphatase (Al. Ph.), a marker of apical membranes. No such co-localisation was seen in controls.

Figure 2: CD71 expression and co-localization with IgA and p31-49 in duodenal biopsies

A. Expression of CD71 (immunoperoxidase labeling) on duodenal biopsies from controls, patients with active or treated CD patients, and patients with refractory celiac sprue. Compared to controls, CD71 was overexpressed in patients with active CD and in patients with refractory celiac sprue (magnification x10). At higher magnification (x40), strong CD71 expression was observed all over the surface epithelium in patients with active CD, whereas in controls and treated CD patients CD71 expression was only observed at the basal pole of villous epithelial cells and in crypt cells.

B. CD71 overexpression by surface epithelium of patients with active CD was confirmed by immunofluorescent labeling. The fluorophore was a cy5-conjugated secondary Ab (blue staining). Results (A and B) are representative of three control subjects, four treated CD patients, eight patients with active CD, and two patients with refractory celiac sprue.

C. Double immunofluorescence labeling of IgA/CD71 in duodenal biopsies. Co-localization (white) of IgA (green) and CD71 (blue) was observed at the apical surface of the epithelium in active CD (n=5), but not in controls or in treated CD patients (n=3).

D. Immunogold electron microscopy with double labeling of IgA (10-nm particles, arrowheads) and CD71 (15-nm particles, arrows). In active CD, IgA and CD71 were expressed in the brush border membrane and subepithelial compartments, and IgA/CD71 co-localization was frequent. No such co-localisation was observed in controls (see additional photographs of one control subject and two patients with active CD in Fig. S2).

E. Secretory IgA can bind CD71 at the cell surface of a B cell line (Daudi cells) known to express CD71 as the only IgA receptor. Cells were incubated for 30 min at 4°C with SIgA or pIgA1 (500 µg/ml) in the presence or absence of soluble CD71 (500 µg/ml). IgA was revealed with biotinylated anti-IgA and allophycocyanin (APC)-labeled streptavidin (green line). Both pIgA1

and SIgA specifically bound CD71, as the binding was inhibited by soluble CD71 receptors (red line). Black line: isotope control.

F. Co-localization (yellow) of IgA (green) and peptide 31-49 (red) in duodenal biopsies from two patients with active CD, mounted in Ussing chambers and exposed to peptide 31-49-TAMRA on the apical side for 15 min (37°C) before being fixed, cryosectioned and stained with anti-IgA-FITC antibodies. No co-localization was found in two treated CD patients or in a control (not shown).

Figure 3: IgA involvement in intestinal transport and processing of ^{3}H-p31-49

A. Transport and processing of p31-49: typical RP-HPLC elution pattern of ^{3}H-material in the basal compartment of duodenal biopsies incubated for 3 hours after apical addition of ^{3}H-p31-49. In controls and treated CD patients, p31-49 was almost completely degraded during transport, as over 95% of the total radioactivity was eluted as free ^{3}H-proline in the basal compartment. In contrast, in patients with active CD (n=7), a large fraction of p31-49 was found on the basal side of the intestinal mucosa, mainly in intact form or as active fragments. Interestingly, in three patients with refractory celiac sprue ~~a complicated form of CD associated with persistent~~ (flat mucosa and absence of anti-gliadin IgA), near-complete degradation of the peptide was observed after intestinal transport, suggesting that a flat mucosa is not responsible for the transport of intact peptide observed in patients with active CD.

B. Mean percentage of tritiated intact p31-49, active fragments and proline found in the basal compartment after intestinal transport of p31-49 by duodenal biopsies from controls, patients with treated CD and patients with active CD. The percentage of intact p31-49 + active fragments crossing the duodenal biopsies was significantly higher in active CD (mean ±SD: 57±18 %, n=17) than in treated CD (23±23 %, n=8) and controls (26±4 %, n=4).*: $p<0.007$.

C. Inhibitory effect of polymeric IgA (pIgA), secretory IgA (SIgA), and monomeric IgA (mIgA) on the transport of intact p31-49. To test the involvement of IgA in the transport of intact p31-49, we performed competitive inhibition experiments with different forms of IgA. Typical RP-HPLC elution profile of ^{3}H-p31-49 obtained in biopsies from the patient with active CD shown in (A): 85% of p31-49 was transported intact in basal conditions, whereas this proportion fell sharply in the presence of pIgA and SIgA but not mIgA (mIgA does not bind significantly to CD71[31]).

D. Intestinal transport of p31-49 in patients with active CD: percentage of intact p31-49 + active fragments found in the basal compartment of Ussing chambers after blockade with mIgA, dIgA, pIgA or SIgA. Compared to "peptide alone" (median= 50, n=7), dIgA (23, n=3), pIgA (0, n=3) and SIgA (0, n=5), but not mIgA (60, n=3) significantly inhibited the intestinal transport of p31-49. *: Significantly different from "peptide alone", $p < 0.01$.

E. Effect of IgG and transferrin (Tf) on the transport of intact p31-49. IgG (50 µg/ml) and Tf (10 µg/ml) were pre-incubated for 30 min on the apical side of duodenal biopsies mounted in Ussing chambers, before adding ^{3}H-p31-49. The basal compartment was collected after 3 hours and analyzed by radio RP-HPLC to detect p31-49 and its metabolites. No inhibitory effect on p31-49 transport was observed.

Figure 4: Inhibitory effect of soluble CD71 on the transport of intact p31-49

A. Typical HPLC elution profiles of p31-49 after intestinal transport across duodenal biopsies from patients with active CD. ^{3}H-radioactive material present in the basal compartment of the duodenal biopsies in Ussing chambers 3 hours after adding ^{3}H-p31-49 to the apical compartment. In basal conditions, intact p31-49 or active fragments were present in the basal compartment. Soluble CD71 (sCD71) reduced the transport of intact p31-49, whereas soluble CD89 (sCD89) had no effect.

B. Inhibition of intestinal transport of ^{3}H-p31-49 in the presence of sCD71. The histogram shows the mean percentage of intact p31-49 and its active fragments found in the basal compartment after intestinal transport. Compared to peptide alone (n=6, median: 50), significant inhibition was observed in the presence of 30 µg/ml sCD71 (n=6, median: 21) but not sCD89 (n=4, median: 46). The horizontal solid lines indicate median values, and dotted lines join paired results from the same patient.

*: Significantly different from "peptide alone" ($p<0.01$).

Figure 5: Duodenal transport of 33-mer in patients with active CD, and competitive inhibition by IgA and soluble CD71.

A. Typical RP-HPLC elution profile of ^{3}H-33-mer after intestinal transport across duodenal biopsies from a control individual and a patient with active CD, mounted in Ussing chambers: ^{3}H-radioactive material present in the basal compartment 3 hours after adding ^{3}H-33-mer to the apical compartment. The percentages of the different eluted fractions (proline, small and large fragments and intact 33-mer) were quantified with Radiostar software. The control tissue almost totally degraded the 33-mer peptide, while digestion was incomplete in the sample from the patient with active CD.

B. (Left panel): Mean percentages of 33-mer and its fragments after intestinal transport. The duodenal mucosa of patients with active CD does not fully degrade 33-mer, as 38% and 22% of intact peptide and large fragments, respectively, were recovered in the basal compartment, compared to 4% and 9% in control subjects. Treated CD patients had an intermediate profile (12% and 8%). **(Right panel):** Dimeric IgA (dIgA), polymeric IgA (pIgA) and soluble CD71 (sCD71) significantly inhibited the transport of intact 33-mer but not of large fragments. *: percentage of intact peptide significantly different from control, $p<0.04$. #: percentage of intact peptide significantly different from "peptide alone", $p< 0.04$

Figure 6: Overview of postulated transferrin receptor (CD71)-mediated transport of IgA-gliadin complexes in celiac disease.

In healthy individuals, gliadin peptides (resistant to luminal degradation) are taken up non specifically by enterocytes and are degraded by lysosomal acid proteases during fluid-phase transcytosis. Very few toxic peptides are delivered into the intestinal *lamina propria*. In patients with active CD, abnormal expression of CD71 (transferrin receptor) at the apical pole of enterocytes allows receptor-mediated uptake of SIgA/gliadin peptide complexes and their "protected" transport toward the *lamina propria* and, thus, towards the local immune system. The exact part of the SIgA molecule involved in CD71 binding is not known. Blockade of gliadin peptide entry into the intestinal mucosa might serve as the basis for a novel therapeutic strategy in celiac disease.

Online Supplemental Material

Figure S1: Comparison of IgA expression in crypt and surface epithelium of control subjects and patients with active CD. In controls, apical IgA is mainly observed in the crypt epithelium, with virtually no labelling at the villous surface epithelium. In contrast, in patients with active CD, IgA is abundant in both crypt and surface epithelium. As illustrated by the histogram, the mean score for apical IgA immunofluorescence of surface epithelium, measured with Image J software, was significantly higher in patients with active CD (n=6, $p<0.0001$) than in the surface (villous) epithelium of controls (n=3) and treated CD patients (n=7).

Figure S2: Immunogold electron microscopy of IgA (10-nm particles) and CD71 (15-nm particles) in duodenal biopsies: sections representative of the crypt or villous epithelium from one control subject and of the crypt or surface epithelium from two patients with active CD. In the

control, IgA was present in the brush border membrane and in subapical endosomes, especially in the crypt epithelium as expected, but little CD71 expression was observed. In active CD, cells of the crypts and surface epithelium labeled positively for IgA and CD71, and IgA/CD71 co-localization was frequent, including at the brush border membrane.

Figure S3: Attempted inhibition of duodenal transport of intact ^{3}H-p31-49 in patients with active CD. (**A)** Anti-CD71 mAb (A24) did not block the transport of intact ^{3}H-p31-49 (n=4 patients), indicating that A24, like transferrin, does not bind CD71 at the same site as IgA[33]. As expected, a monoclonal antibody to secretory component SC was unable (in one patient) to inhibit the transport of intact p31-49 **(B)** The anti-transglutaminase II mAb 6B9 and the transglutaminase inhibitor dansyl-cadaverin 100 µM were unable to block the transport of ^{3}H-p31-49 in active CD (two patients) **(C)** Triangles represent values obtained with single and duplicate duodenal fragments from patients with active celiac disease.

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