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Distinct and Synergistic Contributions of Epithelial Stress and Adaptive Immunity to Functions of Intraepithelial Killer Cells and Active Celiac Disease

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Abstract

Background & Aims—The mechanisms of tissue destruction during progression of celiac disease are poorly defined. It is not clear how tissue stress and adaptive immunity contribute to activation of intraepithelial cytotoxic T cells and development of villous atrophy. We analyzed epithelial cells and intraepithelial cytotoxic T cells in family members of patients with celiac disease, who are without any signs of adaptive anti-gluten immunity, and in potential celiac disease patients, who have antibodies against tissue transglutaminase 2 in absence of villous atrophy.

Methods—We collected blood and intestinal biopsies from 268 patients at tertiary medical centers in the US and Italy from 2004 to 2012. All subjects had had normal small intestinal histology. Study groups included healthy individuals with no family history of celiac disease or antibodies against tissue transglutaminase 2 (controls), healthy family members of patients with celiac disease, and potential celiac disease patients. Intraepithelial cytotoxic T cells were isolated and levels of inhibitory and activating natural killer (NK) cells were measured by flow cytometry. Levels of heat shock protein (HSP) and interleukin-15 (IL15) were measured by immunohistochemistry and ultrastructural alterations in intestinal epithelial cells (IEC) were assessed by electron microscopy.

Results—IEC from subjects with a family history of celiac disease, but not from subjects who already have immunity to gluten, expressed higher levels of HS27, HSP70, and IL15 than controls; their IEC also had ultrastructural alterations. Intraepithelial cytotoxic T cells from relatives of patients with celiac disease expressed higher levels of activating NK receptors than cells from controls, although at lower levels than patients with active celiac disease, and without loss of inhibitory receptors for NK cells. Intraepithelial cytotoxic T cells from potential celiac disease patients failed to upregulate activating NK receptors.

Conclusions—A significant subset of healthy family members of patients with celiac disease with normal intestinal architecture has epithelial alterations, detectable by immunohistochemistry and electron microscopy. The adaptive immune response to gluten appears to act in synergy with epithelial stress to allow intraepithelial cytotoxic T cells to kill epithelial cells and induce villous atrophy in patients with potential celiac disease.

Keywords

cytotoxic intraepithelial lymphocytes; interleukin-15; heat shock protein; natural killer receptors

Introduction

CD is a systemic immune-mediated disorder with autoimmune features triggered by the ingestion of gluten and gluten-related dietary proteins present in wheat, rye and barley in genetically susceptible HLA-DQ2 and/or -DQ8 individuals^{1, 2}. Although it is driven by an

exogenous antigen, CD shares many common features with organ-specific autoimmune disorders, principally type-1 diabetes^{3, 4, 5}. In particular, CD is a T cell-mediated immune disorder associated with autoantibodies directed against tissue-transglutaminase 2 (TG2)^{6, 7} and characterized by a specific destruction of surface intestinal epithelial cells (IEC)⁵. Destruction of IEC is thought to be mediated by cytotoxic intraepithelial lymphocytes (IE-CTL). The mechanisms underlying the licensing of IE-CTL to kill IEC are not completely understood⁸, and whether and how adaptive anti-gluten immunity impacts the ability of IE-CTL to induce villous atrophy remains to be determined^{1, 3, 9}.

Potential CD is a form of CD characterized by the preservation of a normal intestinal morphology despite the presence of anti-TG2 antibodies^{10–19}. Because the presence of anti-TG2 antibodies is dependent on the occurrence of HLA-DQ2 or -DQ8 restricted gluten specific CD4⁺ T cells^{7, 20, 21}, the question arose as to whether CD4⁺ T cells may be required but not sufficient to activate IE-CTL and cause tissue damage^{1, 22, 23}. In accordance with the hypothesis that anti-gluten T cell immunity may not be sufficient to induce villous atrophy, humanized HLA-DQ8 mice retained a normal histology despite developing an interferon (IFN)- γ anti-gluten T cell response with presence of anti-gluten and anti-TG2 antibodies²⁴. Even when HLA-DQ2 or -DQ8 mice underwent drastic gluten immunization protocols^{25, 26} and harbored high frequencies of gluten-specific T cells²⁶, villous atrophy did not develop. Based on these observations, we proposed that IE-CTL were the effector cells mediating tissue damage and that for adaptive immunity to induce tissue damage, epithelial innate signals communicating that the tissue was distressed were required^{9, 23, 27}. This was supported by studies showing that IL-15, a cytokine upregulated in distressed tissues^{28–30}, promoted expression of activating NK receptors in effector CTL^{27, 28, 31} and conferred lymphokine killer activity to IE-CTL *in vitro*^{27, 31, 32}. To assess the respective role of adaptive anti-gluten immunity and tissue stress in the licensing of IE-CTL to kill, we investigated the NK phenotype of IE-CTL and the presence of innate epithelial alteration in family members of CD patients who did not display any signs of adaptive anti-gluten immunity and in potential CD patients (Supplementary Fig. 1A). We refer to both these study groups as “At Risk” because they may have inherited CD predisposing genes and/or may display signs of adaptive anti-gluten immunity. Family members lacking anti-TG2 antibodies were classified as “At Risk TG2^{neg}”, and potential CD patients were referred to as “At Risk TG2^{pos}”. In addition, active CD patients, CD patients on a gluten-free diet (GFD) and non-CD controls who do not have a family history of CD and were undergoing endoscopy for clinical reasons unrelated to CD were included in the study. Importantly, controls, At Risk and GFD CD patients all had normal intestinal architecture. In these different patient groups, the expression of activating and inhibitory NK receptors in freshly-isolated IE-CTL was determined by flow cytometry. Furthermore, IL-15, Heat shock protein (Hsp)70 and Hsp27 expression in IEC and presence of ultrastructural alterations were assessed by immunohistochemistry and electron microscopy, respectively (Supplementary Fig. 1B). We interpret the combined presence of high IL-15 expression, high Hsp expression, and ultrastructural abnormalities in IEC as evidence for epithelial stress. Determining whether and how adaptive anti-gluten immunity and epithelial alterations contribute to the licensing of IE-CTL to kill IEC will advance our understanding of the

natural history of CD and help identify criteria that can be used to identify patients at risk of developing overt CD (Supplementary Fig. 1B).

Material and Methods

Patients

268 subjects (Supplementary Fig. 1A) were recruited from 2004 to 2012 at four tertiary institutions: University of Chicago (S. Guandalini, C. Semrad and S. Kupfer), Columbia University (P. H. Green), Mayo Clinic (J. Murray) and University of Naples Federico II (R. Troncone). The review board of each institution approved this research. Control subjects had normal small intestinal histology, no family history of CD and no TG2 antibodies, and underwent gastrointestinal endoscopy for diagnostic workup (anemia, abdominal discomfort, intestinal disorders of non-celiac origin or failure to thrive). The second group (called "At Risk") included individuals with normal intestinal histology who were either family members of CD patients with negative serum anti-TG2 antibodies (At Risk TG2^{neg} n=48) or potential CD patients, with positive serum anti-TG2 antibodies and no villous atrophy (At Risk TG2^{pos} n=52). The third group comprised active CD with anti-TG2 antibodies and small intestinal villous atrophy, and CD patients on a GFD (37/39>1 year) with no detectable anti-TG2 antibodies at the time of the intestinal biopsy. Serum IgA antibodies against TG2 were measured by ELISA. As previously reported¹⁷, potential CD patients displayed lower levels of anti-TG2 IgA antibodies as compared to active CD patients (Supplementary Fig. 2). Controls included a significantly higher number of patients with gastrointestinal symptoms (symptomatic) as compared to At Risk individuals (Supplementary Fig. 3).

Immunohistochemistry

Epithelial expression of Hsp proteins and IL-15 was assessed by immunohistochemistry (IHC) and scored using a semi-quantitative scoring system from 0 to 3 and 0 to 4, respectively. Typically, staining was present in enterocytes at the villus tip, with progressively less intense staining towards the crypt and almost no specific staining within the epithelial cells of the crypt. Staining of the tip of the villi or faint staining was scored as 1 with progressive increases to 4 for degree of extension of staining towards the crypts or intense staining of villus enterocytes. Intensity of staining was used for active CD who by definition have villous atrophy. Representative images are shown in supplementary figure 4. IL-15 staining within lamina propria mononuclear cells was scored separately, on a scale of 0 to 4, based on the distribution and intensity of staining.

Electron microscopy

The most notable features observed by electron microscopy were: 1) reduced microvillus (MV) density and variable MV length; 2) irregular lateral MV membranes that were no longer straight and parallel, resulting in variable MV width; 3) clustered MV cytoskeletal rootlets that created small 'bouquets' of MV that disrupted the normal parallel arrangement of MV relative to one another; 4) focally-increased density at the termination of the MV cytoskeletal rootlets within the apical cytoplasm; 5) irregular spacing of MV and, in some cases, increased presence of organelles, primarily mitochondria and dilated endoplasmic

reticulum, within the apical cytoplasm. Some of these features have been previously described in active CD³³, but have never been reported in subjects with normal histology. These criteria were used to develop a semi-quantitative grading system (score from 1 to 4), with 1 representing completely normal ultrastructure and 4 representing the most severe disruption.

For more detail, see the Supplementary Materials and Methods section.

Results

IE-CTL from potential CD patients and family members of CD patients do not show the “fully activated” NK phenotype displayed by active CD patients

Classically, CD94 dimerizes with NKG2 molecules to form activating or inhibitory heterodimers, depending on the nature of the associated NKG2 molecule. In particular, NKG2A confers inhibitory properties while the other NKG2 molecules promote activating NK properties. To simplify the reading henceforth, NKG2A will refer to inhibitory CD94⁺NKG2A⁺ and CD94 to activating CD94⁺NKG2A⁻ NK receptors.

In contrast to normal IE-CTL, which predominantly express inhibitory NKG2A receptors^{34, 35}, low levels of NKG2D^{27, 31}, and lack activating CD94 receptors^{28, 31}, IE-CTL of active patients were found to express low levels of inhibitory NKG2A (Fig. 1A) and high levels of activating CD94 (Fig. 1B) and NKG2D (Fig. 1C) NK receptors, as previously shown^{28, 31, 35}. Thus, acquisition of this NK profile by IE-CTL (subsequently referred to as “fully activated” killer phenotype) is characteristic of overt CD^{1, 23}. To test the hypothesis that potential CD and family members of CD patients who have a normal intestinal villous structure might lack IE-CTL with a fully activated NK phenotype, we compared the expression of relevant NK receptors in potential CD patients (At Risk TG2^{pos}), CD family members without anti-TG2 and anti-gluten antibodies (At Risk TG2^{neg}) and active CD patients. Remarkably, At Risk patients, as a group, retained inhibitory NKG2A receptors similar to controls (Fig. 1A). Further analysis revealed that among At Risk subjects, potential CD patients (At Risk TG2^{pos}) had significantly lower proportions of IE-CTL with inhibitory NKG2A receptors compared to At Risk TG2^{neg} ($p=0.0181$) (Fig. 1A). Conversely, however, At Risk TG2^{pos} patients show levels of activating CD94 and NKG2D receptors similar to controls, and significantly lower as compared to Active CD ($p=0.0005$ for CD94/NKG2A⁻, and $p=0.0044$ for NKG2D) and to At Risk TG2^{neg} patients ($p=0.0072$ for CD94/NKG2A⁻, and $p=0.033$ for NKG2D) (Fig. 1B and C).

Taken together these data suggest that IE-CTL of At Risk patients failed to acquire the “fully activated” NK phenotype of active CD. In keeping with this conclusion, we also found that expression of perforin and granzyme were not significantly upregulated in IE-CTL of At Risk patients (Supplementary Fig. 5).

At Risk TG2^{neg} family members but not potential CD patients display signs of epithelial stress that correlate with upregulation of activating NK receptors in IE-CTL

Since *in vitro* studies suggest that IEC alterations, particularly IL-15 upregulation^{28–30}, may be critical for the acquisition of cytolytic properties by IE-CTL in active CD^{28, 30–32}, we

postulated that the increase in activating NK receptors in At Risk TG2^{neg} but not in At Risk TG2^{pos} individuals might correlate with the presence and absence of intestinal epithelial stress, respectively. To test this hypothesis we investigated by immunohistochemistry the expression of IL-15^{30, 36} and inducible Hsp27 and Hsp70³⁷ in IEC (Supplementary Fig. 1B), with the rationale that these 3 innate molecules are poorly expressed in healthy small bowel IEC but are induced under conditions of stress. The study of inducible Hsp is particularly relevant to detect early signs of stress before tissue damage and overt inflammation begins^{37, 38}. Furthermore, IL-15 was reported to upregulate *in vitro* activating NKG2D^{27, 31} and CD94²⁸ NK receptors in IE-CTL. Because our goal was to determine the early events responsible for IE-CTL activation and villous atrophy, we focused our analysis on patients and control groups with normal intestinal histological architecture. Criteria for the analysis of innate IEC markers are detailed in materials and methods and supplementary figure 4.

The number of epithelial stress markers present in IEC was significantly increased in At Risk TG2^{neg} individuals with a family history of CD ($p=0.002$), but not in potential CD patients (At Risk TG2^{pos}) ($p=0.41$) as compared to controls (Fig. 2A and B). Notably, 80% of potential CD patients had normal levels of IL-15 expression in IEC. Potential CD subjects lacked evidence of epithelial stress regardless of whether there was a family history of CD (Supplementary Fig. 6). In contrast, and even though they also had a normal intestinal architecture, all At Risk TG2^{neg} family members had IEC that expressed at least one innate stress marker and a significant proportion of them (roughly 20%) had IEC that displayed all three immunohistochemical markers of ongoing epithelial distress. Importantly, the observed difference in the expression of IEC stress markers between At Risk TG2^{neg} and At Risk TG2^{pos} patients was not due to a difference in their clinical presentation, as there was no significant difference in the frequency of subjects with or without gastrointestinal symptoms (Supplementary Fig. 3). Intriguingly, our data also suggest that CD-predisposing HLA-DQ molecules may play a role in the dysregulation of IL-15 but not of Hsp27 (Supplementary Fig. 7) and Hsp70 (data not shown) expression in IEC. Importantly, HLA-DQ2 and/or -DQ8 positive controls did not display an increase in IL-15 expression in IEC (data not shown), suggesting that the mere presence of the predisposing CD haplotype is not sufficient to upregulate IL-15 in IEC. Finally, similar to At Risk TG2^{neg} individuals, GFD patients were significantly more likely to express epithelial stress markers, relative to controls ($p=0.0037$) and At Risk TG2^{pos} ($p=0.017$) subjects (Fig. 2A and 2B). Consistent with previous reports²⁹, IL-15 overexpression in IEC persisted after gluten exclusion (Supplementary Fig. 8A and 8C). However, and in line with a study suggesting that IL-15 expression can be induced by gluten in intestinal organ cultures of GFD patients³⁹, IL-15 overexpression in lamina propria cells was reduced in subjects on a GFD (Supplementary Fig. 8B and 8C).

Having determined the expression pattern of innate epithelial markers in At Risk CD patients, we next wanted to assess how expression of activating and inhibitory NK receptors segregated with IL-15 expression in IEC and the presence or absence of adaptive anti-gluten immunity. In agreement with previous studies^{28, 27, 31} upregulation of activating CD94 and NKG2D NK receptors was only observed in IE-CTL of At Risk TG2^{neg} family members with high levels of IL-15 in IEC (Fig. 2C). Conspicuously, this was not accompanied by a

loss of inhibitory NKG2A receptors (Fig. 2C), which were only decreased in rare individuals who had both adaptive anti-gluten immunity (At Risk TG2^{pos}) and high levels of IL-15 expression in IEC (Fig. 2C).

Altogether, these findings suggest that neither adaptive anti-gluten immunity nor epithelial stress alone are sufficient to induce a fully activated killer phenotype in IE-CTL. Furthermore, the data suggest that epithelial stress can exist in absence of adaptive anti-gluten immunity and may have a genetic component.

At Risk TG2^{neg} patients showed marked ultrastructural alterations of IEC

Intrigued by the finding that At Risk TG2^{neg} family members of CD patients showed signs of epithelial distress, we analyzed the morphology of IEC of those patients by EM and distinct structural abnormalities of the microvillus (MV) were used to score the samples as indicated in supplementary materials and methods (Fig. 3A). The most remarkable difference between patients and control subjects was noted in the MV brush border and subjacent apical cytoplasm. As reported previously³³, the ultrastructure of villus enterocytes from patients with active CD demonstrated abnormal MV structure. Specifically, the densely packed microvilli present in control subjects were relatively rare in enterocytes from active CD patients, suggesting loss of microvilli. Moreover, rather than the strictly parallel lateral membranes seen in microvilli from control subjects, the lateral MV membranes from active CD patients were irregular and the MV cytoskeletal rootlets were often of increased electron density and were clustered. Finally, the dense network of cortical acto-myosin that normally excludes organelles from the apical cytoplasm was disrupted in active CD, as mitochondria and dilated endoplasmic reticulum were present in this location. The semi-quantitative ultrastructural analysis of intestinal samples from all groups showed significant differences compared to controls, with the exception of At Risk TG2^{pos} (Fig. 3B). The ultrastructure of IEC of At Risk TG2^{neg} patients was also distinguishable from that of control subjects in whom enterocytes expressed Hsp70 and Hsp27, suggesting that ultrastructural changes might be a more sensitive indicator of cellular stress than Hsp expression. There was no difference in the mean of MV length between At Risk CD patients and controls, however there was heterogeneity in MV lengths across patient groups that was amenable to quantitative analysis using the interquartile range (IQR). The average IQR was significantly increased in At Risk TG2^{neg} patients as compared to all other patients' groups (Fig. 3C). In particular there was a marked difference between IQR of At Risk TG2^{neg} and control groups ($p=0.0039$). There was also a significant difference between At Risk TG2^{neg} and active CD and GFD groups ($p<0.05$). Notably, IQR was elevated in 2 out of 4 At Risk TG2^{pos} patients, both of whom had a family history of CD (Fig. 3C [b]). Together, the qualitative, semi-quantitative, and quantitative analyses of epithelial and, specifically, brush border ultrastructure document the presence of alterations in family members of CD patients that are independent of both tissue damage and adaptive anti-gluten immunity. This reinforces the conclusion that a subset of CD patient family members has abnormalities in the intestinal epithelial compartment despite the absence of clinical disease and, in some cases, anti-TG2 antibodies. However, it is important to recognize that interpretations of these ultrastructural data are based on group data, that the number of subjects studied is insufficient for analyses

of sensitivity and specificity, and therefore, that diagnostic application of these criteria is not appropriate at this time.

Discussion

Analysis of asymptomatic individuals at an early stage of the disease (potential CD) and family members of CD patients before tissue damage occurs is important to understand the natural history and early pathogenesis of CD. Altogether, our findings are consistent with the idea that IE-CTL must acquire a fully activated NK phenotype to drive the induction of villous atrophy, and further implicate a distinct and synergistic role for epithelial alteration and adaptive anti-gluten immunity to license IE-CTL to mediate tissue damage (Fig. 4).

Despite characteristic expansion of IE-CTL within the epithelium, the role of IE-CTL in CD pathogenesis has been questioned. This is primarily, because, in contrast to lamina propria CTL⁴⁰, the specificity of IE-CTL for gluten has not been demonstrated¹. An explanation for this apparent enigma was provided when it was shown that IE-CTL expressed activating NK receptors that had the potential to kill epithelial cells based on recognition of stress signals present on IECs in active CD^{31, 35, 41}. If epithelial cell destruction in CD requires that IE-CTL become licensed to kill, one can predict that the absence of villous atrophy in At Risk patients is associated with IE-CTL that lack the fully active NK phenotype. In agreement with this model, potential CD patients had IE-CTL that expressed low levels of activating NK receptors. Hence, the increase in the number of IE-CTL frequently observed in these patients⁴² (Supplementary Fig. 9) is not associated with their transformation into killer cells and not secondary to IL-15 overexpression in IEC, but linked to the presence of adaptive anti-gluten immunity (Supplementary Fig. 9). Other potential explanation for the absence of villous atrophy in potential CD patients resides in the nature⁴³ and magnitude² of the adaptive anti-gluten T cell immune response. Accordingly, as previously reported¹⁷, the levels of circulating anti-TG2 antibodies are lower in potential relative to active CD patients (Supplementary Fig. 2). In contrast to potential CD patients, At Risk TG2^{neg} family members of CD patients had IE-CTL with increased expression of activating NK receptors that correlated with an upregulation of IL-15 in IEC. These data provide further support for *in vitro*^{27, 28, 31} and *ex vivo*^{28, 31} studies suggesting a critical role for epithelial IL-15 in the induction of activating NK receptors in IE-CTL. However, IE-CTL in these patients conspicuously preserved expression of inhibitory NKG2A receptors, providing a basis as to why these individuals did not develop villous atrophy. How the interplay between epithelial stress and inflammatory CD4⁺ T cell responses regulate CD94/NKG2A receptor expression in IE-CTL remains to be determined. Altogether, it is likely that anti-gluten T cell responses influence the licensing of IE-CTL to become killer cells, and reciprocally that epithelial stress and IE-CTL activation enhance inflammatory anti-gluten CD4⁺ T cells responses and anti-TG2 antibody levels. The importance of a cross-talk between CD4 T cells and IL-15 has been discussed in the context of CD by Frits Koning and colleagues⁴⁴ and suggested by mouse studies looking at the impact of IL-15 overexpression in CD4 T cell specific for ovalbumin⁴⁵.

The other important finding of this study is that a significant number of At Risk TG2^{neg} individuals, defined as having a family history of CD, display signs of epithelial stress with

ultrastructural changes in the absence of adaptive anti-gluten immunity. These epithelial alterations are different from the previously reported increased MHC class II and CD25 expression in IEC from CD family members⁴⁶ that has been linked to subclinical potential CD⁴⁷. In the presence of adaptive anti-gluten immunity, significant upregulation of IL-15 and Hsp27 in IEC was absent in potential CD patients; this result argues strongly against the possibility that undetected adaptive anti-gluten immunity is responsible for the induction of epithelial stress in At Risk TG2^{neg} individuals. Furthermore, the observations that At Risk TG2^{neg} family members of CD patients display signs of epithelial stress and that a subset of CD patients retain features of IEC stress even on GFD, suggest that this IEC stress response may be constitutive, and possibly inherited. Intriguingly, upregulation of IL-15 (but not Hsp27 or Hsp70) was only observed in IEC of At Risk TG2^{neg} family members who had the CD-predisposing HLA molecules, implying that IL-15 dysregulation may be linked to the CD-predisposing haplotype. Although IL-15 has not been identified as a direct risk factor³, it was linked to CD genes in genome-wide association studies (GWAS). Whether any of the numerous immune genes in the MHC Class III region, which is in linkage disequilibrium with the MHC class II region, may contribute to IL-15 regulation in CD and contains a yet undefined CD locus remains to be explored. In addition to the potential for a genetic component, constitutive epithelial stress in TG2^{neg} family members could be secondary to dysbiosis⁴⁸ or acute challenges, such as of viral infection⁴⁹ that disrupt lastingly intestinal immune homeostasis^{3, 5}. Furthermore, our findings predict that small intestinal villous atrophy and active CD may develop in potential CD patients subjected to environmental hits that have the means to induce epithelial stress accompanied by IL-15 upregulation^{3, 5, 9}. Of note, our results showing that potential CD patients lack epithelial stress markers are not incompatible with findings suggesting that potential CD patients may have other forms of epithelial alterations associated with defects in tight junctions⁵⁰. Changes in intestinal permeability in these patients could in fact be explained by the increase in anti-gluten CD4⁺ T cells producing IFN- γ or TNF- α ^{43, 51, 52}.

Overall, our study supports the concept that IE-CTL are the key effector T cells mediating villous atrophy in CD and that, in order to become pathogenic licensed killer cells, IE-CTL require complementary signals generated by both adaptive anti-gluten immunity and epithelial stress (see model depicted in Fig. 4). Our findings suggest more specifically that IEC stress and in particular IL-15 drive upregulation of activating NK receptors in IE-CTL, while adaptive anti-gluten immunity drives the expansion of IE-CTL. Both adaptive anti-gluten immunity and epithelial stress seem to be involved in the downregulation of the inhibitory NKG2A receptors and likely in the induction of non-classical MHC class I molecules in IEC. How adaptive immunity impacts on IE-CTL and IEC and synergizes with epithelial stress to induce villous atrophy remains to be determined. The notion that CTL need to be licensed in tissues to become effective killer cells and that this licensing is dependent on the presence of IL-15 is further supported by the finding that beta islet cells display increased levels of IL-15 expression in overt type-1 diabetes but not in latent autoimmune diabetes⁵³. Finally, our study suggests that in some subjects, epithelial stress may be a genetic component that lowers the barrier to development of CD. Identification of genes regulating epithelial stress will require further analysis of populations sub-phenotyped for the presence or absence of epithelial stress. It is however difficult to eliminate that CD

could be triggered in individuals with the at risk HLA by an environmental factor able to upregulate IL-15, and to induce danger signals in epithelium, independently of any genetic predisposition. Future studies will also determine whether epithelial stress as identified in the TG2^{neg} family members drives symptoms in a subset of patients with irritable bowel syndrome or the as yet poorly defined non-celiac gluten sensitivity syndrome^{13, 54, 55}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AI	autoimmune
CD	celiac disease
EM	electron microscopy
FH	family history
GFD	gluten free diet
GWAS	genome wide association studies
HLA	Human Leukocyte Antigen
Hsp	Heat shock protein
IE-CTL	intra-epithelial cytotoxic T lymphocytes
IEC	intestinal epithelial cells
IHC	immunohistochemistry
IQR	interquartile range
MHC	major histocompatibility complex
MV	microvilli
NK	natural killer
TCR	T cell receptor

TG2 tissue transglutaminase 2

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Figure 1. Potential CD patients both fail to upregulate activating and downregulate inhibitory NK receptors to the same extent as active CD patients

Representative flow cytometry analysis of NK receptors on freshly isolated TCR- $\alpha\beta$ ⁺CD8⁺ intra-epithelial cytotoxic T lymphocytes (IE-CTL) in control, At Risk, and active CD patient groups. **(A)** At Risk TG2^{neg} subjects conserve similar levels of inhibitory NKG2A receptors expression as compared to controls. In contrast, At Risk TG2^{pos} (potential CD) patients ($p=0.0181$) and active CD patients ($p<0.0001$) have lower proportions of IE-CTL with inhibitory NKG2A receptors as compared to At Risk TG2^{neg} and controls ($p<0.0001$). **(B)** At Risk TG2^{neg} ($p=0.0156$) and active CD patients ($p=0.0002$), but not At Risk TG2^{pos} patients ($p=0.38$), showed an increased proportion of IE-CTL with activating CD94 receptors as compared to controls and to At Risk TG2^{pos} patients ($p=0.0072$ and $p=0.0005$ respectively). **(C)** At Risk TG2^{neg} ($p=0.0015$) and active CD patients ($p<0.0001$) showed an increased proportion of IE-CTL with activating NKG2D receptors as compared to controls and to At Risk TG2^{pos} ($p=0.033$ and $p=0.0044$ respectively). Percentage of positive cells for

each patients' group is shown in A and B, mean fluorescence intensity (MFI) of NKG2D is shown in C. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Mann Whitney test).

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Figure 2. At Risk TG2^{neg} family members but not At Risk TG2^{pos} patients express innate epithelial stress markers and upregulate activating NK receptors

(A) Hsp27, Hsp70 and IL-15 expression was assessed in intestinal epithelial cells (IEC) by immunohistochemistry (IHC). The distribution of the number of positive innate stress markers for each group is represented on a Box plot (left) and the relative proportion of controls, At Risk TG2^{neg}, At risk TG2^{pos} and GFD CD patients within the groups of individuals presenting 0, 1, 2 or 3 positive innate markers is shown (right). IEC from controls and At Risk TG2^{pos} subjects show a significantly lower number of innate immune markers as compared to IEC from At Risk TG2^{neg} and GFD subjects. Pairwise Wilcoxon rank tests were performed between patient groups: control vs. At risk TG2^{pos} $p=0.41$, vs. At Risk TG2^{neg} $p=0.002$, vs. GFD $p=0.0037$; At risk TG2^{pos} vs. At Risk TG2^{neg} $p=0.0005$, vs. GFD $p=0.0017$; At Risk TG2^{neg} vs. GFD $p=0.4$. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. (B) Representative IHC images of Hsp27, Hsp70 and IL-15 staining are shown for each category. (C) At Risk TG2^{pos} potential CD subjects who have high levels of IL-15 expression (IL-15^{pos}) in IEC tend to have lower levels of inhibitory NKG2A receptors (left panel) ($p=0.03$) but have also low levels of the activating NKG2D (right panel), while At Risk TG2^{neg} who have high levels of IL-15 expression show an increase in IE-CTL with high NKG2D (right panel). However, these individuals retain high levels of inhibitory NKG2A receptors (left panel). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (Mann Whitney test).

Figure 3. At Risk TG2^{neg} family members but not At Risk TG2^{pos} subjects display ultrastructural alterations of intestinal epithelial cells

Ultrastructural examination of small intestinal absorptive enterocytes from 5 control subjects, 5 At Risk TG2^{pos} and 4 TG2^{neg} subjects, 5 active CD and 3 GFD CD patients was performed by transmission electron microscopy (EM). **(A)** Representative images from two patients in each patient group are shown. Below each image the semi-quantitative EM score (1 to 4) and the expression of innate epithelial stress markers are indicated. **(B)** The semi-quantitative EM scoring for all subjects examined within each group was significantly different for all groups compared to controls (vs. TG2^{neg} $p=0.05$, vs. Active CD $p=0.029$ vs. GFD $p=0.023$) except for potential CD patients (At Risk TG2^{pos} subjects). [a] Out of the two potential patients showing an EM score of 3, one has a family history of CD and the other had an associated autoimmune condition (type-1 diabetes). **(C)** A significant increase of interquartile range (IQR) of MV length was found in At Risk TG2^{neg} patients relative to controls ($p=0.0039$). [b] The two patients with the highest IQR had a family history of CD, partially overlapping with the IQR of At Risk TG2^{neg} individuals.

Figure 4. Innate epithelial stress and adaptive immune responses are both required for IE-CTL to acquire a “fully active” killer phenotype and induce villous atrophy

Proposed “two-hit” model of CD pathogenesis. The adaptive immune response in CD is characterized by anti-TG2 antibodies (1) that are induced in patients with adaptive anti-gluten immunity (2). Epithelial stress is characterized by the expression of innate epithelial stress markers (i.e. Hsp27, Hsp70 and IL-15) and the presence of ultrastructural alterations in the small intestinal enterocytes (3). Each of these responses is necessary but not sufficient to induce full activation of IE-CTL, characterized by high levels of activating and low levels of inhibitory NK receptors (4), and epithelial destruction resulting in villous atrophy (5). At Risk TG2^{neg} subjects show signs of intestinal epithelial stress, but lack the adaptive immune response. Although they upregulate activating NK receptors, the expression of high levels of inhibitory NK receptors on IE-CTL prevents extensive IEC killing. In contrast, Potential CD

patients show the typical adaptive immune response of CD, but lack the epithelial stress response. Hence, they fail to upregulate activating NK receptors and often maintain inhibitory NK receptors to some extent on IE-CTL. Only active CD patients, who have anti-gluten specific adaptive immunity and innate epithelial alterations develop villous atrophy as a result of their IE-CTL acquiring a fully activated NK phenotype characterized by the loss of inhibitory NK receptors and upregulation of activating NK receptors.

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