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

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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 the activation of intraepithelial cytotoxic T cells and the development of villous atrophy. We analyzed epithelial cells and intraepithelial cytotoxic T cells in family members of patients with celiac disease, who were without any signs of adaptive antigluten immunity, and in potential celiac disease patients, who have antibodies against tissue transglutaminase 2 in the absence of villous atrophy. METHODS: We collected blood and intestinal biopsy specimens from 268 patients at tertiary medical centers in the United States and Italy from 2004 to 2012. All subjects 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 receptors were measured by flow cytometry. Levels of heat shock protein (HSP) and interleukin-15 were measured by immunohistochemistry, and ultrastructural alterations in intestinal epithelial cells (IECs) were assessed by electron microscopy. RESULTS: IECs from subjects with a family history of celiac disease, but not from subjects who already had immunity to gluten, expressed higher levels of heat shock protein 70 (HSP70), and interleukin-15 than controls; their IECs also had ultrastructural alterations. Intraepithelial cytotoxic T cells were isolated from relatives of patients with celiac disease and expressed higher levels of activating 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 up-regulate activating receptors. CONCLUSIONS: A significant subset of healthy family members of patients with celiac disease with normal intestinal architecture had 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 active celiac disease.

Keywords: Cytotoxic Intraepithelial Lymphocytes; Interleukin-15; Heat Shock Protein; Natural Killer Receptors.

Celiac disease (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 HLA-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.3–5 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 (IECs).8 Destruction of IECs is thought to be mediated by cytotoxic intraepithelial lymphocytes (IE-CTL). The mechanisms underlying the licensing of IE-CTL to kill IECs are not completely understood,8 and whether and how adaptive antigluten immunity impacts the ability of IE-CTLs 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 HLA-DQ8–restricted gluten-specific CD4+ T cells,20,21 the question arose as to whether CD4+ T cells may be required, but not sufficient, to activate IE-CTLs and

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Abbreviations used in this paper: CD, celiac disease; GFD, gluten-free diet; Hsp, heat shock protein; IE-CTL, intraepithelial cytotoxic T lymphocytes; IEC, intestinal epithelial cell; IEL, intraepithelial lymphocyte; IL, interleukin; IQR, interquartile range; MHC, major histocompatibility complex; MV, microvilli; NK, natural killer; TG2, tissue transglutaminase 2.

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cause tissue damage. In accordance with the hypothesis that antigluten T-cell immunity may not be sufficient to induce villous atrophy, humanized HLA-DQ8 mice retained a normal histology despite developing an interferon-γ antigluten T-cell response with the presence of antigluten and anti-TG2 antibodies. Even when HLA-DQ2 or HLA-DQ8 mice underwent drastic gluten immunization protocols and harbored high frequencies of gluten-specific T cells, villous atrophy did not develop. Based on these observations, we proposed that IE-CTLs 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. This was supported by studies showing that interleukin-(IL)15, a cytokine up-regulated in damaged tissues, promoted expression of activating natural killer (NK) receptors in effector CTLs and conferred lymphokine killer activity to IE-CTLs in vitro. To assess the respective role of adaptive antigluten immunity and tissue stress in the licensing of IE-CTLs to kill, we investigated the NK phenotype of IE-CTLs and the presence of innate epithelial alteration in family members of CD patients who did not display any signs of adaptive antigluten immunity and in potential CD patients (Supplementary Figure 1A). We refer to both of these study groups as at-risk because they may have inherited CD-predisposing genes and/or may show signs of adaptive antigluten immunity. Family members lacking anti-TG2 antibodies were classified as at-risk TG2neg, and potential CD patients were referred to as at-risk TG2pos. In addition, active CD patients, CD patients on a gluten-free diet (GFD), and non-CD controls who did 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-CTLs was determined by flow cytometry. Furthermore, IL-15, heat shock protein (Hsp)70, and Hsp27 expression in IECs and the presence of ultrastructural alterations were assessed by immunohistochemistry and electron microscopy, respectively (Supplementary Figure 1B). We interpret the combined presence of high IL-15 expression, high Hsp expression, and ultrastructural abnormalities in IECs as evidence for epithelial stress. Determining whether and how adaptive antigluten immunity and epithelial alterations contribute to the licensing of IE-CTLs to kill IECs 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 Figure 1B).

Materials and Methods

Patients

A total of 268 subjects (Supplementary Figure 1A) were recruited from 2004 to 2012 at 4 tertiary institutions: University of Chicago (S.G., C.S., and S.K.), Columbia University (P.H.G.), Mayo Clinic (J.M.), and University of Naples Federico II (R.T.). 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 a diagnostic work-up (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 TG2neg; n = 48) or potential CD patients, with positive serum anti-TG2 antibodies and no villous atrophy (at-risk TG2pos; n = 52). The third group comprised active CD with anti-TG2 antibodies and small intestinal villous atrophy, and CD patients on a GFD (37 of 39 for >1 year) with no detectable anti-TG2 antibodies at the time of the intestinal biopsy. Serum IgA antibodies against TG2 were measured by enzyme-linked immunosorbent assay. As previously reported, potential CD patients showed lower levels of anti-TG2 IgA antibodies as compared with active CD patients (Supplementary Figure 2). Controls included a significantly higher number of patients with gastrointestinal symptoms (symptomatic) compared with at-risk individuals (Supplementary Figure 3).

Immunohistochemistry

Epithelial expression of Hsp proteins and IL-15 was assessed by immunohistochemistry 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 toward 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 toward the crypts or intense staining of villus enterocytes. Intensity of staining was used for patients with 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 as follows: (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; and (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 described previously in active CD. These criteria were used to develop a semiquantitative grading system (score, 1–4), with 1 representing a completely normal ultrastructure and 4 representing the most severe disruption.

For more details, see the Supplementary Materials and Methods section.
Results

**IE-CTLs From Potential CD Patients and Family Members of CD Patients Do Not Show the Fully Activated NK Phenotype Found in 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 whereas the other NKG2 molecules promote activating NK properties. To simplify the reading henceforth, NKG2A will refer to inhibitory CD94^+ NKG2A^+ and CD94 will refer to activating CD94^+ NKG2A^- NK receptors.

In contrast to normal IE-CTLs, which predominantly express inhibitory NKG2A receptors,^24,35^ low levels of NKG2D^27,31^ and lack activating CD94 receptors,^28,31^ IE-CTLs of active patients were found to express low levels of inhibitory NKG2A (Figure 1A) and high levels of activating CD94 (Figure 1B) and NKG2D (Figure 1C) NK receptors, as previously shown.\(^{28,31,35}\) Thus, acquisition of this NK profile by IE-CTLs (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-CTLs with a fully activated NK phenotype, we compared the expression of relevant NK receptors in potential CD patients (at-risk TG2^pos^), CD family

![Figure 1](image-url)

**Figure 1**. Potential CD patients both fail to up-regulate activating and down-regulate inhibitory NK receptors to the same extent as active CD patients. Representative flow cytometry analysis of NK receptors on freshly isolated T-cell receptor-αβ^+^CD8^+^ IE-CTLs in control, at-risk, and active CD patient groups. (A) At-risk TG2^neg^ subjects conserve similar levels of inhibitory NKG2A receptor expression as compared with controls. In contrast, at-risk TG2^pos^ (potential CD) patients (\(P < .0001\)) and active CD patients (\(P < .0001\)) have lower proportions of IE-CTLs with inhibitory NKG2A receptors as compared with at-risk TG2^neg^ and controls (\(P < .001\)). (B) At-risk TG2^neg^ (\(P = .0156\)) and active CD patients (\(P = .0002\)), but not at-risk TG2^pos^ patients (\(P = .38\)), showed an increased proportion of IE-CTLs with activating CD94 receptors as compared with controls and with at-risk TG2^pos^ patients (\(P = .0072\) and \(P = .0005\), respectively). (C) At-risk TG2^neg^ (\(P = .0015\)) and active CD patients (\(P < .0001\)) showed an increased proportion of IE-CTLs with activating NKG2D receptors as compared with controls and at-risk TG2^pos^ (\(P = .033\) and \(P = .0044\), respectively). The percentage of positive cells for each patient group is shown in panels A and B, and the mean fluorescence intensity (MFI) of NKG2D is shown in panel C. *\(P < .05\), **\(P < .01\), ***\(P < .001\), ****\(P < .0001\) (Mann–Whitney test).
members without anti-TG2 and antigluten antibodies (at-risk TG2\textsuperscript{neg}), and active CD patients. Remarkably, at-risk patients, as a group, retained inhibitory NKG2A receptors similar to controls (Figure 1A). Further analysis showed that among at-risk subjects, potential CD patients (at-risk TG2\textsuperscript{pos}) had significantly lower proportions of IE-CTLs with inhibitory NKG2A receptors compared with at-risk TG2\textsuperscript{neg} (P = .0181) (Figure 1A). Conversely, however, at-risk TG2\textsuperscript{pos} patients show levels of activating CD94 and NKG2D receptors similar to controls, and significantly lower levels as compared with active CD (P = .0005 for CD94/NKG2A\textsuperscript{and} P = .0044 for NKG2D) and with at-risk TG2\textsuperscript{neg} patients (P = .0072 for CD94/NKG2A\textsuperscript{and} P = .033 for NKG2D) (Figure 1B and C).

Taken together, these data suggest that IE-CTLs 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 up-regulated significantly in IE-CTLs of at-risk patients (Supplementary Figure 5).

At-Risk TG2\textsuperscript{neg} Family Members but Not Potential CD Patients Show Signs of Epithelial Stress That Correlate With Up-Regulation of Activating NK Receptors in IE-CTLs

Because in vitro studies have suggested that IEC alterations, particularly IL-15 up-regulation,\textsuperscript{28–30} may be critical for the acquisition of cytolytic properties by IE-CTLs in active CD,\textsuperscript{28,30–32} we postulated that the increase in activating NK receptors in at-risk TG2\textsuperscript{neg} but not in at-risk TG2\textsuperscript{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\textsuperscript{30,36} and inducible Hsp27 and Hsp70\textsuperscript{37} in IECs (Supplementary Figure 1B), with the rationale that these 3 innate molecules are expressed poorly in healthy small-bowel IECs 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.\textsuperscript{37,38} Furthermore, IL-15 was reported to up-regulate in vitro activating NKG2D\textsuperscript{27,31} and CD94\textsuperscript{48} NK receptors in IE-CTLs. 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 histologic architecture. Criteria for the analysis of innate IEC markers are detailed in the Materials and Methods section and in Supplementary Figure 4.

The number of epithelial stress markers present in IECs was increased significantly in at-risk TG2\textsuperscript{neg} individuals with a family history of CD (P = .002), but not in potential CD patients (at-risk TG2\textsuperscript{pos}) (P = .41) as compared with controls (Figure 2A and B). Notably, 80% of potential CD patients had normal levels of IL-15 expression in IECs. Potential CD subjects lacked evidence of epithelial stress regardless of whether there was a family history of CD (Supplementary Figure 6). In contrast, and even though they also had a normal intestinal architecture, all at-risk TG2\textsuperscript{neg} family members had IECs that expressed at least 1 innate stress marker and a significant proportion of them (roughly 20%) had IECs that displayed all 3 immunohistochemical markers of ongoing epithelial distress. Importantly, the observed difference in the expression of IEC stress markers between at-risk TG2\textsuperscript{neg} and at-risk TG2\textsuperscript{pos} patients was not owing to a difference in their clinical presentation because there was no significant difference in the frequency of subjects with or without gastrointestinal symptoms (Supplementary Figure 7). 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 Figure 8) and Hsp70 (data not shown) expression in IECs. Importantly, HLA-DQ2– and/or HLA-DQ8–positive controls did not show an increase in IL-15 expression in IECs (data not shown), suggesting that the mere presence of the predisposing CD haplotype is not sufficient to up-regulate IL-15 in IECs. Finally, similar to at-risk TG2\textsuperscript{neg} individuals, GFD patients were significantly more likely to express epithelial stress markers, relative to controls (P = .0037) and at-risk TG2\textsuperscript{pos} (P = .017) subjects (Figure 2A and B). Consistent with previous reports,\textsuperscript{25} IL-15 overexpression in IECs persisted after gluten exclusion (Supplementary Figure 8A and C). However, and in line with a study suggesting that IL-15 expression can be induced by gluten in intestinal organ cultures of GFD patients,\textsuperscript{49} IL-15 overexpression in lamina propria cells was reduced in subjects on a GFD (Supplementary Figure 8B and C).

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 IECs and the presence or absence of adaptive antigluten immunity. In agreement with previous studies,\textsuperscript{27,28,51} up-regulation of activating CD94 and NKG2D NK receptors was observed only in IE-CTLs of at-risk TG2\textsuperscript{neg} family members with high levels of IL-15 in IECs (Figure 2C). Conspicuously, this was not accompanied by a loss of inhibitory NKG2A receptors (Figure 2C), which were decreased only in rare individuals who had both adaptive antigluten immunity (at-risk TG2\textsuperscript{pos}) and high levels of IL-15 expression in IECs (Figure 2C).

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

At-Risk TG2\textsuperscript{neg} Family Members Showed Marked Ultrastructural Alterations of IECs

Intrigued by the finding that at-risk TG2\textsuperscript{neg} family members of CD patients showed signs of epithelial distress, we analyzed the morphology of IECs of those patients by electron microscopy and distinct structural abnormalities of the MV were used to score the samples as indicated in the Supplementary Materials and Methods section (Figure 3A). The most remarkable difference between patients and control subjects was noted in the MV brush border and
Figure 2. At-risk TG2neg family members but not at-risk TG2pos patients express innate epithelial stress markers and up-regulate activating NK receptors. (A) Hsp27, Hsp70, and IL-15 expression was assessed in IECs by immunohistochemistry. 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 TG2neg, at-risk TG2pos, and GFD CD patients within the groups of individuals presenting 0, 1, 2, or 3 positive innate markers is shown (right). IECs from controls and at-risk TG2pos subjects show a significantly lower number of innate immune markers compared with IECs from at-risk TG2neg and GFD subjects. Pairwise Wilcoxon rank tests were performed between patient groups: control vs at-risk TG2pos, \( P = .41 \); vs at-risk TG2neg, \( P = .002 \); vs GFD, \( P = .0037 \); at-risk TG2pos vs at-risk TG2neg, \( P = .0005 \); vs GFD, \( P = .0017 \); at-risk TG2neg vs GFD, \( P = .4 \). *\( P < .05 \), **\( P < .01 \), ***\( P < .001 \) (Mann-Whitney test).

(B) Representative immunohistochemistry images of Hsp27, Hsp70, and IL-15 staining are shown for each category. (C) At-risk TG2neg potential CD subjects who have high levels of IL-15 expression (IL-15pos) in IECs tend to have lower levels of inhibitory NKG2A receptors (left) \( P = .03 \) but also have low levels of the activating NKG2D (right), whereas at-risk TG2neg who have high levels of IL-15 expression show an increase in IE-CTLs with high NKG2D (right). However, these individuals retain high levels of inhibitory NKG2A receptors (left). *\( P < .05 \), **\( P < .01 \), ***\( P < .001 \) (Mann-Whitney test).
subsequent apical cytoplasm. As reported previously, the ultrastructure of villous enterocytes from patients with active CD showed 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 actomyosin that normally excludes organelles from the apical cytoplasm was disrupted in active CD because 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 with controls, with the exception of at-risk TG2-pos subjects (Figure 3B). The ultrastructure of IECs of at-risk TG2-pos patients also was 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 increased significantly in at-risk TG2neg patients as compared with all other patient groups (Figure 3C). In particular, there was a marked difference between the IQR of at-risk TG2neg and control groups (P = .0039). There was also a significant difference between at-risk TG2pos and active CD and GFD groups (P < .05). Notably, the IQR was increased in 2 of 4 at-risk TG2pos patients, both of whom had a family history of CD (Figure 3C, b). Together, the qualitative, semiquantitative, 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 antiglutem 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-CTLs 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 antiglutem immunity to license IE-CTLs to mediate tissue damage (Figure 4).

Despite characteristic expansion of IE-CTLs within the epithelium, the role of IE-CTLs in CD pathogenesis has been questioned. This is primarily because, in contrast to lamina propria CTLs, the specificity of IE-CTLs for gluten has not been shown. An explanation for this apparent enigma was provided when it was shown that IE-CTLs expressed activating NK receptors that had the potential to kill epithelial cells based on recognition of stress signals present on IECs in active CD. If epithelial cell destruction in CD requires that IE-CTLs become licensed to kill, one can predict that the absence of villous atrophy in at-risk patients is associated with IE-CTLs that lack the fully active NK phenotype. In agreement with this model, potential CD patients had IE-CTLs that expressed low levels of activating NK receptors. Hence, the increase in the number of IE-CTLs frequently observed in these patients (Supplementary Figure 9) is not associated with their transformation into killer cells and not secondary to IL-15 overexpression in IECs, but is linked to the presence of adaptive antiglutem immunity (Supplementary Figure 9). Other potential explanations for the absence of villous atrophy in potential CD patients resides in the nature and magnitude of the adaptive antiglutem T-cell immune response. Accordingly, as previously reported, the levels of circulating anti-TG2 antibodies are lower in potential relative to active CD patients (Supplementary Figure 2). In contrast to potential CD patients, at-risk TG2 pos family members of CD patients had IE-CTLs with increased expression of activating NK receptors that correlated with an up-regulation of IL-15 in IECs. These data provide further support for in vitro and ex vivo studies suggesting a critical role for epithelial IL-15 in the induction of activating NK receptors in IE-CTLs. However, IE-CTLs 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

![Figure 3](image-url)
regulate CD94/NKG2A-receptor expression in IE-CTLs remains to be determined. Altogether, it is likely that antigen T-cell responses influence the licensing of IE-CTLs to become killer cells, and reciprocally that epithelial stress and IE-CTL activation enhances inflammatory antigen gluten CD4 T-cell responses and anti-TG2 antibody levels. The importance of a cross-talk been CD4 T cells and IL-15 has also been discussed in the context of CD by van Bergen J et al and suggested by mouse studies looking at the impact of IL-15 overexpression in IECs from CD family members that have been linked to subclinical potential CD.47 In the presence of adaptive antigluten immunity, significant up-regulation of IL-15 and Hsp27 in IECs was absent in potential CD patients; this result argues strongly against the possibility that undetected adaptive antigluten immunity is responsible for the induction of epithelial stress in at-risk TG2neg individuals. Furthermore,

Figure 4. Innate epithelial stress and adaptive immune responses are both required for IE-CTLs to acquire a fully active killer phenotype and induce villous atrophy. A proposed 2-hit model of CD pathogenesis is shown. The adaptive immune response in CD is characterized by anti-TG2 antibodies (1) that are induced in patients with adaptive antigluten immunity (2). Epithelial stress is characterized by the expression of innate epithelial stress markers (ie, 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-CTLs, characterized by high levels of activating and low levels of inhibitory NK receptors (4), and epithelial destruction resulting in villous atrophy (5). At-risk TG2neg subjects show signs of intestinal epithelial stress, but lack the adaptive immune response. Although they up-regulate activating NK receptors, the expression of high levels of inhibitory NK receptors on IE-CTLs 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 up-regulate activating NK receptors and often maintain inhibitory NK receptors to some extent on IE-CTLs. Only active CD patients, who have anti–gluten-specific adaptive immunity and innate epithelial alterations develop villous atrophy as a result of their IE-CTLs acquiring a fully activated NK phenotype characterized by the loss of inhibitory NK receptors and up-regulation of activating NK receptors.
the observations that at-risk TG2<sup>neg</sup> family members of CD patients show signs of epithelial stress and that a subset of CD patients retain features of IEC stress even on a GFD suggest that this IEC stress response may be constitutive, and possibly inherited. Intriguingly, up-regulation of IL-15 (but not Hsp27 or Hsp70) was observed only in IECs of at-risk TG2<sup>neg</sup> 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 factor, it was linked to CD genes in genome-wide association studies. 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 an as yet undefined CD locus remains to be explored. In addition to the potential for a genetic component, constitutive epithelial stress in TG2<sup>neg</sup> family members could be secondary to dysbiosis<sup>10</sup> or acute challenges, such as viral infection<sup>49</sup> that disrupts lasting intestinal immune homeostasis.<sup>3,5</sup>

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 up-regulation.<sup>3,5,9</sup> 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 junctions.<sup>50</sup> Changes in intestinal permeability in these patients could in fact be explained by the increase in anti-gluten CD<sup>+</sup> T cells producing interferon-γ or tumor necrosis factor-α.<sup>43,51,52</sup>

Overall, our study supports the concept that IE-CTLs are the key effector T cells mediating villous atrophy in CD and that to become pathogenic licensed killer cells, IE-CTLs require complementary signals generated by both adaptive antigluten immunity and epithelial stress (model is shown in Figure 4). Our findings suggest more specifically that IEC stress and in particular IL-15 drive up-regulation of activating NK receptors in IE-CTLs, whereas adaptive antigluten immunity drives the expansion of IE-CTLs. Both adaptive antigluten immunity and epithelial stress seem to be involved in the down-regulation of the inhibitory NKG2A receptors and likely in the induction of nonclassic MHC class I molecules in IECs. How adaptive immunity impacts IE-CTLs and IECs 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 supported further by the finding that β-islet cells show increased levels of IL-15 expression in overt type 1 diabetes but not in latent autoimmune diabetes.<sup>53</sup>

Finally, our study suggests that in some subjects, epithelial stress may be a genetic component that lowers the barrier to CD development. Identification of genes regulating epithelial stress will require further analysis of populations subphenotyped for the presence or absence of epithelial stress. However, it is difficult to eliminate that CD could be triggered in individuals with the at-risk HLA by an environmental factor able to up-regulate IL-15, and to induce danger signals in epithelium, independently of any genetic predisposition. Future studies also will determine whether epithelial stress as identified in the TG2<sup>neg</sup> family members drives symptoms in a subset of patients with irritable bowel syndrome or the as yet poorly defined non-celiac gluten sensitivity syndrome.<sup>13,54,55</sup>

### Supplementary Material
Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2015.05.013.

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Conflicts of interest
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Supplementary Materials and Methods

Intraepithelial Cytotoxic T-Lymphocyte Isolation and Flow Cytometry

IE-CTLs were purified from duodenal biopsy specimens as previously described, and analyzed for surface expression of CD3, CD103, T-cell receptorαβ, CD8, CD94, NKG2D, and NKG2A (eBioscience, San Diego, CA) by flow cytometry on a 6-color FACs Canto (BD Biosciences, Palo Alto, CA) using FlowJo Software (Treestar, Inc, Ashland, OR).

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed and paraffin-embedded duodenal biopsy sections (5-μm thick). Slides were stained for IL-15 (MAB647, clone 34505, 5 μg/mL; R&D Systems, Minneapolis, MN), Hsp70 (W27, sc-24, 10 μg/mL; Santa Cruz, Santa Cruz, CA), and Hsp27 (rabbit polyclonal, 1:200 dilution; Stressgen, Ann Arbor, MI). Antibody binding was detected using secondary antibody conjugated to horseradish-peroxidase–labeled polymer (EnVision system; Dako [Carpinteria, CA]) and 3,3′-diaminobenzidine tetra hydrochloride chromogen. To verify specificity, staining with horseradish-peroxidase–labeled secondary antibody in the absence of the primary antibody was performed systematically. Sections were scored in a double-blinded manner by a gastrointestinal pathologist (J.R.T.). Staining intensity and, more particularly, the location of positive enterocytes were taken into consideration. For all antigens, 10 representative patients from each group (control, at-risk, active CD, and GFD) were analyzed using the ACIS Automated Cellular Imaging System (Dako). Results of this quantitative analysis correlated with the semiquantitative scoring, providing further validation for the semiquantitative scales developed.

Electron Microscopy

Electron microscopy was performed on duodenal biopsy specimens kept in 2.5% glutaraldehyde and 4% paraformaldehyde for up to 24 hours, stained en bloc with 1% uranyl acetate, and embedded and polymerized in Spurr resin (Sigma-aldrich, MO) for 1–2 days. Plastic sections (90-nm-thick) were stained with uranyl acetate and lead citrate and examined under 300 KV with an FEI Tecnai F30 transmission electron microscope (Hillsboro, OR). Images were photographed using a Gatan CCD digital micrograph (Pleasanton, CA). A gastrointestinal pathologist (J.R.T.) analyzed the electron micrographs in a double-blinded manner. The most notable features observed were scored in the following way for Figure 3A: (1) reduced 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; and (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 described previously in active CD, but have never been reported in subjects with normal histology. The 5 criteria listed earlier were used to develop a semiquantitative grading system that took each factor into account and provided a total score of 1–4, with 1 representing completely normal ultrastructure and 4 representing the most severe disruption.

Statistical Analysis

The Mann–Whitney test was performed to test for differences between pairs of groups with regard to the percentage of IE-CTLs expressing the different NK receptors and for differences in the number of IE-CTLs per 100 IECs. To test for differences in the mean number of positive epithelial stress markers, pair-wise Wilcoxon rank tests were computed between pairs of experimental groups. The Fisher exact test was used to check for differences in epithelial stress markers between groups and to compare the rate of symptomatic vs asymptomatic subjects across groups.

Quantitative Polymerase Chain Reaction

Quantitative polymerase chain reaction was performed using the light cyclor 480 (Roche Diagnostics, Branchburg, NJ). SYBR Advantage Quantitative Polymerase Chain Reaction Premix (Clontech; Mountain View, CA) and the following primers were used: glyceraldehyde-3-phosphate dehydrogenase forward, 5'-ATGGGGAAGGTGAAGGTCG-3' and reverse 5'-GGGGTCATTGATGGCAACAATA-3'; and Granzym B forward, 5'-CCCTGGGAAAAACACTACACA-3' and reverse 5'-GCA-CAAATGCTACTGTGCG-3'. Relative expression levels were determined using the ΔΔCt-method.
Supplementary Figure 1. Study design and aims. (A) A total number of 268 subjects were included in this study, divided into 3 main groups: controls, at-risk, and CD patients. The first group comprised 42 control subjects with normal small intestinal histology and negative anti-TG2 antibodies, 32 without and 10 with autoimmune (AI) disorders other than CD. The second group included 100 at-risk subjects as defined by individuals with normal small intestinal histology who were either family members of CD patients showing no signs of adaptive antigluten immunity and lacking anti-TG2 antibodies (at-risk TG2<sup>neg</sup>; n = 48), with (n = 25) or without (n = 23) the CD-predisposing HLA haplotype, or potential CD patients, with positive serum anti-TG2 antibodies (at-risk TG2<sup>pos</sup>; n = 52), 18 with a family history (FH<sup>pos</sup>) or 34 without a family history (FH<sup>neg</sup>) of CD. The third group comprised 126 CD patients, including 87 active CD and 39 patients following a GFD. (B) This study was performed to determine the respective impact of the adaptive antiglutens immune response and innate epithelial alterations on the induction of a fully activated killer phenotype by IE-CTLs and the development of villous atrophy. The proxy for ongoing adaptive antigluten T- and B-cell immunity is the presence of antiglutens and anti-TG2 antibodies. The presence of epithelial alterations was assessed by determining the expression of IL-15, Hsp27, Hsp70, and IL-15. Licensing of IE-CTL to become killer cells led to loss of the inhibitory NKG2A receptor and up-regulation of the activating NKG2D receptor.
Supplementary Figure 2. Potential CD patients (at-risk TG2pos) show significantly lower levels of serum anti-TG2 IgA antibodies compared with active CD patients. Fold-increase in anti-TG2 IgA antibody titers compared with the upper limit of the normal range is shown. ****P < .0001 (unpaired 2-tailed Student t test).
Supplementary Figure 3. At-risk TG2^{pos} do not differ from at-risk TG2^{neg} in their clinical presentation. The percentage of subjects with (symptomatic) and without (asymptomatic) gastrointestinal symptoms among controls, at-risk TG2^{neg} and at-risk TG2^{pos} groups. No differences were found between at-risk TG2^{neg} and at-risk TG2^{pos} in terms of symptom presentation. An increased percentage of symptomatic patients was found in controls compared with each of the 2 at-risk groups (*P = .01 vs at-risk TG2^{neg}, ***P < .001 vs at-risk TG2^{pos}).
Supplementary Figure 4. Reference immunohistochemistry images of innate stress markers in intestinal epithelial cells. Hsp70 (upper) and Hsp27 (middle) epithelial expression was scored from 0 to 3, taking into account both the intensity and the distribution of the staining. Typically, the most intense staining was within enterocytes at the villus tip, with progressively less intense staining toward the crypt and almost no specific staining within crypt epithelial cells. IL-15 epithelial expression (lower) was assessed, taking into account the surface epithelium, but not crypt epithelial cells, and graded from 0 to 4 based on both intensity and localization of the staining.
Supplementary Figure 5. Absence of significant increase in perforin and granzyme in at-risk CD patients. Representative flow cytometry analysis of cytotoxic molecules (perforin and granzyme B) on freshly isolated T-cell receptor-αβ⁺ CD8⁺ IE-CTLs in control, at-risk, and active CD patient groups. (A) At-risk TG2neg and active CD patients (P < .05) showed an increased proportion of IE-CTLs expressing perforin as compared with controls and with at-risk TG2pos. The mean fluorescence intensity (MFI) is shown. (B) Increased expression of perforin in IE-CTLs from at-risk TG2neg IL-15pos individuals as compared with at-risk TG2neg IL-15neg individuals. (C) Granzyme B gene expression was increased significantly in IE-CTLs from active CD patients as compared with controls (P = .003), GFD (P = .0008), and at-risk TG2pos (P = .011) individuals. Relative expression to glyceraldehyde-3-phosphate dehydrogenase is shown (relative expression level [REL]). *P < .05, ***P < .001 (One-way analysis of variance test).
Supplementary Figure 6. At-risk TG2\textsuperscript{neg} but not at-risk TG2\textsuperscript{pos} (potential CD) family members express innate epithelial stress markers. Expression of Hsp27, Hsp70, and IL-15 in IECs was compared in family members of CD patients (at-risk FH\textsuperscript{pos}) with (orange, TG2\textsuperscript{pos}) and without (green, TG2\textsuperscript{neg}) serum anti-TG2 antibodies. Box plot represents the number of positive markers for each subgroup. **$P = .003$ (pairwise Wilcoxon rank test). FH, family history.
Supplementary Figure 7. Increased epithelial IL-15 expression is found only in at-risk family members carrying the CD-associated HLA-DQ alleles. Intestinal epithelial expression of (A) IL-15 and (B) Hsp27 across different patients subgroups is shown. At-risk subjects are divided on the basis of the presence (FHpos) or absence (FHneg) of a family history of CD, and the presence (HLApos) or absence (HLAneg) of CD-predisposing alleles. (A) Significant increases in epithelial IL-15 expression as compared with controls is found in GFD (P = .0023) and at-risk TG2negHLApos (P < .001), but not in at-risk TG2negHLAneg individuals. Furthermore, the frequency of at-risk TG2negHLApos subjects with high IL-15 expression is increased significantly as compared with at-risk TG2negHLAneg (P = .007), and with at-risk TG2posFHneg (P = .002) and TG2posFHpos (P < .001) individuals. (B) Increased Hsp27 expression is found in at-risk TG2neg subjects independently from their HLA haplotype (controls vs HLAneg, P = .004, controls vs HLApos, P = .02). *P < .05, **P < .01, ***P < .001 (the Fisher exact test). FH, family history.
Supplementary Figure 8. Lamina propria but not epithelial IL-15 expression decreases on a GFD in CD patients. IL-15 expression in the (A) epithelium and in the (B) lamina propria, as assessed by immunohistochemistry scoring, by duration of GFD (years) is shown. (C) Percentage of subjects showing overexpression of IL-15 either in the epithelium (light grey) or in the lamina propria (dark grey) in control, active, and GFD patients. Active CD patients show a higher expression of IL-15 in both compartments, whereas GFD patients maintain high levels of IL-15 only in the epithelium. *P < .05, **P < .01 (Fisher exact test). LP, lamina propria.
Supplementary Figure 9. Intraepithelial lymphocyte counts are increased in at-risk TG2<sup>pos</sup> patients independently of epithelial IL-15 expression. (A) Box plots showing the number of intraepithelial lymphocytes (IELs) per 100 IECs in at-risk subjects. A significantly higher IEL infiltrate can be found in at-risk TG2<sup>pos</sup> compared with at-risk TG2<sup>neg</sup> (**p < .001) subjects. The Mann–Whitney test was performed. (B) Number of IELs/100 IECs by epithelial IL-15 expression is shown in at-risk TG2<sup>neg</sup> (left) and at-risk TG2<sup>pos</sup> (right). The Pearson correlation coefficient within each at-risk group did not show any significant correlation between IEL counts and IL-15 epithelial expression ($r^2 = 0.054$ in at-risk TG2<sup>neg</sup>; $r^2 = 0.015$ in at-risk TG2<sup>pos</sup>).