EPITHELIUM-DERIVED INTERLEUKIN-15 REGULATES INTRAEPITHELIAL LYMPHOCYTE TH1 CYTOKINE PRODUCTION, CYTOTOXICITY AND SURVIVAL IN COELIAC DISEASE

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Abbreviations: CD, coeliac disease; FITC, fluorescein isothiocyanate; IFN, interferon; IL, interleukin; IEL, intraepithelial lymphocyte; LPMC, lamina propria mononuclear cell; PI, propidium iodide; TCR-γ, T-cell receptor γ chain; TNF, tumour necrosis factor.

Keywords: apoptosis, coeliac disease, cytotoxicity, enterocyte, interleukin-15, intraepithelial lymphocyte, T-cell lymphoma.

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Abstract
Background & Aims: Epithelium-derived interleukin (IL)-15 signaling via IL-15Rα is critical for the development, activation and survival of intraepithelial lymphocytes (IEL). We aimed to better understand the IL-15-driven effects on IEL underlying mucosal damage and lymphomagenesis in coeliac disease (CD). Methods: Enterocytes, IEL and lamina propria mononuclear cells (LPMC) were isolated from 46 patients with uncomplicated CD (25 untreated and 21 treated) and 22 controls. IL-15 and IL-15Rα expression were determined by immunoblotting. Secretion of IL-15, IFN-γ, TNF-α and granzyme B into cell culture supernatants was assessed by ELISA. The ability of IL-15 to regulate IEL proliferation, perforin/granzyme-dependent cytotoxicity and apoptosis was tested by adding different combinations of IL-15 or IL-15 blocking antibody or chloroquine to IEL cultured alone or with Caco-2 cells as target. IL-15 mucosal levels were determined by ELISA also in five patients with complicated CD (two ulcerative jejunoileites, one refractory sprue, and two enteropathy-associated T-cell lymphomas) tested for T-cell receptor γ chain clonality. Results: IL-15 was overexpressed in untreated CD enterocytes and LPMC, and in the mucosa of complicated CD patients and uncomplicated untreated CD patients, where its levels correlated with the degree of mucosal damage. Enterocytes from untreated, but not treated, CD patients and controls secreted IL-15. Untreated CD IEL, characterised by a higher IL-15Rα expression, showed increased proliferation, production of IFN-γ and TNF-α, and perforin/granzyme-dependent cytotoxicity and a decreased propensity to apoptosis in response to IL-15. Conclusions: Our findings suggest that IL-15 plays a crucial role in the generation of epithelial damage in active CD. Its promotion of IEL survival in CD may predispose to the emergence of T-cell clonal proliferations. Blocking IL-15, by suppressing uncontrolled IEL activation and survival, has the potential to provide new therapeutic tools to prevent tissue damage and lymphomagenesis in CD.
Introduction
The discovery that interleukin (IL)-15 may be produced by intestinal epithelial cells\(^1\) and is a potent stimulant of intraepithelial lymphocytes (IEL)\(^2\) focused the attention in mucosal immunity on the role of IL-15 in the interplay between enterocytes and IEL.\(^3,4\) IL-15, a 14-kDa glycoprotein mainly produced by macrophages as well as non-lymphoid cells, is a key regulatory cytokine which supports the homeostasis between innate and adaptive immunity.\(^5\) The occurrence of lymphocytopenia and specific lack of natural killer cells, natural killer T cells, CD8\(^+\) T cells and \(\gamma/\delta\) IEL in IL-15-deficient (IL-15\(-/-\)) and IL-15R\(\alpha\)-deficient (IL-15R\(\alpha\)\(-/-\)) transgenic mice\(^6,7\) suggest that IL-15 signals transmitted via IL-15R\(\alpha\) are critical for the development, activation and/or survival of these cells.\(^8\)

As IL-15 expression is strictly regulated at multiple distinct levels, including transcription, translation and intracellular trafficking,\(^5\) and a removal of these negative control mechanisms results in an increased IL-15 production, which may predispose to the risk of excessive autoreactive T cell survival and abnormal lymphocyte activation, thus leading to the development of autoimmune or chronic inflammatory diseases.\(^9,10\) Indeed, IL-15 overexpression is associated with an array of immune-mediated intestinal disorders, such as inflammatory bowel disease\(^11,12\) and coeliac disease (CD).\(^13-16\) In this latter condition, IL-15 has been shown to be involved in the generation of villous atrophy,\(^13,14\) in favouring the selective expansion of CD94\(^+\) IEL,\(^15\) and in promoting the emergence of T-cell clonal proliferations in refractory sprue.\(^16\)

In the present study, we aimed to clarify the role of IL-15 in modulating the interactions between enterocytes and IEL in CD.

Patients and Methods
Patients and tissues. Size-appropriate and well-oriented endoscopic biopsy specimens were obtained from the second part of the duodenum in 25 uncomplicated untreated CD patients (mean age 39.2 yrs, range 20-65). The histopathological diagnosis was based on typical mucosal lesions with crypt cell hyperplasia, villous atrophy and increased number of intraepithelial lymphocytes. All untreated CD patients tested positive for antiendomysial antibodies at the time of diagnosis. Biopsies from 18 of them showed a Marsh IIIc lesion (total villous atrophy), while biopsies from the remaining seven patients showed a Marsh IIIb lesion (subtotal villous atrophy). In all of them there was histological improvement of duodenal mucosa following gluten withdrawal. Biopsies were also collected from 21 uncomplicated CD patients on a gluten-free diet for at least 12 months (mean age 41.5 yrs, range 21-63) who were in clinical and histological remission, and negative for antiendomysial antibodies. Twenty-two subjects undergoing upper gastrointestinal endoscopy for functional dyspepsia (mean age 43.2 yrs, range 21-68), who tested negative for antiendomysial antibody and had normal IgA levels and normal histology, were also studied. Some of the biopsy samples were processed according to standard methods for routine histology, others were homogenised for the determination of IL-15 or used to obtain suspensions of purified enterocytes, IEL and lamina propria mononuclear cells (LPMC). IL-15 was also determined in tissue samples from the small intestine of five patients with complicated CD (mean age 49.0 yrs, range 34-66). Two of these patients were affected by ulcerative jejunoileitis, one by refractory sprue and two by an enteropathy-associated T-cell lymphoma. In the mucosal samples of complicated CD patients the clonality of T-cell receptor \(\gamma\) chain (TCR-\(\gamma\)) gene was investigated by PCR amplification of TCR-\(\gamma\) using an adapted protocol with five different primers in two reactions, as described by McCarthy et al.\(^17\) Clinical and pathological features of complicated CD patients are reported in Table 1. Each subject gave informed consent to the study.
Table 1  
Clinical and pathological features of complicated coeliac patients

<table>
<thead>
<tr>
<th>Pt No</th>
<th>Sex</th>
<th>HLA status</th>
<th>Complication</th>
<th>Age at diagnosis of CD (y)</th>
<th>Age at diagnosis of complication (y)</th>
<th>Duration of GFD (y)</th>
<th>Marsh type lesion</th>
<th>Monoclonal TCR-γ gene rearrangement</th>
<th>Therapy</th>
<th>Outcome</th>
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<tr>
<td>1</td>
<td>F</td>
<td>DQ2</td>
<td>Ulcerative jejuno-ileitis</td>
<td>51</td>
<td>52</td>
<td>6</td>
<td>IIIb</td>
<td>No</td>
<td>Steroids</td>
<td>Alive</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>DQ2</td>
<td>Refractory sprue</td>
<td>27</td>
<td>33</td>
<td>8</td>
<td>IIIb</td>
<td>Yes</td>
<td>None</td>
<td>Alive</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>DQ2</td>
<td>Ulcerative jejuno-ileitis</td>
<td>32</td>
<td>38</td>
<td>8</td>
<td>IIIa</td>
<td>Yes</td>
<td>Steroids</td>
<td>Alive</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>DQ2</td>
<td>T-cell lymphoma</td>
<td>59</td>
<td>65</td>
<td>6</td>
<td>IIIb</td>
<td>Yes</td>
<td>AZA</td>
<td>Alive</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>DQ2</td>
<td>T-cell lymphoma</td>
<td>53</td>
<td>55</td>
<td>2</td>
<td>IIc</td>
<td>Yes</td>
<td>Steroids</td>
<td>Dead</td>
</tr>
</tbody>
</table>

AZA, azathioprine; CD, coeliac disease; GFD, gluten-free diet; TCR-γ, T-cell receptor γ chain

Cell isolation. Enterocytes, IEL and LPMC were isolated as previously described. Briefly, the epithelial layer was removed with 1 mM ethylenediamine-tetraacetic acid (EDTA, Sigma, St. Louis, MO) and 1 mM dithiothreitol (DTT, Sigma). After stirring for 1 h at 37°C, the single cell suspension was pelleted from the supernatant and IEL and enterocytes were then separated on a Percoll density gradient (Pharmacia, Uppsala, Sweden). A discontinuous density gradient (25%, 40% and 75%) was used. The cells obtained between the 40% and 75% fractions were collected as IEL, whereas the cells that were found between the 40% and 25% interface were collected as enterocytes, respectively. To release LPMC, the remaining tissue was treated with type 1A collagenase 128 U/ml (Sigma) for 2 h with stirring at 37°C. The crude cell suspension was allowed to stand for 5 min to permit the sedimentation of debris. Cells from the supernatant were washed twice with RPMI-1640 medium containing 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. Single cell suspensions were resuspended in 1 ml PBS and kept on ice until used. Cells were not used if viability did not exceed 90%. The purity of enterocyte and IEL populations, determined by staining cytocentrifuge preparations with anticytokeratin pan antibody (Boehringer Mannheim, Indianapolis, IN) at a dilution of 1:400, was found to exceed 93% and 90% respectively. The purity of the LPMC population was 90%, as assessed by flow-cytometric analysis with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD45 monoclonal antibody and its isotype control (FITC-conjugated IgG1) (Becton Dickinson, San Jose, CA).

Cell culture. IL-15 was measured by ELISA in the supernatants of freshly isolated enterocytes (1 x 10^5/ml) cultured for 24 h, and in the supernatants of freshly isolated LPMC (1 x 10^5/ml) cultured for 24 h in the presence or absence of either 5 µg/ml LPS or 1000 U/ml interferon (IFN)-γ (R&D Systems, Minneapolis, MN). IFN-γ and tumour necrosis factor (TNF)-α were measured by ELISA in the supernatants of IEL (1 x 10^5/ml) cultured for 24 h in the presence or absence of recombinant human IL-15 (Chemicon, Temecula, CA) at a concentration of 50 ng/ml, which has been shown to be optimal for lymphocyte activation. Flow cytometric FITC-Annexin V and propidium iodide (PI) staining was used to quantify apoptosis in IEL incubated for 18 h in the presence or absence of either IL-15 (1, 10 and 50 ng/ml) or 10 µg/ml IL-15 blocking antibody (R&D Systems) or an isotype-matched control antibody (mouse IgG1). Granzyme B concentrations in the supernatants of IEL cultured in the presence or absence of 50 ng/ml IL-15 or 10 µg/ml IL-15 blocking antibody or control IgG1 were determined by ELISA.

Proliferation assay. For the proliferation assay, 1 x 10^5 IEL were plated out in 96-well microtitre plates in a total volume of 0.2 ml per well in the presence of IL-15 (1, 10 or
100 ng/ml). After 72 h of culture, 1 µCi [³H] thymidine was added to each well for the last 8 h to determine DNA synthesis. IEL lysates were collected on a glass filter and radioactivity was measured. Values were calculated as stimulation index of mean cpm in the presence of IL-15 divided by the mean cpm of unstimulated cultures.

Cytotoxicity assay. A ⁵¹Cr release assay was performed using cells of the human enterocyte-like cell line Caco-2 as target. Caco-2 cells were labelled with 30 µCi of ⁵¹Cr (Amersham, Arlington Heights, IL) for 1 h, then cocultured with IEL at different effector target ratios (0.6, 1.2, 2.5 and 5) for 4 h in the presence or absence of either 50 ng/ml IL-15 or IL-15 blocking antibody (1, 5 and 10 µg/ml) or control IgG. In parallel experiments, IEL preincubated for 1 h with different concentrations (0, 10 and 50 µg/ml) of chloroquine (Sigma), an inhibitor of granzyme/perforin-dependent cytotoxicity, were cocultured with Caco-2 cells at the effector target ratio of 5. After incubation, 100 µl medium from each well of labelled cocultures were carefully removed and radioactivity measured in a gamma counter. The percentage of specific ⁵¹Cr release was calculated according to the following formula: 100 x (experimental release – spontaneous release)/(total content – spontaneous release), where total content was determined by lysing target cells with 1N HCl.

Evaluation of apoptosis by FITC-Annexin V and PI binding. Apoptosis was quantified using FITC-Annexin V and PI according to the manufacturer’s protocol (ApoDETECT FITC-Annexin V Kit, Zymed Laboratories, San Francisco, CA). Cells were stained with 5 µl of FITC-Annexin V diluted 1:10 in buffer, and 2.5 µl of PI. After incubation for 15 min, the cells were analyzed by flow cytometry with standard FACScan equipment (Becton Dickinson). The following controls were used to set up compensation and quadrants: unstained cells, cells stained with FITC-Annexin V alone, and cells stained with PI alone. Cells undergoing the early stage of apoptosis were stained with FITC-Annexin V alone (% of apoptosis). Cells permeabilized with 0.1% saponin were used as the control for PI-positive cells.

ELISA. IL-15, IFN-γ, TNF-α and granzyme B levels were measured by ELISA using respectively the Quantikine Human IL-15 Immunoassay (R&D Systems), the Human IFN-γ ELISA Kit (Chemicon), the Human TNF-α ELISA Kit (Chemicon) and the Pelikine Compact Human Granzyme B ELISA Kit (CLB, Amsterdam, The Netherlands) according to the manufacturers’ instructions. For the determination of IL-15 concentration in the mucosa, tissue samples were added to 0.3 ml of Tris-HCl 20 mmol/L pH 7.4, containing 1 µmol/L phenylmethylsulfonyl fluoride, 10 µmol/L leupeptin, 10 µmol/L pepstatin, 1 µmol/L aprotinin and homogenised using a hand-held-Potter-Elvehjem tissue grinder and by sonication (5W, 80% output, 1 min and 50 sec, alternating 10 sec sonication and 10 sec pause) with a sonicator (Vibracell, Sonic & Materials, Danbury, CT).

Immunoblotting. Cell lysates were prepared by lysing cells in ice-cold lysing buffer containing 20 mM pH 7.6 Tris-HCl, 150 mM sodium chloride, 1mM phenylmethylsulfonyl fluoride, 1% Triton-X, 1 mM EDTA, 2 mM sodium orthovanadate, 10 µg/ml pepstatin, 10 µg/ml leupeptin and 10 µg/ml aprotinin (Sigma). Protein concentrations in cell lysates were determined using the Pierce protein assay (Pierce, Rockford, IL). Equivalent amounts of protein (12 µg) were separated on 10-15% SDS-PAGE gels and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked with 5% non-fat milk, followed by incubation for 90 min at room temperature with mouse anti-IL-15 or anti-IL-15Rα antibodies (both from R&D Systems). Membranes were washed several times with TTBS and then incubated for 90 min at room temperature with appropriate HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). After extensive washing, the reaction was developed by enhanced chemiluminescent staining (Super Signal, Pierce). Each blot was stripped and analyzed for β-actin, as an internal loading control, using an anti-human β-actin antibody (Santa Cruz
Biotechnology). Bands were quantified by scanning densitometry using an LKB Ultrascan XL Laser Densitometer (Kodak Ltd., Hemel Hempstead, UK).

Mucosal morphometry. In order to quantify the degree of mucosal flattening in CD, the surface area to volume ratio was estimated in biopsy specimens using a Weibel graticule (Graticules Ltd, Tunbridge Wells, UK) inserted into the microscope eyepiece, according to the method of Dunnill and Whitehead as standardized by us. A record was made of the number of times the lines cut the mucosal surface (c) and the number of hits (h) -that is, end points of the lines falling on the lamina propria. At a constant magnification (×125) the length (l) of each line of the graticule was 1.7 x 10⁻² cm. The ratio c/lh was then calculated and regarded as a measure of surface area to volume ratio.

Statistical analysis. Data were analyzed in the GraphPad Prism statistical PC program (GraphPad Software, San Diego, CA) by means of the non-parametric Mann-Whitney U-test. One-way analysis of variance (ANOVA test) was used to define differences in the percentage of cytotoxic or apoptotic cells obtained in cultures treated with various concentrations of stimuli. A level of p<0.05 was considered statistically significant.

Results

IL-15 production by enterocytes. As shown in Figure 1A, IL-15 protein was significantly (p<0.0001) higher in enterocytes from untreated CD patients in comparison to treated CD patients and controls. No significant difference was found between treated CD patients and controls. Figure 1B shows that IL-15 was undetectable in the supernatants of enterocytes from treated CD patients and controls, but it was secreted by enterocytes from untreated CD patients (mean 19.3 ± 4.6 pg/ml).

IL-15Rα expression by IEL. We investigated by immunoblotting whether IEL expressed IL-15Rα required for IL-15-triggered signal transduction. Figure 2A shows significantly (p<0.001) lower IL-15Rα expression in IEL isolated from controls in comparison to untreated and treated CD patients, without any significant difference between untreated and treated CD patients.

Effect of IL-15 on the secretion of IFN-γ and TNF-α by IEL. To examine the role of IL-15 in regulating Th1 cytokine production by IEL, IFN-γ and TNF-α were measured in the supernatants of both unstimulated and IL-15-stimulated IEL. As shown in Figure 2B, IL-15 stimulation significantly (p<0.001) enhanced IFN-γ and TNF-α secretion by IEL in untreated CD patients (from mean 88.4 ± 10.7 to 758.1 ± 122.7 and from 12.8 ± 5.6 to 121.7 ± 34.2 pg/ml, respectively), in treated CD patients (from mean 10.4 ± 1.6 to 98.3 ± 36.2 and from 0 to 22.4 ± 7.3 pg/ml, respectively) and in controls (from mean 0 to 79.4 ± 22.8 and from 0 to 18.6 ± 6.9 pg/ml, respectively). In the supernatants of IL-15-stimulated IEL, IFN-γ and TNF-α levels were significantly (p<0.001) higher in untreated CD patients in comparison to treated CD patients and controls, while no significant difference was found between treated CD patients and controls.

ProLiferative responses of IEL to IL-15. Since IL-15 induces IEL proliferation, the proliferation-inducing effects of IL-15 on IEL from untreated and treated CD patients and controls were compared. As shown in Figure 2C, in untreated CD patients the proliferative stimulation index was raised significantly (p<0.0001) by incubation with increasing concentrations (1, 10 and 100 ng/ml) of IL-15. As for treated CD patients and controls, the proliferative stimulation index was increased by IL-15, but only after 100 ng/ml IL-15 did this difference reach a statistically significant level (p<0.001). At each IL-15 concentration, the proliferative stimulation index of IEL from untreated CD patients was significantly (p<0.001) higher than that of IEL from treated CD patients and controls.

Effect of IL-15 on the cytotoxicity of IEL against epithelial cells. To test whether exogenous IL-15 influenced the killing activity of IEL against epithelial cells, IEL isolated...
from untreated CD patients and controls were cocultured with Caco-2 cells in the presence or absence of 50 ng/ml IL-15, and a cytotoxicity assay was performed to determine the extent of target cell death (Figure 3A-B). Figure 3A shows that control IEL, which had no killing activity in the absence of exogenous IL-15, manifested a strong cytotoxicity against Caco-2 cells when cultured with IL-15. Additionally, the cytotoxicity of CD IEL was significantly (p<0.001) greater than that of control IEL, and was increased further by IL-15. At each effector target ratio, the killing activity of IL-15-stimulated IEL from both untreated CD patients and controls was significantly higher (p<0.001) than that observed when IEL were cultured in unstimulated conditions. Figure 3B shows that the cytotoxicity of IL-15-stimulated CD and control IEL and of unstimulated CD IEL was abrogated when cells were preincubated with chloroquine, an inhibitor of granzyme/perforin-dependent cytotoxicity.19

To investigate the role of endogenous IL-15 in modulating IEL cytotoxicity in CD, IEL isolated from untreated CD patients were cocultured with Caco-2 cells after treatment with IL-15 blocking antibody. As shown in Figure 3C, cytotoxicity against Caco-2 cells was reduced in a concentration-dependent manner when IEL were treated with increasing concentrations of IL-15 blocking antibody.

To further explore the role of perforin/granzyme molecules in the cytotoxic activity of IEL in CD, granzyme B was measured in the supernatants of IEL cultured with IL-15 or IL-15 blocking antibody. Figure 3D shows that control IEL secreted detectable amounts of granzyme B only when stimulated with IL-15 (43.4 ± 8.8 U/ml) but not when cultured in the presence of RPMI 1640 medium only. In untreated CD patients IL-15 stimulation significantly (p<0.001) enhanced the granzyme B release by IEL (from 32.1 ± 6.5 to 174.2 ± 24.1 U/ml). Additionally, neutralisation of IL-15 by culturing IEL from untreated CD patients with IL-15 blocking antibody significantly reduced (p<0.001) the release of granzyme B from 30.1 ± 5.9 to 7.4 ± 2.1 U/ml.

Effect of IL-15 on apoptosis of IEL. To investigate the role of IL-15 in regulating IEL apoptosis, control IEL were cultured with IL-15, while IEL from untreated CD patients were cultured with IL-15 blocking antibody or control IgG1. Apoptosis was analyzed by flow cytometry with FITC-Annexin V and PI staining. In Figure 4, representative results of control subjects (A-B) show that, in comparison to unstimulated IEL (A), IEL cultured in the presence of 50 ng/ml IL-15 (B) showed a lower percentage of apoptotic cells (FITC-Annexin V-positive/PI-negative). Additionally, representative results of untreated CD patients (C-D) show that in comparison to IEL cultured with IgG1 (C), IEL treated with 10 µg/ml IL-15 blocking antibody (D) showed a higher percentage of apoptosis. As shown in Figure 4E, the percentage of apoptotic control IEL (mean 32.1 ± 6.7%) was significantly reduced by incubation with increasing concentrations of 1, 10 and 50 ng/ml IL-15 to 19.3 ± 4.9% (p<0.01), 12.8 ± 3.2% (p<0.001) and 8.8 ± 1.9% (p<0.001), respectively. Consistent with this, blocking IL-15 significantly (p<0.001) enhanced the percentage of apoptosis of CD IEL from 4.2 ± 1.3% to 17.7 ± 3.9%.

IL-15 production by LPMC. As shown in Figure 5A, IL-15 protein was significantly (p<0.001) higher in LPMC from untreated CD patients in comparison to treated CD patients and controls, while no significant difference was found between treated CD patients and controls. Figure 5B shows that only LPMC from untreated CD patients released IL-15 in the absence of stimulation (20.8 ± 4.7 pg/ml). When LPMC were stimulated with LPS or IFN-γ, significantly (p<0.001) higher IL-15 levels were detected in the supernatants of untreated CD patients (mean 82.4 ± 15.1 and 68.3 ± 12.7 pg/ml, respectively) in comparison to treated CD patients (mean 17.8 ± 4.6 and 13.6 ± 3.9 pg/ml, respectively) and controls (mean 15.9 ± 3.7 and 14.1 ± 2.4 pg/ml, respectively). No significant difference was found between treated CD patients and controls.

IL-15 expression in the small intestinal mucosa. Figure 6 shows that the mucosal IL-15 concentration was significantly higher both in patients with uncomplicated untreated CD
(median 11.6 pg/ml, range 11.0-14.1) and in patients with complicated CD (median 12.6 pg/ml, range 11.3-16.1) than in patients with uncomplicated treated CD (median 7.7 pg/ml, range 4.0-11.5, p<0.001) and controls (median 6.5 pg/ml, range 3.9-10.6, p<0.001). There was no significant difference between uncomplicated untreated CD patients and complicated CD patients, and between treated CD patients and controls. In the complicated CD group, the two patients with enteropathy-associated T-cell lymphoma showed the highest IL-15 levels. Figure 7 shows that in uncomplicated untreated CD a significant inverse correlation ($r_s = -0.85; p<0.005$) between IL-15 mucosal levels and surface area to volume ratio was found.

Discussion
The results presented in the current study suggest the involvement of IL-15 in the pathogenesis of CD. Firstly, we confirmed that IL-15 is overexpressed in active CD mucosa, where its level correlated with the degree of mucosal damage according to morphometric analysis, and showed that enterocytes and LPMC represent the major source of this cytokine. Further, we found that IL-15 induces IEL to proliferate and release proinflammatory cytokines, promotes IEL perforin/granzyme-dependent cytotoxicity against epithelial cells, and inhibits IEL apoptosis. In addition, we have shown that the concentration of IL-15 in the mucosa is significantly higher in patients with complicated CD and in untreated CD patients in comparison to patients with treated CD and controls.

It has been suggested that macrophages and dendritic cells are the main producers of IL-15. However, recently attention has been focused on intestinal epithelial cells as source of this cytokine. Immunohistochemical studies have shown conflicting results concerning IL-15 expression by CD epithelium, which has been reported as either devoid of staining or intensely stained from surface to the bottom of the crypts. Our immunoblotting analysis on isolated enterocytes showed an increase of IL-15 protein in untreated CD in comparison to controls. In keeping with previous flow cytometric findings, enterocyte expression of IL-15 returned to normal level in treated CD. Interestingly, although IL-15 is reported to be a poorly secreted cytokine hardly detectable by ELISA, we were able to measure it in the supernatants of enterocytes from untreated CD patients cultured for 24 h. These findings contrast with those of Mention et al., who reported that IL-15, rather than being released, is retained in functional form on the surface of epithelial cells in active CD. However, in that study the IL-15 secretion was not tested directly on enterocytes isolated from untreated CD patients but on T84 intestinal epithelial cell line and on organ culture of intestinal biopsy specimens from treated CD patients incubated with gliadin. The very high affinity binding between IL-15 and IL-15R$\alpha$, the IL-15 receptor $\alpha$ chain that we found upregulated on CD IEL, might explain the apparent lack of secreted IL-15 in the organ culture supernatants of CD biopsies challenged with gliadin. Our data, together with the demonstration that Th1 cytokines, which are known to be overexpressed in active CD mucosa, are capable of inducing the activation of a specific protease that cleaves cell surface IL-15, support the hypothesis that IL-15 may be secreted by enterocytes in untreated CD. On this basis, and in agreement with findings on human peripheral blood monocytes, we suggest that, under physiological conditions, IL-15 exerts its biological effects mainly as a membrane-bound form at epithelial level. In contrast to this, in active CD IL-15 may be released by enterocytes, either as a consequence of Th1 cytokine-induced proteolytic shedding of the membrane-anchored form or upon cell death.

IL-15 is a potent activator of normal IEL, stimulating both their proliferation and IFN-$\gamma$ secretion. Our findings provide evidence that IEL from untreated CD patients are more susceptible to stimulation by IL-15 than those from treated CD patients and controls, showing in response to IL-15 treatment an increased proliferation and a higher release of
IFN-γ and TNF-α. In particular, of these two cytokines IFN-γ seems to be much more involved in the generation of gluten-driven mucosal damage in CD, as indicated by the efficacy of anti-IFN-γ antibodies in preventing villous atrophy.\textsuperscript{27}

In the normal intestine, where enterocytes are exposed to a wide array of pathogens, IL-15 has a role in enhancing IEL cytotoxicity and in modulating perforin/granzyme and NFG2D signaling pathways in order to maintain a healthy epithelium by eliminating infected cells.\textsuperscript{2,28,29} Additionally, IL-15 is known to induce the lymphokine-activated killer activity of human peripheral blood lymphocytes and the killing of murine intestinal epithelial cells by a perforin-dependent pathway.\textsuperscript{30,31} In a pathological condition like CD, characterized by an overexpression of IL-15 by enterocytes associated with upregulation of IL-15Rα on IEL, the promotion of perforin/granzyme-mediated killing might lead to epithelial cell damage and, then, villous atrophy. Since we previously reported a higher infiltration of perforin+ IEL in active CD, which significantly correlated with the degree of enterocyte apoptosis,\textsuperscript{32} we investigated \textit{in vitro} whether IL-15 was capable of activating the perforin/granzyme-dependent cytotoxicity provided by IEL against epithelial cells. Our results, in accordance with those reported in refractory sprue,\textsuperscript{16} show that in untreated CD the IL-15-induced killing action of IEL against epithelial cells is higher than in treated CD and controls. The fact that blocking perforin pathway with chloroquine, an inhibitor of granzyme/perforin-dependent cytotoxicity,\textsuperscript{19} completely suppressed the IEL cytotoxic activity against Caco-2 cells clearly indicates that this cytolytic mechanism is perforin-dependent. Consistent with this, blocking IL-15 reduced in a concentration-dependent manner the cytotoxic activity of untreated CD IEL against Caco-2 cells and significantly decreased the granzyme B release in IEL supernatants. Taken together, these findings are in keeping with those of Meresse et al.\textsuperscript{33} who showed that IL-15 is able to convert cytotoxic T lymphocytes into lymphokine-activated killer cells through the induction of a NKG2D signaling pathway both \textit{in vitro} and \textit{in vivo} in CD.

To our knowledge, this is the first study to explore the \textit{in vitro} LPMC secretion of IL-15 in CD. Previous immunohistochemical studies of IL-15 expression in CD lamina propria, both affected by the subjective quantification of immunostaining, produced discrepant results, showing either positivity of some scattered mononuclear cells or massive upregulation of LPMC expression.\textsuperscript{13,16} We observed a higher LPMC production of IL-15 in active CD, as demonstrated by immunoblotting and by culture of LPMC stimulated with LPS or IFN-γ. IL-15 protein, not measurable in the supernatants of unstimulated control and treated CD LPMC, was detectable in unstimulated LPMC from untreated CD patients, allowing us to infer that this cytokine is secreted in the lamina propria of active CD patients. Since IL-15 plays a crucial role in the maturation and activation of dendritic cells and macrophages,\textsuperscript{5} it is conceivable that in CD, in addition to its role in activating the innate response by promoting the TCR-independent activation and cytotoxicity of IEL, IL-15 might amplify the adaptive response to gluten by enhancing the ability of dendritic cells to present gliadin peptides to CD4+ T cells (Figure 8).

Activated T cells undergo apoptosis upon repeated stimulation. This activation-induced cell death is an important mechanism underlying T cell homeostasis after an immune response,\textsuperscript{34} particularly in the intestine where IEL activation through TCR by foreign and self antigens is likely to occur constantly.\textsuperscript{35} In the present study, we demonstrated that exogenous IL-15 prevents apoptosis of normal IEL, whilst decreased propensity of IEL to undergo apoptosis in active CD, shown in a previous study of ours,\textsuperscript{18} is reverted by blocking endogenous IL-15 with a neutralizing antibody. The antiapoptotic effect of IL-15 reported in this study is consistent with previous observations in which IL-15 promoted the survival of human peripheral blood B and T cells upon cross-linking of Fas or antigenic receptors,\textsuperscript{36} and of activated intestinal murine γδ IEL.\textsuperscript{37} The IL-15-induced resistance to apoptosis that we found in CD IEL is particular intriguing when one considers
that in refractory sprue, an established complication of CD characterized by an abnormal IEL population with a clonal rearrangement of the TCR-γ, IL-15 promotes the in vitro survival and expansion of clonal CD103+sCD3- IEL, which may ultimately transform a refractory form into an aggressive T-cell lymphoma. Accordingly, we found high IL-15 content in the intestinal mucosa of patients with complicated CD, four of them characterized by a clonal rearrangement of the TCR-γ.

In conclusion, our findings suggest that IL-15 plays a pivotal role in the development of CD. Overexpression of IL-15 in active CD may increase the number of inflammatory cells involved in the generation of epithelial damage by stimulating proliferation, Th1 cytokine production and cytotoxicity of IEL and by protecting these cells from apoptosis. These findings together with the recent demonstration by Maiuri et al. that the non-immunodominant peptide p31-43, but not the immunodominant epitopes, is able to induce rapid expression of IL-15 in tissues from patients with CD, strongly suggest that this cytokine is involved in modulating the innate immune response to gliadin in CD. Moreover, the pro-survival effect of IL-15 on IEL may predispose to the emergence of T-cell clonal proliferations in CD, thus promoting the transformation of low grade/"cryptic" enteropathy-associated T-cell lymphomas -refractory sprue and ulcerative jejuno-ileitis- into high grade lymphomas sharing the same clonality.

Dissecting the complex machinery of the IL-15/IL-15R system will provide interesting answers to the numerous questions raised by IL-15 involvement in the pathogenesis of CD and will open up new possibilities for the development of therapeutic strategies aimed at suppressing uncontrolled IEL activation and survival by blocking IL-15 or its receptor, thus preventing both tissue damage and lymphomagenesis that occur in CD.
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Competing Interest
None declared.
References


**Figure Legends**

Figure 1. IL-15 production by enterocytes. A. Expression of IL-15 and β-actin in enterocytes isolated from an untreated CD patient, a treated CD patient and a control subject. The example is representative of experiments performed in 10 subjects for each group. Bottom panel indicates densitometry of Western blots. Horizontal bars represent median value (*p<0.0001 vs treated CD patients and controls). B. IL-15 release by enterocytes from 10 untreated CD patients, 9 treated CD patients and 10 controls. Freshly isolated enterocytes were cultured for 24 h and IL-15 was measured by ELISA in the enterocyte supernatants. Results are mean (SD).

Figure 2. IL-15Rα expression by IEL and IL-15-driven Th1 cytokine production and proliferation of IEL. A. Expression of IL-15Rα and β-actin in IEL isolated from an untreated CD patient, a treated CD patient and a control subject. The example is representative of experiments performed in 10 subjects for each group. Lower panel shows densitometry of Western blots. Horizontal bars represent median value (*p<0.0001 vs untreated and treated CD patients). B. IFN-γ and TNF-α secretion by IEL from 9 untreated CD patients, 8 treated CD patients and 8 controls after IL-15 stimulation. Freshly isolated IEL were cultured for 24 h in the absence (white bars) or presence (black bars) of 50 ng/ml IL-15. IFN-γ and TNF-α levels were measured by ELISA in the cell culture supernatants. Results are mean (SD). C. Proliferation of IEL in response to IL-15. IEL from 8 untreated CD patients (square), 7 treated CD patients (triangle) and 7 controls (circle) were cultured in the presence of different concentrations of IL-15 (1, 10 and 100 ng/ml) for 72 h. The cultures were then pulsed with 1 μCi [3H] thymidine for the last 8 h and [3H] thymidine incorporation was measured. Data are expressed as stimulation index. Results are mean (SD).

Figure 3. Effects of IL-15 on perforin/granzyme-dependent cytotoxicity of IEL. A. Caco-2 target cells labelled with 51Cr were cocultured with IEL from 8 untreated CD patients (circle) and 8 control subjects (square) in the presence (black simbols) or absence (grey simbols) of 50 ng/ml IL-15 at various effector target ratios. B. IEL from 7 untreated CD patients (circle) and 7 controls (square) were pretreated with different concentrations of chloroquine, and then cocultured at an effector target ratio of 5 with Caco-2 target cells in the presence (black simbols) or absence (grey simbols) of 50 ng/ml IL-15. C. IEL from 7 untreated CD patients were cocultured at an effector target ratio of 5 with Caco-2 target cells in the presence of increasing concentrations of IL-15 blocking antibody or control IgG1. Cytotoxic activity against Caco-2 target cells was tested by 51Cr release assay. Results are mean (SD). D. Granzyme B release by IEL from 8 untreated CD patients (black bars) and 8 controls (white bars) cultured for 24 h in RPMI 1640 medium only or in the presence of 50 ng/ml IL-15 or 10 μg/ml IL-15 blocking antibody or control IgG1. Granzyme B was measured by ELISA in the cell culture supernatants. Results are mean (SD).

Figure 4. Effect of IL-15 on IEL apoptosis. IEL isolated from a control subject were cultured in the absence (A) or presence (B) of 50 ng/ml IL-15, while IEL isolated from an untreated CD patient were cultured with control IgG1 (C) or 10 μg/ml IL-15 blocking antibody (D). The percentage of apoptotic cells was assayed by flow cytometric analysis of fluorescein isothiocyanate-Annexin V and PI binding. The lower right quadrant represents apoptotic cells (FITC-Annexin V-positive/PI-negative). Numbers within the dot plots represent the percentages of apoptotic cells. Data are representative of experiments performed in 9 untreated CD patients and 8 controls. E. Percentage of apoptotic IEL from controls subjects cultured with increasing concentrations of IL-15 and blocking effect of 10 μg/ml IL-15 neutralizing antibody on CD IEL apoptosis. Results are mean (SD).

Figure 5. IL-15 production by LPMC. A. Expression of IL-15 and β-actin in LPMC isolated from an untreated CD patient, a treated CD patient and a control subject. The
example is representative of experiments performed in 10 subjects for each group. Bottom panel shows densitometry of Western blots. Horizontal bars represent median value (*p<0.001 vs treated CD patients and controls). B. LPMC isolated from 11 untreated CD patients, 10 treated CD patients and 9 controls were cultured for 24 h in the absence (white bars) or presence of 5 μg/ml LPS (grey bars) or 1000 U/ml IFN-γ (black bars). IL-15 was measured by ELISA in the cell culture supernatants. Results are mean (SD).

Figure 6. IL-15 levels in small intestinal mucosa of five patients with complicated CD, 10 uncomplicated untreated CD patients, 10 uncomplicated treated CD patients and 10 controls. Among the five patients with complicated CD, two were affected by ulcerative jejuno-ileitis (triangle), one by refractory sprue (square) and two by an enteropathy-associated T-cell lymphoma (open circle). IL-15 mucosal concentration was measured by ELISA. Horizontal bars represent median values.

Figure 7. Correlation between the degree of mucosal damage, morphometrically evaluated and expressed as surface area to volume ratio, and the IL-15 mucosal levels in uncomplicated untreated CD patients.

Figure 8. Possible model of the pathogenic role of IL-15 in modulating both innate (grey panel) and adaptive arms of the immune response in CD. APC, antigen presenting cell; DC, dendritic cell; IEL, intraepithelial lymphocyte; LPMC, lamina propria mononuclear cell; TCR, T-cell receptor.
Figure 1
Figure 2
Figure 3
Figure 4

- **A**: Control IEL
- **B**: Control IEL + IL-15 (1 ng/ml)
- **C**: Control IEL + medium
- **D**: Control IEL + IL-15 (10 ng/ml)
- **E**: Control IEL + IL-15 (50 ng/ml)
- **F**: Untreated CD IEL
- **G**: Untreated CD IEL + anti-IL-15 (10 µg/ml)

**Propidium iodide fluorescence**

**Annexin V fluorescence**

**% Apoptosis**
Figure 5
Figure 6

IL-15 mucosal levels (pg/ml)

Complicated CD  |  Untreated CD  |  Treated CD  |  Controls

0  |  4  |  8  |  12  |  16  |  20

Figure 6
Figure 7

Surface area to volume ratio

IL-15 mucosal levels (pg/ml)

$r_s = -0.85$
$p < 0.005$
Epithelium-derived interleukin-15 regulates intraepithelial lymphocyte Th1 cytokine production, cytotoxicity and survival in coeliac disease

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