Concomitant increase of IL-10 and pro-inflammatory cytokines in intraepithelial lymphocyte subsets in celiac disease

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Abstract

Celiac disease (CD) is a small intestinal enteropathy caused by permanent intolerance to wheat gluten. Active disease is characterized by a prominent cytokine response of intraepithelial lymphocytes (IELs) to gluten-containing diet with concomitant increase in expression of pro-inflammatory IFN-γ and down-regulatory IL-10 without increase in tumor necrosis factor-α (TNF-α) or transforming growth factor-β1 (TGF-β1). The aim was to understand the local immune reaction by determining which intraepithelial T cell subsets produce the different cytokines. The three major IEL-subsets γδIELs, CD4⁺αβIELs and CD8⁺αβIELs, as well as CD4⁺CD8⁺αβIELs, selectively expanded in active CD, were retrieved from small intestinal biopsies of children with active CD and controls and analyzed quantitatively for cytokine mRNA expression. In active CD, CD8⁺αβIELs showed a significant increase in expression levels of both IFN-γ and IL-10. CD8⁺αβIELs were also the IEL subset with highest expression level per cell of both cytokines and constituted the cellular source for almost all IFN-γ and most IL-10. Expression levels of both cytokines were higher in CD4⁺CD8⁺αβIELs than CD94⁺CD8⁺αβIELs. TNF-α levels were only increased in CD4⁺αβIELs, which also showed the highest expression level per cell and constituted the major source of this cytokine. Interestingly, IL-10 was increased also in CD4⁺αβIELs. Cytokine levels were low in γδIELs. ‘Classical’ CD94⁺CD8⁺αβ T cells within the epithelium are responsible for the excessive production of IFN-γ, believed to drive the formation of intestinal lesions in active CD. Production of IL-10 may be a common feature of IELs producing pro-inflammatory cytokines, thereby attempting to limit inflammation in an autocrine fashion.

Introduction

Celiac disease (CD) is a chronic inflammatory disease of the small intestine caused by inappropriate immune reactions to dietary prolamins in wheat, barley and rye, in particular wheat gliadin, in genetically susceptible individuals carrying the HLA-DQ2 and/or HLA-DQ8 alleles (1). In active disease, there is an intestinal lesion characterized by villous atrophy, crypt hyperplasia and increased numbers of leucocytes both within the epithelium and in the lamina propria. Clinical and histological improvements are seen upon withdrawal of gluten from the diet (2). A central role for T lymphocytes in CD has long been recognized by the observation that the frequency of activated CD4⁺T cells increases in lamina propria in active disease (3). Moreover, gliadin-specific, HLA-DQ2-restricted CD4⁺T cell clones and HLA-A2-restricted CD8⁺T cell clones have been isolated from the small intestinal mucosa of CD patients (4, 5). Profound changes in the frequency, composition and activation stage of intraepithelial lymphocytes (IELs) are a hallmark in CD. Small intestinal IELs are composed of multiple T lymphocyte subsets (6). The frequencies of both αβ- and γδIELs are increased in the small intestine of CD patients and αβIELs were shown to vary with disease activity. It is still not settled whether the frequency of γδIELs also varies with disease activity or is constantly elevated (7–9). Active CD is associated with a selective expansion of the otherwise rare NKT cell-like CD94⁺CD103⁺CD8⁺ IEL subtype (10). Two cytokines, pro-inflammatory IFN-γ and down-regulatory IL-10, are produced in significantly higher amounts in IELs of patients with active disease compared with both treated CD and controls (11). Both cytokine responses were more pronounced in IELs compared with T cells localized to the lamina propria and the frequency of IFN-γ-positive IELs was 10-fold higher.
in active CD as compared with controls. Active CD is also associated with IL-15 production by intestinal epithelial cells (IECs) (12). IL-15 in turn affects IELs (13–16). We have observed that bacteria are frequently associated with the epithelial surface of CD patients, but not with normal controls (17). Perhaps the combination of bacteria and gluten adversely affects IECs in CD patients promoting inflammation. These results all point towards important roles for IELs in the pathogenesis of CD.

To further our understanding of the role of IELs in the pathogenesis of the disease, the cellular sources of the different cytokines produced were investigated. To this end, we compared the cytokine mRNA expression levels in freshly isolated IEL subsets of children with newly diagnosed, active CD. Comparisons with expression levels in IEL subsets of controls with no food-intolerance were also performed.

**Methods**

**Patients and biopsy sampling**

Intestinal biopsies were collected from children admitted to the Department of Pediatrics at the University Hospital of Northern Sweden, Umeå, on suspicion of CD. One or two adjacent biopsies were collected from distal duodenum/proximal jejunum at the region of the ligament of Treitz using a Watson pediatric capsule. Part of one biopsy was used for routine pathology examination and grading by the Alexander score (18). The rest of the biopsy material was immediately placed in ice-chilled HEPES-buffered RPMI1640 containing antibiotics and used for cytokine mRNA analysis. The cell isolation procedure was started within 45 min. Patients were from two diagnostic groups, newly diagnosed CD patients with active disease and controls with no known food intolerance. CD patients were seven boys and five girls [3.2 (0.8–12.5) years (median and range)] showing elevated anti-endomysium antibodies of IgA isotype (EMA) and/or anti-gliadin antibodies of IgA isotype titers and a small intestinal mucosa with elevated numbers of immune cells and villous atrophy (Alexander 3 or 4). Controls were five boys and five girls [5.9 (1.7–16.5) years] with EMA titers below 1/20 and normal small intestinal histology (Alexander 1). Informed consent was obtained from the parents. The study was approved by the local Research Ethics Committee of the Medical Faculty, Umeå University, Umeå, Sweden.

**Cell isolation procedures**

IELs were isolated from jejunal biopsies by combination and modification of previously described procedures (11, 19, 20). Briefly, the biopsies were treated with dithiotreitol under vigorous shaking at room temperature. Freed cells, i.e. IELs and iECs, were collected by centrifugation and pretreated with paramagnetic beads (Dynabeads M-450 coated with goat-anti-mouse IgG, Dynal, Oslo, Norway) charged with anti-CD11b mAb (clone OKM1, American Type Culture Collection, Rockville, MD, USA) in order to remove possible sticky cells and contaminating cells of myeloid lineage. TCR-γδ+ cells (γδIELs) were thereafter retrieved by treatment with paramagnetic beads charged with a mixture of anti-δ-chain mAb clones Immun510 (Immunotech, Marseille, France) and 5A6.E9 (Serotec, Oxford, UK) and anti-γ-chain mAb γ3.20 (Serotec). CD4+IELs (TCR-γδ− CD4+ cells) were retrieved from the unbound TCR-γδ− cells by treatment with Dynabeads M-450 directly coated with anti-CD4 mAb (Dynal). Finally, CD8+IELs (TCR-γδ− CD4− CD8+ cells) were retrieved from the unbound TCR-γδ− CD4− cells by treatment with Dynabeads M-450 directly coated with anti-CD8 mAb (Dynal). In six samples from CD patients, TCR-γδ− CD4− cells were divided into CD94+ and CD94− cells by retrieval of cells binding to paramagnetic beads charged with anti-CD94 mAb (clone HP-3D9, BD PharMingen, San Diego, CA, USA) (CD94+CD8+IELs) prior to retrieval of cells binding to anti-CD8 charged beads (CD94−CD8+IELs). Bound cells were washed in RNase-free PBS and stored at −80°C until RNA extraction.

**RNA preparation and real-time quantitative reverse transcriptase–PCR**

Total RNA was extracted from positively selected IEL subpopulations by the acid guanidium thiocyanate–phenol–chloroform method and suspended in RNase-free water containing 1 kU/ml RNase inhibitor (Promega, Madison, WI, USA) as described (20). Levels of mRNA for IL-2, IL-10, IFN-γ, tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1) were determined by using real-time quantitative reverse transcriptase (qRT)–PCR assays with RNA copy standards previously constructed at the laboratory. These assays all utilize the TaqMan EZ technology ((PerkinElmer Biosystems, Foster City, CA, USA) in which the thermostable Thermus thermophilus DNA polymerase is used throughout the RT and PCR reactions and the 3′-primer is used for reverse transcription. Specific primer pairs are placed in different exons and used in combination with a 5′-fluorescent reporter dye-labeled internal probe hybridizing over the exon boundary in the mRNA sequence. For sequences of primers and probes and preparation of RNA copy standards, see (11, 21, 22). Emission from released reporter dye was monitored by an ABI Prism 7700 Sequence Detection System (PE Biosystems). Samples were analyzed in triplicate and mRNA concentrations determined from parallel RT–PCR analysis of serial dilutions of the total RNA extracted from activated PBMC. 18S rRNA was previously found to be the most stable housekeeping gene when comparing resting and activated T lymphocytes (23) and was therefore chosen for normalization. All samples were analyzed for their content of 18S rRNA by preparing cDNA using random hexamers followed by specific realtime qPCR according to the manufacturer’s instructions (PerkinElmer Applied Biosystems). 18S rRNA concentrations were expressed as arbitrary units per microliter as defined from a standard curve obtained by parallel reverse transcription and qPCR of triplicate serial dilutions of a pool of total RNA extracted from activated PBMC. One unit was defined as the signal obtained by 10 pg of this RNA pool and corresponded to ~100 lymphocytes (23). Results are given as cytokine mRNA copies per 18S rRNA unit as an estimation of the average cytokine mRNA content per 100 cells or as percentage of the total amount of a given cytokine mRNA species. Percentage was calculated as (amount of the cytokine mRNA in a given IEL subset retrieved from one biopsy/the...
amount of the cytokine mRNA in γδIELs + CD4*IELs + CD8*IELs from the same biopsy) × 100.

**Statistical analyses**

GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used for statistical analysis and graphic representation. One-way analysis of variance (ANOVA) with post hoc analysis using the Bonferroni multiple comparison test was used for statistical analysis of differences between IEL subtypes in cytokine mRNA expression levels. Statistical analysis of differences in cytokine mRNA expression levels of IEL subtypes of CD patients compared with controls was performed using Student’s t-test. Analyses of correlation between mRNA expression levels of different cytokines were performed using the Pearson correlation test. The variance of the groups was tested for equality by F-test prior to ANOVA, t-test and correlation analysis. Two-tailed analyses were used in the two latter methods. A P value <0.05 was considered statistically significant.

**Results**

We have previously shown that CD3*IELs of CD patients exhibit a significant increase in expression levels of both IFN-γ and IL-10 mRNAs, with a concomitant significant decrease of TNF-α and IL-2 mRNA levels compared with CD3*IELs of controls (11). Furthermore, there was a strong correlation between IFN-γ mRNA expression levels and number of IFN-γ-positive IELs as detected by immunoperoxidase staining. Moreover, IL-10-positive cells could also be detected within the epithelial compartment by using immunohistochemistry. These results raised the question whether the cellular source of IFN-γ and IL-10 is the same or whether IL-10 production is induced in a different subset. Therefore, freshly isolated IELs from CD patients with active disease were subdivided into γδIELs, CD4*IELs and CD8*IELs by sequential positive selection, and the expression levels of IFN-γ, IL-10, TNF-α, IL-2 and TGF-β1 mRNAs in the different subtypes were determined. For comparison, the same T cell subtypes from IELs of controls with no known food intolerance were also analyzed. For CD patients, the relative proportion of 18S rRNA was 52 ± 17% in the γδIELs, 9 ± 7% in the CD4*IELs and 39 ± 17% in the CD8*IELs.

**CD8* cells are the main producers of IFN-γ in IELs in active CD**

In CD patients, the average expression level of IFN-γ mRNA per cell was significantly higher in CD8*IELs than in both γδIELs and CD4*IELs (Fig. 1a and Table 1). CD8*IELs also contributed with most of the IFN-γ mRNA in each sample (78 ± 10%, mean ± SD), with γδIELs as the second important source (17 ± 11%) contributing significantly less than CD8*IELs but still significantly more than the CD4*IELs (4.7 ± 4.5%) (Fig. 1b). Although the expression levels of IFN-γ differed considerably between IEL subtypes in CD patients, all three subtypes showed a significant increase compared with controls and CD8*IELs reached as high mean expression level as 2544 copies per 18S rRNA unit corresponding to an average expression of ~25 copies per cell (Table 1).

**CD4* cells are the main producers of TNF-α in IELs in active CD**

CD4*IELs exhibited the highest expression levels of TNF-α mRNA and CD4*IELs also constituted the major source of this cytokine in most samples (50 ± 26%; Fig. 1c and d). In active CD, both CD4*IELs and CD8*IELs had significantly higher expression levels of TNF-α mRNA than γδIELs (Fig. 1c and Table 1). However, CD4*IELs were the only IEL subtype that exhibited an increased expression level of TNF-α mRNA compared with controls (Table 1). TNF-α mRNA expression was negligible in γδIELs of both CD patients and controls (Fig. 1c and d and Table 1).

**CD8* cells are the main producers of IL-10 in IELs in active CD**

Active CD also induced drastic changes in expression levels of the down-regulatory cytokine TGF-β1 in all three IEL subtypes (Table 1). This was most marked in CD8*IELs and γδIELs, i.e. from a mean of a few mRNA copies per 18S rRNA unit in controls up to ~100 copies per 18S rRNA unit (Table 1). Although the expression levels of IL-10 mRNA were similar in all three IEL subsets in active CD (Fig. 1e and Table 1), CD8*IELs were the main contributors (62 ± 15%) followed by γδIELs (23 ± 14%) and CD4*IELs (15 ± 14%) (Fig. 1f).

**TGF-β1 mRNA is expressed at high levels in IELs of both CD patients and controls**

The down-regulatory cytokine TGF-β1 exhibited a quite different expression pattern compared with IL-10. TGF-β1 mRNA levels were only moderately increased in IEL subsets of CD patients compared with controls and only for CD4*IELs did this increase reach statistical significance (Fig. 1g and Table 1). Still CD8*IELs contributed with significantly more TGF-β1 mRNA than CD4*IELs and γδIELs (Fig. 1h), which is explained by their relatively high expression level and large proportion of the IELs. TGF-β1 mRNA levels were high compared with the other four cytokines analyzed in both CD patients and controls and its mRNA levels were ~30 times higher than those of IL-10 in CD4*IELs of both groups (Fig. 1g and Table 1).

**Active CD does not increase IL-2 mRNA expression levels in any IEL subtype**

The expression levels of IL-2 mRNA were generally low and several samples from patients with active CD did not have detectable amounts of IL-2 mRNA (7/12, 6/12 and 2/12 for γδIEL, CD4*IEL and CD8*IEL samples, respectively). Expression levels of IL-2 were markedly lower in γδIELs compared with CD4*IELs and CD8*IELs in both CD patients and controls (Table 1).

**Classical CD8*IELs significantly contribute to the elevated levels of both IFN-γ and IL-10 mRNA in active CD**

The CD94*CD8* subset of IELs is expanded in active CD (10). We argued that these cells might constitute the cellular source of the increased amounts of IFN-γ and/or IL-10 mRNAs expressed in active CD. CD94*CD8*IELs of patients with active CD were therefore collected by retrieving CD94*
Cytokines in IEL subsets in celiac disease

(a) IFN-γ

(b) TNF-α

(c) IL-10

(d) TGF-β1

Each graph shows the cytokine mRNA expression levels in IEL subsets (γIELs, CD4+IELs, CD8+IELs) with statistical significance indicated by p-values.
cells from IEL samples depleted of γδIELs and CD4⁺IELs. We subsequently retrieved CD94⁺CD8⁺IELs from the unbound cell fraction. Equal numbers of cells were obtained in the two fractions as estimated by the amount of 18S rRNA (49.5 ± 19% and 50.5 ± 19% in the two fractions, respectively; n = 6).

Contrary to our expectation, the expression levels of IFN-γ mRNA were higher in the CD94⁺CD8⁺IELs in five of the six patients analyzed and the average expression level in CD94⁺CD8⁺IELs was 3.4-fold higher than in the CD4⁺CD8⁺IELs (Fig. 2a). Similarly, the expression levels of IL-10 were higher in most CD94⁺CD8⁺IEL samples than in the corresponding CD94⁺CD8⁺IEL samples (four/six samples) and the average expression level was 5.2-fold higher in CD94⁺CD8⁺IELs (Fig. 2b). The results for TNF-α and TGF-β1 mRNAs showed the same tendency (data not shown).

IL-10 correlates with different cytokines in different IEL subsets

The possibility that active CD causes changes in production of several cytokines in parallel in a particular IEL subtype was addressed by performing correlation analysis of expression levels of mRNA for IFN-γ, TNF-α, IL-10 and TGF-β1 in the three IEL subtypes separately.

Interestingly, these analyses showed several strong positive correlations between pro-inflammatory and down-regulatory cytokines and suggest that (i) γδIELs comprise two subpopulations expressing IL-10 and TGF-β1 (Table 2 and Fig. 3a) and one expressing IFN-γ and TNF-α (Table 2), (ii) CD4⁺IELs appear to comprise one population simultaneously expressing TNF-α, IL-10 and TGF-β1 (Table 2 and Fig. 3b), (iii) CD8⁺IELs finally seem to comprise two cytokine producing populations, one expressing IFN-γ and IL-10 (Table 2 and Fig. 3c) and one expressing TNF-α and TGF-β1 (Table 2).

Discussion

The most striking result from this study is that IFN-γ, the hallmark cytokine of active CD, is to a large extent produced by CD8⁺IELs. The average IFN-γ mRNA content per cell in fact exceeded that of polyclonally activated blood T lymphocytes (23). Previously we showed that ~60% of all IFN-γ mRNA expressed in small intestinal T cells of the patient group analyzed here, i.e. children with newly diagnosed, active CD, is expressed by IELs (11). Here we found that on average, 80% of the IFN-γ mRNA in IELs is expressed by CD8⁺IELs, implying that approximately half of all IFN-γ is derived from CD8⁺ T cells in the epithelium underscoring the importance of the epithelial reaction in the disease. These results are in accordance with those of Olaussen et al. (24) who reported that the majority of IELs that stain positively for intracellular IFN-γ expresses CD8. The nominal antigen for the CD8⁺IELs is not yet determined. However, short-term cultures of MHC class I-restricted, γ-glutamin-specific CD8⁺ cells have been established from small intestinal biopsies of CD patients (5). Interestingly, these cells produced IFN-γ upon in vitro challenge with gliadin peptides. Non-specific mechanisms may also be operating in the celiac lesion. A gliadin peptide that apparently is not a T cell epitope can induce rapid expression of IL-15 in the intestinal mucosa of CD patients (25) and recent studies suggest that IL-15 can stimulate IELs to

![Fig. 1.](https://academic.oup.com/intimm/article-abstract/19/8/993/709672) IFN-γ (a and b), TNF-α (c and d), IL-10 (e and f) and TGF-β1 (g and h) mRNA expression levels in γδIELs, CD4⁺IELs and CD8⁺IELs freshly isolated from small intestinal biopsies of children with active CD (n = 12). Cytokine mRNA and 18S rRNA contents in the samples were determined by real-time qRT-PCR. Results are given either as average expression level calculated as the ratio between the content of a given cytokine mRNA species and the 18S rRNA content in each sample (a, c, e and g) or as the relative contribution by γδIELs, CD4⁺IELs and CD8⁺IELs, respectively. The filled circles in (a, c, e and g) represent the values from single samples and horizontal bars indicate the medians. Whiskers in (b, d, f and h) indicate the ranges, boxes indicate the 25th to 75th percentiles and horizontal bars inside boxes indicate the medians. Statistically significant differences are indicated.

### Table 1. Cytokine mRNA expression levels in subsets of IELs freshly isolated from small intestinal mucosa of CD patients with active disease and controls with no food intolerance

<table>
<thead>
<tr>
<th>Cytokine mRNA</th>
<th>γδIELs</th>
<th>CD4⁺IELs</th>
<th>CD8⁺IELs</th>
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<tbody>
<tr>
<td>CD8⁺IELs</td>
<td>Controls</td>
<td>CD</td>
<td>Fold difference</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>6.6 ± 5.4</td>
<td>320 ± 86</td>
<td>48</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5.2 ± 2.6</td>
<td>6.9 ± 5.6</td>
<td>1.3</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.9 ± 0.2</td>
<td>80 ± 33</td>
<td>84</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>129 ± 50</td>
<td>997 ± 423</td>
<td>7.7</td>
</tr>
<tr>
<td>IL-2</td>
<td>4.1 ± 1.6</td>
<td>2.3 ± 1.1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*Freshly isolated subtypes of IEL retrieved by sequential positive selection using magnetic beads charged with anti-γδ-TCR mAbs (γδIELs), followed by magnetic beads charged with anti-CD4 mAb (CD4⁺IELs) and finally magnetic beads charged with anti-CD8 mAb (CD8⁺IELs).

Expression levels of indicated cytokine mRNA species expressed as mean ± 1 SEM mRNA copies per 18S rRNA unit. Numbers of samples analyzed are 10 for controls and 12 for CD patients.

Fold difference calculated as mean expression level in CD patients over mean expression level in controls.

*P values indicate statistically significant differences between cytokine mRNA expression levels in CD patients compared with controls as determined by two-tailed, unpaired Student’s t-test. NS = not significant.
cytotoxicity by mechanisms that override restriction by TCR recognition (16, 26). The frequency of CD8+ IELs expressing the NK-receptor CD94 is increased in active CD (10) and IL-15 was also reported to induce CD94 expression and IFN-γ production by IELs (14). However, from the present study, it became evident that classical CD8+ IELs contribute most of the IFN-γ production in active CD with CD94+CD8+ IELs also contributing significantly. Thus, gluten intake might cause parallel adaptive and innate reactions, both involving IFN-γ production by CD8+ IELs in CD patients or there might be a gradual transition from specific to TCR-unrestricted activation of CD8+ IELs.

CD4+ IELs were the only subtype that showed increased expression levels of TNF-α in active CD and, hence, the little TNF-α mRNA expressed was derived mainly from these cells. Although the contribution of CD4+ IELs to the IFN-γ production was minimal (≤5%), their expression level of IFN-γ per cell was elevated compared with controls, suggesting an ongoing, weak Th1 response in addition to the massive IFN-γ response by CD8+ IELs. A small population of CD4+ IELs with a Th1 cytokine profile is normally present in the small intestinal mucosa of man (6, 27). Their function is not yet settled but it is conceivable that they regulate immune responses within the epithelial compartment and they could have a role in initiating the anti-gluten reactivity of CD8+ IELs. Whether these cells are gluten specific in CD patients is not known but HLA-DQ is expressed on a small fraction of the iECs of small intestine (6) and IFN-γ can up-regulate the HLA-DQ expression in intestinal epithelial cell lines (28) giving the opportunity for presentation of immunogenic gliadin T cell epitopes on iECs in individuals carrying the predisposing HLA-DQ alleles.

The expression levels of pro-inflammatory cytokines, particularly TNF-α, were low in CD IELs compared with the other two IEL subsets. This is consistent with the results of Léon et al. (29) who reported that the cytokine response to in vitro stimulation is stronger in CD IELs compared with γδ IELs and that in vitro stimulated γδ IELs of CD patients retain their Th1 profile.

One very intriguing finding from the present study is the fact that active CD was associated with significantly elevated expression levels of the down-regulatory cytokine IL-10 in all IEL subsets.

**Table 2.** Correlations between cytokine mRNA expression levels in subsets of IELs freshly isolated from small intestinal mucosa of CD patients with active disease

<table>
<thead>
<tr>
<th></th>
<th>γδ IELs</th>
<th>CD4+ IELs</th>
<th>CD8+ IELs</th>
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<tr>
<td>IFN-γ versus IL-10⁺</td>
<td>0.4</td>
<td>-0.5</td>
<td>0.73</td>
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<tr>
<td>IFN-γ versus TNF-α</td>
<td>0.88</td>
<td>-0.1</td>
<td>-0.4</td>
</tr>
<tr>
<td>IFN-γ versus TGF-β1</td>
<td>0.3</td>
<td>-0.2</td>
<td>-0.1</td>
</tr>
<tr>
<td>IL-10 versus TNF-α</td>
<td>-0.2</td>
<td>0.95</td>
<td>0.3</td>
</tr>
<tr>
<td>IL-10 versus TGF-β1</td>
<td>0.96</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TNF-α versus TGF-β1</td>
<td>-0.30</td>
<td>0.73</td>
<td>0.73</td>
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⁺Freshly isolated subtypes of IEL retrieved by sequential positive selection using magnetic beads charged with anti-γδ-TCR mAbs (γδ IELs), followed by magnetic beads charged with anti-CD4 mAb (CD4+ IELs) and finally magnetic beads charged with anti-CD8 mAb (CD8+ IELs).

r and P values obtained by correlation analyses between indicated cytokine mRNA species as determined by two-tailed Pearson correlation test. NS = not significant.
three major IEL subsets analyzed. Expression levels of IL-10 correlated with the ‘marker cytokine’ of the respective IEL subtype. In the case of CD8⁺IELs, there was a significant correlation between expression levels of the IL-10 and IFN-γ mRNAs. We interpret high degree of correlation between expression levels of two cytokines in the subfractionated IEL populations to mean that the same cell produces the cytokines. With this assumption, our data indicate that IEL contains at least five functionally different T cell populations (Fig. 4).

In active CD, four of these are activated and/or expanded. Only one example of CD8⁺ T cells that produce both IFN-γ and IL-10 has previously been reported, namely, human small intestinal IELs after prolonged stimulation with IL-15 in vitro (14). In the case of CD4⁺IELs, the IL-10 mRNA levels correlated to TNF-α mRNA levels. We are not aware of any report of CD4⁺ T cells simultaneously secreting these two cytokines. In γδIELs finally, IL-10 levels correlated with TGF-β1 levels, a common combination in regulatory cells. Several types of regulatory T cells exert their function in the intestinal mucosa by secretion of IL-10 (30) and addition of IL-10 significantly inhibited responses to gliadin challenge in organ cultures of intestinal mucosa of CD patients (31). Thus, the expression of IL-10 in the IEL subsets is indeed suggestive of a regulatory function and an attempt to counteract the pro-inflammatory features in the mucosal gluten response apparently in an autocrine fashion. Several types of regulatory T cells described to prohibit or attenuate gut inflammation in mouse models are CD4⁺ (30). γδIELs have, however, also been shown to have immunoregulatory function through secretion of IL-10 (reviewed in ref. 32) and co-culture with iECs induced a regulatory function in CD8⁺ T cells suggesting that CD8⁺ suppressive IELs can be generated during activation locally within the epithelium (33). We have previously shown that freshly isolated lymphocytes of both normal and inflamed intestine have the capacity to kill the T cell line Jurkat by a Fas ligand/Fas-mediated TCR-independent mechanism (34, 35) suggesting ongoing activation-induced cell death (AICD). Recently Ellen Ebert (14) showed that IL-10 promotes this Fas ligand-mediated cytotoxicity. Thus, it is likely that local immune reactions in the small intestinal mucosa normally are regulated by a delayed onset of IL-10 production in the activated IEL that shuts down the immune reaction by killing of activated IELs by AICD and that the IL-10 production seen in active CD is a reflection of an AICD that is not sufficient strong to turn off the inflammatory process. To be able to increase the IL-10 production or to administer IL-10 locally may prove to be a useful future therapy by which one might dampen the inflammation and at the same time reduce the number of gluten-reactive T cells by promotion of AICD. This in turn may yield better tolerance to gluten-containing diet.

Taken together, the results from the present study point to a pronounced epithelial reaction in active CD with all three major IEL subsets activated. Apparently, both CD8⁺IELs and CD4⁺IELs contain cell populations that simultaneously secrete a pro-inflammatory cytokine, IFN-γ and TNF-α, respectively, and a down-regulatory cytokine, IL-10, while the γδIELs seem to have a more conventional regulatory role with simultaneous production of IL-10 and TGF-β1. The strong inflammatory reaction, most pronounced in the CD8⁺IELs, is compatible with the notion that gluten is mistaken for a pathogen at the epithelial surface in CD patients yielding innate and adaptive immune responses perhaps triggered or enhanced by bacterial adhesion to the epithelium (17).
Fig. 4. Schematic drawing depicting the cytokine profile of the five functionally different IEL subpopulations suggested by the results in the present study. Filled circle indicates that the cell type is activated and/or expanded in active CD. Arrow indicates production of the indicated cytokine. Thick arrow indicates prominent production.

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**Abbreviations**

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<th>Abbreviation</th>
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<tr>
<td>AICD</td>
<td>activation-induced cell death</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>CD</td>
<td>celiac disease</td>
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<tr>
<td>EMA</td>
<td>anti-endomysium antibodies of IgA isotype</td>
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<tr>
<td>iEC</td>
<td>intestinal epithelial cell</td>
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<tr>
<td>IEL</td>
<td>intraepithelial lymphocyte</td>
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<tr>
<td>qRT</td>
<td>quantitative reverse transcriptase</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor</td>
</tr>
</tbody>
</table>

**References**

14. Ebert, E. C. 2005. IL-15 converts human intestinal intraepithelial lymphocytes to CD94 producers of IFN-gamma and IL-10, the latter promoting Fas ligand-mediated cytotoxicity. *Immunology* 115:118.


