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Cytokine Tissue Levels as Markers of Disease Activity in Pediatric Crohn Disease

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ABSTRACT

The mucosal immune system is overactivated in Crohn disease (CD) and viral infections have been associated with clinical exacerbations. To investigate the potential association between mucosal inflammation and the cytokines involved in the early response to viruses, we analyzed colonic tissue levels of IL-2Rα, interferon-α, and IL-15 in CD. Patients undergoing diagnostic colonoscopy were classified into controls ($n = 22$) and three CD groups based on the histologic severity of inflammation and clinical activity: a) severely active CD ($n = 3$); b) mild to moderately active CD ($n = 14$); and c) quiescent CD ($n = 23$). Rectal biopsies (two per patient) were homogenized and cytokine levels determined by ELISA kits. Statistical analysis was performed by ANOVA with Tukey and Scheffé tests. IL-2Rα levels were increased in the active CD group compared with the quiescent CD group: a) 405 $\pm$ 87, b) 159 $\pm$ 31, and c) 33 $\pm$ 15 pg/mg DNA ($p < 0.001$). The latter group was similar to controls (39 $\pm$ 20 pg/mg DNA). Furthermore, a linear correlation ($r = 0.98$) between IL-2Rα and disease activity (Van Hees index) was observed. IL-15 levels were also higher in active compared with quiescent CD and controls: a) 0.69 $\pm$ 0.23 and b) 0.72 $\pm$ 0.31 *versus* c) 0.28 $\pm$ 0.21 and 0.28 $\pm$ 0.14 pg/mg DNA for controls ($p < 0.05$). Interferon-α levels were undetectable in all samples. Our data suggest that IL-2Rα tissue levels correlate with CD activity. IL-15 is also overproduced in inflamed CD tissue. The lack of a parallel elevation of interferon-α does not support a role for viral induction of IL-15 in inflamed CD samples. **(*Pediatr Res* 54: 456–461, 2003)**

Abbreviations

CD, Crohn disease
IL-2Rα, IL-2 receptor α-chain
IBD, inflammatory bowel disease
IFN-α, interferon-α

IBD, encompassing ulcerative colitis (UC) and CD, are chronic idiopathic disorders in which hereditary and environmental factors interact to produce disease (1). The state of activation of the mucosal immune system is augmented and mediates the mucosal inflammatory response (2).

The IL-2Rα is well established as a marker of *in vivo* immune activation (3). IL-2Rα is primarily expressed by activated T cells and macrophages in intestinal lesions of IBD (4). Circulating IL-2Rα levels are significantly elevated in UC and CD patients with active disease; decreases are parallel to clinical improvement, and increases have been reported to predict CD relapses (5–11). IL-2Rα levels were particularly high in the most severely inflamed tissue homogenates from IBD colonic biopsies compared with less inflamed and control tissue (6, 12, 13). However, no significant correlations were observed with clinical activity indices (12).

IL-15 is a pro-inflammatory cytokine that shares many immunologic properties with IL-2 (14). IL-15 mRNA is expressed in a wide variety of tissues and cells, but its protein is mainly produced by monocytes and macrophages (15–19). Increased IL-15 levels have been reported in peripheral blood mononuclear cells (PBMC) in patients with moderate and severely active UC (20). Also, macrophage production of IL-15 in inflamed mucosal specimens from IBD patients is higher than in control and noninflamed tissues (21). Increased protein expression of IL-15 has also been reported in organ culture supernatants of rectal biopsies from IBD patients (22). However, IL-15 was undetectable in colonic mucosa biopsies from severely inflamed tissue in another

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study (23). Thus, the role of IL-15 in the pathogenesis of IBD remains uncertain.

IL-15 is considered a pivotal antiviral cytokine in the clearance of established viral infections (24, 25), along with other rapidly secreted cytokines, such as type I interferons (IFN-α and IFN-β) (26, 27). IL-15 shares common transcriptional control regions with the IFN regulatory factor (IRF-E) and IFN-2α (28). Viral infections, particularly of the upper respiratory tract, have been proposed as initiators of IBD relapses in children (29). Several studies have reported the detection of cytomegalovirus, Epstein-Barr virus, and human herpes virus 6 in IBD tissue samples (30–33). Although evidence for measles virus infection in CD has also been reported (34), this has not been confirmed by other investigators (35).

To explore the potential correlation between intestinal inflammation and early antiviral cytokines, we analyzed tissue levels of IL-2Rα, IFN-α, and IL-15 in CD colonic mucosa. We found that IL-2Rα and IL-15 tissue levels are overexpressed in inflamed CD tissue. Nevertheless, tissue IFN-α levels were undetectable, suggesting that IL-15 induction in inflamed CD tissue occurs independently of viral infections.

MATERIALS AND METHODS

Patient selection and tissue procurement. Colonic specimens were obtained from consenting patients at the time of colonoscopy for evaluation of gastrointestinal symptoms. Forty pediatric CD patients and 22 controls were included in this study. The average ages for these groups were 13 and 10 y, with a range of 9–20 y and 4–17 y, respectively. Forty percent of the CD patients were male, compared with 54% of the controls. Disease duration for the CD group was 2.2 y on average, with a range of 0–9 y. The diagnosis of CD was made on the basis of established standard clinical, bacteriological, radiologic, endoscopic, and histopathological criteria. Some of the patients were receiving various standard medications at the time of study, including 5-aminosalicylic acid, corticosteroids, and 6-mercaptopurine. Controls included patients undergoing colonoscopy for polyp removal or for the investigation of lower gastrointestinal bleeding. All had normal radiologic, bacteriological, and histopathological results, with the exception of juvenile polyps, when present. An average of eight colonic biopsies were taken for histopathological analysis. Six additional rectal biopsies were obtained for the purposes of the research study. The later specimens were taken from macroscopically diseased areas of the rectal mucosa. If no macroscopic lesions were visible, the rectal specimens were taken randomly. One of these was fixed in formalin for histologic assessment and the five others were promptly transported on ice in Leibovitz L-15 medium (Invitrogen, Carlsbad, CA, U.S.A.) to the laboratory.

Assessment of disease activity. Disease activity for the CD group was assessed using two standard clinical indices: those of Harvey and Bradshaw (36) and Van Hees (37). The severity of inflammation of the eight colonic and of the rectal biopsies taken from each patient was evaluated histologically by light microscopy using 10 and 40 power fields. All slides were scored by an experienced pathologist, who was blinded to the clinical diagnosis. Six histopathological features were assessed: acute inflammatory cell infiltration, the presence of crypt abscesses, mucin depletion, surface epithelial integrity, chronic inflammatory cell infiltration, and crypt architectural irregularities (38, 39). Each feature was graded on a 4-point scale, corresponding to normal (0), mild (1), moderate (2), and severe (3). The final score was the numerical sum of all the partial scores, yielding four categories: normal with scores from 0 to 3, mild from 4 to 8, moderate from 9 to 14, and severe from 15 to 18.

Signed informed consent was obtained from all the patients or their legal guardian. The study protocol and consent form were approved by the Institutional Review Board's Ethics Committee of the Sainte-Justine Research Center before initiation of the study.

Biopsy homogenization. Rectal biopsies (four to five per patient) were blotted dry, weighed (range, 6–29 mg), and then frozen at −80°C until used for assay. Two biopsy samples were placed in glass tubes containing 40 μL of protease inhibitor cocktail (Complete Protease Inhibitor Cocktail Tablets, Roche Diagnostics, Mannheim, Germany), 1 mL of M-PER mammalian protein extraction reagent (Pierce Chemical, Rockford, IL, U.S.A.) and homogenized for 30 s in an ice-cold water bath at 22,000 rpm with an IKA ULTRA-TURRAX T25 homogenizer (IKA Works, Inc., Wilmington, NC, U.S.A.). The homogenates were then centrifuged at 13,000 rpm at 4°C in a microfuge for 15 min, and the supernatants were then transferred to fresh tubes and frozen at −80°C until assay.

DNA quantitation. Cytokine levels in tissue homogenates were expressed as picogram of protein per milligram of total tissue DNA. Total DNA was determined in triplicate, by fluorometry using a PicoGreen dsDNA quantitation kit (Molecular Probes, Eugene, OR, U.S.A.). The assays were performed in accordance with the manufacturer's instructions. In brief, 5 μL of homogenate supernatant was assayed in 2 mL of kit solution. After 20 and 40 min of reaction, cuvettes were excited at 480 nm and fluorescence emission intensity was measured at 520 nm using a spectrofluorometer. Concentrations in the samples were determined by interpolation from standard curves, which were run concurrently with each assay. The detection limit of the assay was 25 pg/mL dsDNA.

Cytokine assays. The concentrations of IL-15, IL-2Rα, and IFN-α were determined in triplicate by ELISA, using commercially available kits (R & D Systems, Minneapolis, MN, U.S.A.), in accordance with the manufacturer's instructions. Results were read by colorimetry on an automatic 96-well plate reader at a wavelength of 450 nm for IL-2Rα and IFN-α. Samples were not diluted for IFN-α assay. For IL-15, results were read by luminometry on an automatic 96-well plate reader. Concentrations in the samples were determined by interpolation from standard curves, which were run concurrently in each plate. The detection limit of the assays was 10 pg/mL for IFN-α and IL-2Rα, and 0.12 pg/mL for IL-15.

Statistical analysis. Data are presented as means ± SD. Statistical analysis was performed using a one-way ANOVA with Tukey and Scheffé tests to determine significance for multiple comparisons. The Kruskal-Wallis test with Bonferroni post hoc test was used for semiquantitative nonparametric data (Harvey Bradshaw index and pathologic scores). A p value of

< 0.05 was considered statistically significant. Statistical analyses were performed using the SPSS 10.0.5 computer program (SPSS Inc., Chicago, IL, U.S.A.).

RESULTS

Clinical and pathologic profile of CD patients. The CD patients were divided into three groups, according to clinical and histopathological disease severity (Table 1). Group A was comprised of cases with severe, active disease profiles. All had pancolitis, with a mean Harvey Bradshaw index of 12 (active = 5–13), and a Van Hees index of 232 (severe = 210–300). The colonic pathologic score was 10 (moderate) and rectal pathologic score 13 (moderate). Group B had a mild to moderate disease profile, with a mean Harvey Bradshaw index of 4 (inactive = 0–4), Van Hees index of 198 (moderate = 150–210), pathologic score of 5 (mild), and rectal pathologic score of 2 (normal). Group C had quiescent disease, with a mean Harvey Bradshaw index of 4, Van Hees index of 163, pathologic score of 6 (mild), and rectal pathologic score of 2 (normal). Significant statistical differences between group A and the other CD groups were observed for the Harvey Bradshaw index and rectal histopathological scores (Table 1).

The distribution of patients receiving drug therapy in each CD group was also different. In groups A and B, 33% and 36% of the patients were receiving medications for their CD at the time of the study, compared with the 78% of group C ($p < 0.05$ *versus* group B). This difference is related to the fact that the 67% of patients in group A and the 57% in group B were newly diagnosed, hence untreated, compared with only 39% in group C. Among the cases in group C, the 69% were receiving prednisone or 5-aminosalicylic acid, compared with only 33% in group A and 14% in group B. In contrast, all the patients on therapy in group A and the 80% of those in group B were receiving 6-mercaptopurine, compared with the 22% of patients on therapy in group C.

Tissue DNA determination. The results of rectal biopsy DNA content are shown in Figure 1. In CD groups A and B, the mean values were 0.10 ± 0.03 and 0.09 ± 0.04 mg/mg tissue, respectively. In group C, the mean value was 0.19 ± 0.12 mg/mg tissue, whereas the control group's mean value was 0.12 ± 0.06 mg/mg tissue. No statistical differences were noted between the four groups.

Tissue cytokine levels. The rectal mucosal cytokine profiles in the three CD and control groups are illustrated in Figure 2, *A* and *B*. IL-2Rα mean values were significantly increased in all active CD groups (A and B), with the highest levels seen in the most severely affected Group A (405 ± 87 pg/mg DNA). In the CD group B, with mild to moderate activity, IL-2Rα levels (159 ± 31 pg/mg DNA) were also higher than in the quiescent and control groups, but was significantly less than group A. The IL-2Rα levels in the quiescent CD group C were not different from controls (33 ± 15 *versus* 39 ± 20 pg/mg DNA, respectively).

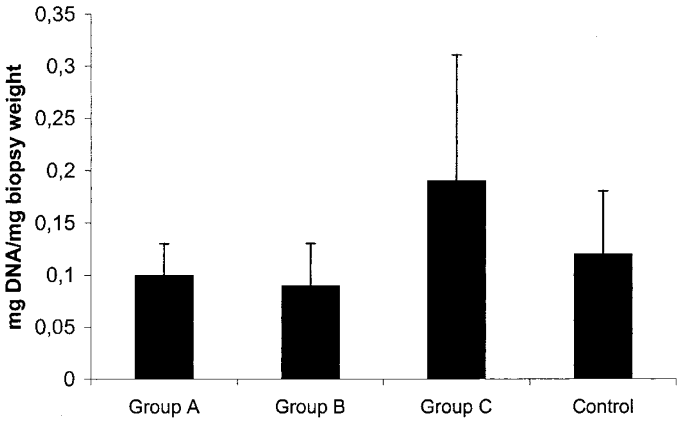

Figure 1. DNA tissue levels are shown for each CD group, based on disease severity: group A (severely active), B (mild-moderately active), and C (quiescent). No statistical differences were found between the control group and the three CD groups. Each bar represents the mean ± SD of each group.

IL-15 results were quite similar in groups A and B, with the highest mean tissue values observed (0.69 ± 0.16 and 0.72 ± 0.31 pg/mg DNA, respectively). These values were both significantly higher ($p < 0.05$) than those observed in the quiescent CD and control groups (0.28 ± 0.21 *versus* 0.28 ± 0.14 pg/mg DNA, respectively).

IFN-α levels were consistently below the detection limit of the assay for all patients' rectal specimens. Thus, statistically significant differences were observed between groups A, B, and C for IL-2Rα ($P < 0.001$), as well as IL-15, which was significantly higher in groups A and B, compared with group C and the control group ($p < 0.05$).

Influence of medical treatment. We also examined the effect of therapy on tissue cytokine profiles (Table 2). The mean values of IL-2Rα levels for treated ($n = 5$) and nontreated ($n = 9$) patients in group B were 167 ± 38 and 154 ± 30 pg/mg DNA, respectively. In group C, IL-2Rα levels for treated ($n = 18$) and nontreated ($n = 5$) patients were 29 ± 14 and 48 ± 18 pg/mg DNA. No significant statistical differences were observed. For IL-15, the mean values for treated and nontreated patients in group B were 0.70 ± 0.47 and 0.65 ± 0.15 pg/mg DNA, respectively. In group C, IL-15 levels for treated and nontreated patients were 0.30 ± 0.17 and 0.22 ± 0.12 pg/mg DNA, respectively. No statistical significant differences were found.

Table 1. *CD patients grouped according to clinical and histopathological disease severity*

	Group A: severe CD ($n = 3$)		Group B: mild to moderate CD ($n = 14$)		Group C: quiescent CD ($n = 23$)		
	Score	SD	Score	SD	Score	SD	*p* Value
Harvey and Bradshaw index	12	4	4	3	4	2	0.001 *vs* B, C
Van Hees index	232	18	198	37	163	40	0.05 *vs* C
Colonic histopathologic score	10	4	5	4	6	5	NS
Rectal histopathologic score	13	2	2	3	2	4	0.001 *vs* B, C

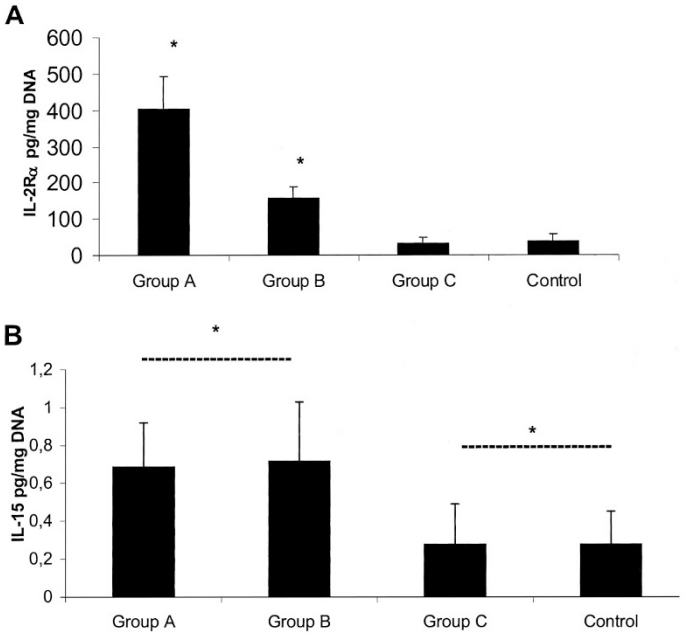

Figure 2. Colonic tissue cytokine profile in CD patient groups according to disease severity. (*A*) IL-2αR tissue levels are shown for biopsy homogenates. Differences between CD group A (severely active), B (mild-moderately active), and C (quiescent) are significant ($p < 0.001$). No differences were found between the control group and group C. (*B*) IL-15 tissue levels are shown for biopsy homogenates. Significant differences were found between groups A and B in comparison with group C and the control group ($p < 0.05$). Each bar represents the mean ± SD of each group. Asterisks indicate significant differences between groups. Statistical evaluation was performed using a one-way ANOVA for nonparametric data with Tukey and Scheffé tests.

Table 2. *Effect of therapy on tissue cytokine profiles*

	Group B (mild to moderately active)		Group C (quiescent disease)	
	DNA (pg/mg)	SD	DNA (pg/mg)	SD
With drug therapy				
IL-2Rα	167	38	29	14
IL-15	0.70	0.47	0.30	0.17
Without drug therapy				
IL-2Rα	154	30	48	18
IL-15	0.65	0.15	0.22	0.12

Correlation between clinical activity and mucosal cytokine data. The association between IL-2Rα and IL-15 with the values of Van Hees clinical index for each CD group is shown in Figure 3. A linear correlation ($r = 0.98$) was observed for IL-2Rα and the Van Hees index. Higher IL-15 levels were observed for increased disease activity in the Van Hees range of 200, after which a plateau was reached.

DISCUSSION

Diverse criteria for ascertaining clinical-pathologic disease activity have been used to delineate the aberrant immune response at various stages of CD (40). However, there is a lack of correlation between clinical parameters of remission and endoscopic remission, as evidenced by the large number of clinically inactive patients with mucosal lesions visible endoscopically (41, 42). Thus, correlations between bioclinical

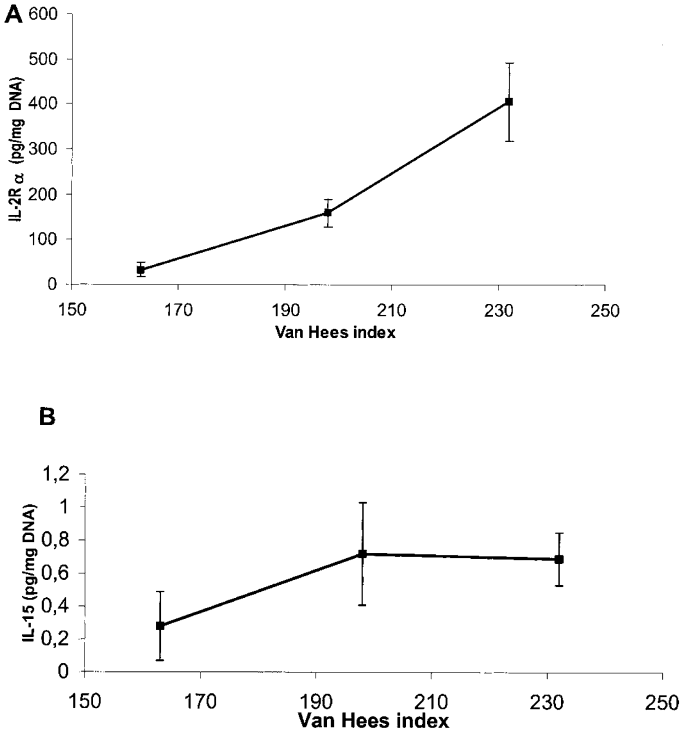

Figure 3. Correlation between clinical disease activity, expressed by Van Hees index, with cytokine tissue levels in CD patient groups: group A (severely active), B (mild-moderately active), and C (quiescent). (*A*) IL-2αR mean tissue levels ± SD *vs* Van Hees index mean value for each CD group. A linear correlation ($r = 0.98$) was found. (*B*) IL-15 mean tissue levels ± SD *vs* Van Hees index mean values for each CD group. An increase in IL-15 tissue levels was observed between Van Hees scores of 170 and 200, above which IL-15 tissue levels reached a plateau.

activity indices and mucosal cytokine levels have been difficult to establish.

In this study, CD patients were classified into three groups, based on the severity of their disease activity, using a detailed, semiquantitative categorization system that evaluates both clinical as well as histopathological criteria (Table 1). The two clinical indices used include the Harvey Bradshaw score, a simple symptom-based clinical index without laboratory criteria. The other is the Van Hees index, which includes six clinical variables and two standard laboratory criteria of inflammation. The Van Hees index has been shown to classify patients similarly to the pediatric CD activity index (PCDAI) (43). The PCDAI requires a more detailed journal of symptoms collected prospectively. This was not feasible for this study, which relied on colonoscopy to recruit potential subjects. The Harvey Bradshaw score only differentiated between severely active and quiescent disease. On the other hand, the Van Hees index more readily differentiates between severe, moderate, and quiescent activity profiles. Our results revealed that the pathologic analysis was less sensitive to differentiate between mild-moderate and quiescent CD groups, compared with the tissue cytokine levels, particularly for IL-2Rα levels.

In this study, we elected to express tissue cytokine levels in terms of the DNA content of each specimen, rather than biopsy weight or protein content. In preliminary studies, the latter values were hampered by confounding elements in the extra-

cellular matrix and the presence of edema, yielding inconsistent values in the presence of variable degrees of inflammation. We observed that tissue DNA is a more constant indicator of cell mass, compared with dry weight and tissue protein (preliminary data not shown). This is important given that IL-2Rα is a transmembrane protein such that its distribution and amount are restricted to the membrane surface and finally to the number of cells present. Figure 1 illustrates the DNA quantitation in the three CD and control groups, expressed in terms of biopsy weight. All groups had similar results, supporting the expression of mucosal cytokine content in terms of DNA, as reported herein.

We found that elevated histologic scores of inflammation (group A) correlated very highly with increased IL-2Rα tissue levels. Control biopsies were found to have low, measurable amounts of IL-2Rα, reflecting the physiologic state of immune activation in the mucosa. These results are consistent with previous studies (6, 12). Most notable in the present study was the strong linear correlation ($r = 0.98$) between tissue IL-2Rα levels and the Van Hees index (Fig. 3*A*). In previous reports, the highest correlation observed between IL-2Rα levels and a clinical index ($r = 0.72$) was obtained with serum samples in CD patients and the Harvey Bradshaw index (8). Methodological differences in the homogenization procedure used and expression of the cytokine results in terms of tissue DNA likely explain the very high correlation observed in our study. IL-2Rα tissue levels of treated and untreated patients in groups B and C, respectively, were not different (Table 2). Overall, the data thus suggest that drug therapy did not influence tissue IL-2Rα levels, but rather that increased levels are related to the presence of active tissue inflammation. However, biopsies would need to be analyzed prospectively, before and after treatment, to resolve this issue.

IL-15 is a pro-inflammatory cytokine with numerous key effects on the immune response. However, difficulty in detecting IL-15 protein has also been described in supernatants of cells that express the mRNA because of the strong inhibitory posttranscriptional control at the level of mRNA translation (44). In the present study, we detected IL-15 tissue levels in rectal biopsies using a high-sensitivity ELISA assay. We observed that IL-15 levels were 2-fold higher in severe and mild-moderate CD tissue, in comparison with tissue from the controls and quiescent CD groups (Fig. 2*B*). These results are in agreement with the reports of Kirman and Nielsen (20) and Liu *et al.* (21), where a low percentage of PBMC and lamina propria macrophages from normal and quiescent adult CD patients were IL-15+, in comparison with a higher percentage in moderate and severely active CD. Also, IL-15 tissue levels of treated and untreated patients in group B and C were similar (Table 2), again suggesting that IL-15 tissue levels are non-drug-therapy dependant.

We found also that IL-15 and IL-2Rα tissue levels did not increase in the same proportion when comparing groups with different degrees of severity of inflammation. IL-15 levels were similar in the severe and mild-moderate groups, whereas IL-2Rα levels increased linearly with the Van Hees clinical index of CD (Fig. 3). It is conceivable that IL-15 plays an as yet undetermined secondary role in the pathogenesis of inflammation in pediatric CD patients, compared with other key pro-inflammatory cytokines such as IL-1β, tumor necrosis factor-α, γ-IFN, or IL-6 (45–47). More information is needed to clarify the immunoregulatory processes involved in the overexpression of IL-15 and its role in the inflammatory response in IBD.

A functional role of increased levels of IL-15 in CD inflamed tissue might be attributable to viral infections. IL-15 was reported to be secreted in herpes virus–infected PBMC (24, 25), and herpes virus has been reported to be associated with IBD. Also, high serum levels of IFN-α on samples from children with viral illness have been reported (48) and common transcriptional control regions for IL-15 and IFN-2α have been described. Therefore, we hypothesized that CD patients with excessive IL-15 levels might also have increased IFN-α production, suggesting that a concomitant active viral infection was present. However, IFN-α levels were not detected in any of our CD patient specimens. Otherwise, the number of samples is too small to speculate about the role of IFN-α in the pathogenesis of CD, but we would suggest that this antiviral cytokine is not likely related to IL-15 overexpression.

Despite the reliance on assays of protein levels of cytokines alone, and the limited number of patients, especially for group A, our results clearly show that IL-2Rα tissue levels and clinical disease activity are linearly correlated in pediatric CD patients. IL-15 is also overexpressed in inflamed CD tissue. However, the lack of a parallel elevation of IFN-α does not support a role for viral induction of IL-15 in inflamed CD tissue. More direct approaches, using PCR methodology, are currently underway in our laboratory to search for viruses as a cause of disease initiation or relapse in pediatric CD.

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