Analysis of interleukin-4 and interleukin-10 and their association with the lymphocytic infiltrate in the small intestine of patients with coeliac disease

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Abstract

Background—Concentrations of proinflammatory cytokines are raised in the small intestine of patients with coeliac disease after ingestion of gluten but there are equivalent data on interleukin-4 (IL-4) and interleukin-10 (IL-10) producing cells. These cytokines are known to exert important regulatory effects on proinflammatory cytokine production from lymphocytes and macrophages.

Aims—To investigate whether there is a primary deficiency of IL-4 and IL-10 producing cells and their site of production in the small intestine of patients with coeliac disease in relation to the changes in inflammatory cell infiltrate.

Patients—Jejunal biopsy specimens from patients with coeliac disease (11 untreated, 10 treated) and nine disease controls were studied.

Methods—Immunohistochemical staining of sections for IL-4 and IL-10 cytokines and the cell phenotypic markers CD3 (T lymphocytes) and CD45 (total inflammatory cell infiltrate) was carried out using monoclonal antibodies. Expression of IL-4 and IL-10 messenger RNA was detected by in situ hybridisation with oligonucleotide probe cocktails for each cytokine.

Results—IL-4 and IL-10 mRNA and protein were detected in the lamina propria of treated and untreated coeliac patients and disease controls but not in the epithelium. A significant increase in the number of CD45 ($p<0.005$) and CD3 ($p<0.05$) positive cells was found in the lamina propria of patients with untreated coeliac disease compared with treated coeliac patients and disease controls but there were no differences in IL-4 or IL-10 between these groups with either method.

Conclusions—There is no primary deficiency of IL-4 and IL-10 producing cells in the small intestine of patients with coeliac disease. Detectable concentrations of IL-4 and IL-10 were found in control patients which suggests that these cytokines are involved in normal mucosal immunoregulation. The increased number of T lymphocytes but not IL-4 or IL-10 producing cells in the lamina propria of patients with untreated than in those with treated disease suggests not only that the lamina propria is the major mucosal compartment for cytokine production but that newly recruited mucosal T lymphocytes are directed to a predominant Th1 and not a Th2 cytokine response in coeliac patients on a diet containing gluten.

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Keywords: coeliac disease, interleukin-4, interleukin-10, lymphocytes.

Coeliac disease is a gluten induced enteropathy that occurs in genetically predisposed subjects. The lesions found in the small intestine of patients affected consists of villus flattening and crypt cell hyperplasia.[1] These changes revert towards normal on removal of gluten from the diet. There is mounting evidence that T lymphocytes are central to the disorder[2] and that the production of cytokines by inflammatory cells, especially T cells and macrophages, are responsible for the histological changes.

T lymphocytes can be defined according to the profile of cytokines that they secrete.[3] This has been clearly shown in murine T cells but is less obvious in human tissue[4]: however, it is still a useful way of delineating cytokine responses. The Th1 response is predominantly of interferon-γ (IFN-γ) and interleukin-2 (IL-2) and drives cell mediated responses whereas the Th2 cytokines, which include IL-4 and IL-10, control antibody mediated processes. Th0 cells are characterised by the production of cytokines of both Th1 and Th2 types and are thought to be precursors of the Th1 and Th2 cells. These cytokines exert regulatory effects on each other which may be important in disease states.

A role for IL-4 and IL-10 in gastrointestinal disease is suggested by the fact that IL-10 knockout mice develop a chronic enterocolitis.[5] There are also reduced numbers of IL-4 secreting cells in the intestine of children with inflammatory bowel disease.[6]

In coeliac disease it has already been shown that there is a Th1 response with increased concentrations of the cytokines IL-2 and IFN-γ[7][8] as well as IL-6 and tumour necrosis factor-α (TNF-α), which are predominantly produced by macrophages[9] in the lamina propria of patients with the untreated condition. These findings have been reinforced by the fact that gluten specific HLA-DQ restricted T cells have been isolated from the small intestine of patients with coeliac disease.[10] Supernatant fluids from these T cell clones activated with gluten caused a reduction in

enterocyte height – an indicator of epithelial cell damage – when cultured with normal small intestinal biopsies and this effect could be blocked with anti-TFN-γ antibodies.[11] These findings raise the possibility that a lack of regulatory cytokines could be responsible for raised concentrations of proinflammatory cytokines in response to gluten in patients with coeliac disease.

The aim of the study was to investigate IL-4 and IL-10 mRNA expressing and protein producing cells in the small intestine of patients with treated and untreated coeliac disease compared with disease controls in relation to the changes in inflammatory cells, particularly T lymphocytes.

These experiments should show whether there is a primary deficiency of these regulatory cytokines in coeliac disease and identify their site of production. They should also permit the dissection of the balance of Th1 and Th2 cytokine profiles in the mucosa of the small intestine of patients with coeliac disease and their change in response to a diet containing gluten.

Patients and methods
The patient groups comprised of following:

Untreated coeliac disease
Eleven adult patients were studied (eight women, three men, mean age 38·2, range 16–67).

Treated coeliac disease
Ten adult patients were studied (seven women, three men, mean age 47·6, range 24–84). These patients had been shown to have pathological changes in biopsy sections of the small intestine consistent with a diagnosis of coeliac disease which later improved after at least six months of treatment with a gluten free diet (according to the European Society for Paediatric Gastroenterology criteria [12]).

Control group
Nine adult patients were studied (five women, four men, mean age 45·5, range 24–74). These had undergone biopsy of the small intestine as part of routine investigation and had a final diagnosis of irritable bowel syndrome with normal histology.

BIOPSY SPECIMENS
Three biopsy specimens were obtained from the second part of the duodenum, with an Olympus IT20 endoscope using FK13 forceps (8 mm). One specimen was fixed in formalin whereas the other two specimens were oriented villous side uppermost, coated in Optimum Cutting Compound (Cryo-M-bed; Bright's Huntington, UK), snap frozen in isopentane cooled in liquid nitrogen, and stored in liquid nitrogen until processed. At least 12 cryostat sections were cut at 5 μm on a Bright's open top cryostat at –20°C and collected on to glass slides that had been washed with distilled water rendered RNAase free by diethylpyrocarbonate (DEPC) treatment and precoated with poly-L-lysine. Sections were briefly fixed in acetone for five minutes and stored at –20°C.

IMMUNOHISTOCHEMISTRY
Sequential sections were stained in duplicate with an indirect immunoperoxidase technique. They were fixed in acetone, preincubated with 0·1% sodium azide/0·3% hydrogen peroxide for 10 minutes, then normal rabbit serum for 20 minutes to block endogenous peroxidase staining, 0·01% avidin for 20 minutes, and 0·01% biotin for 10 minutes to block endogenous biotin. The primary layer antibodies were then added and the mixture incubated for one hour at room temperature. The primary monoclonal antibodies used underwent initial titration experiments to determine the optimal dilution of each antibody. The following were used.

(a) Murine monoclonal antihuman CD45 (Serotec MCA669) at 1:200 dilution. This recognises the common leucocyte antigen but not haemopoetic cells and gives an estimate of total inflammatory cell infiltrate.

(b) Murine monoclonal antihuman CD3 (Dako M0835) at 1:80 dilution. This recognises T lymphocytes. The cell phenotype antibodies were diluted with phosphate buffered saline (PBS).

(c) Murine monoclonal antihuman IL-4 (Serotec MCA952) at 1:75 dilution.

(d) Murine monoclonal antihuman IL-10 (Serotec MCA1110) at 1:400 dilution. The cytokine antibodies were diluted in a 5% solution of foetal calf serum.

The secondary layer comprised biotinylated rabbit antimouse immunoglobulins (Dako E354) at 1:200 dilution for 30 minutes. The tertiary layer comprised avidin-biotin complex (Dako K391) at 1:100 dilution for a further 30 minutes. A further step was used between the secondary and tertiary layers which involved treatment with 0·5 mg/ml glucose oxidase for 30 minutes at 37°C in glucose stock solution which blocked non-specific staining of eosinophils. After 10 minutes of treatment with aminoethyl carbazole (Sigma A6926) the cells were counterstained with Mayer's haematoxylin and mounted with glycerol mounting medium. Sections were thoroughly washed with PBS between steps.

The controls omitted the primary antibody and used a murine monoclonal antibody WB6/8 (1:20 dilution) raised against a gliadin peptide, of the same isotype as the antihuman antibodies employed.

IN SITU HYBRIDISATION
DNA oligonucleotide probes were obtained commercially (R and D systems). Cytokine mRNA detection was achieved with a cocktail of three probes for each cytokine, each 30 bases long, with cDNA sequences coded by three different exon regions of the IL-4 (BPR 22) and IL-10 (BPR 237) genes.

Each oligonucleotide (4 pmol) was labelled by the addition of an a ^{35}S dATP 'tail' to the 3' termini using terminal deoxynucleotidyl transferase, according to the supplier's protocol (Promega). After the labelling reaction, probes were purified by filtration through Sephadex G-50 DNA grade columns (Pharmacia) and their specific activity measured with a Rackbeta liquid scintillation counter. The specific activity was measured with a Rackbeta liquid scintillation counter. The specific activity of the probes was greater than 1×10^8 cpm/μg.

All solutions were prepared with DEPC treated water. Fixation was with 4% (w/v) paraformaldehyde (PFA) in PBS pH 7·4 for 15 minutes, followed by two washes in 15% (w/v) sucrose in PBS for 10 minutes each. Pre-treatments were (a) 0·3% (v/v) Triton-X-100 in PBS for 10 minutes, (b) 1 μg/ml proteinase K for 30 minutes at 37°C, (c) postfixation in 0·4% (w/v) PFA for 10 minutes, and (d) 0·1 M triethanolamine containing 0·25% (v/v) acetic anhydride for 10 minutes. Duplicate sections were prehybridised in a mixture of 2× saline-sodium citrate buffer (SSC), 50% (v/v) form-amide, and 1×Denhardt's solution at 37°C for one hour and then hybridised overnight with 2 ng labelled probe at 37ηC in a solution containing: 2×SSC, 50% (v/v) formamide, 0·4 mg/ml denatured sheared salmon sperm DNA, and 10% (w/v) dextran sulphate. Post-hybridisation washes were carried out at 39°C, for 30 minutes each, in 2×SSC, 1×SSC+50% formamide, and 0·1×SSC. Sections were dehydrated serially in 60%, 90%, and 95% ethanol and dried at room temperature. Auto-radiography consisted of dipping sections in Ilford K5 photographic gel emulsion that had been diluted 1:1 in prewarmed distilled water. The sections were left to dry in a dark room for about two hours and then incubated at 4°C in a lightproof box containing silica gel for five days. Slides were developed in 4·4% (w/v) Dektol solution (Kodak) for two minutes, washed briefly in distilled water, and fixed in 30% (w/v) sodium thiosulphate at 4°C for 15 minutes. Finally the slides were thoroughly rinsed in running tap water and counterstained with Mayer's haematoxylin.

Slides treated with either RNAase-free DNAase (no effect on hybridisation) or DNAase-free RNAase (no hybridisation) were used as controls. After the proteinase K treatment, sections were incubated at 37°C with either of the two enzymes at a concentration of 100 mg/ml for 30 minutes.

Oligonucleotides containing sequences complementary to the cytokine probes (sense probes) were used as negative controls. An oligo dT probe was also used for the detection of total mRNA as a positive control.

QUANTIFICATION AND STATISTICAL ANALYSIS
All assessments were performed by two observers who were blinded to patient identity and cytokine/cell phenotype. The number of positive cells within the lamina propria were counted per unit area with an eyepiece graticule under ×400 magnification. The lamina propria was assessed from the tip of the villus down to the deeper lamina propria on duplicate sections for each cytokine/cell phenotype. The eyepiece graticule consisted of 100 squares which enabled the villous lamina propria to be counted as a fraction of a complete grid and it enabled the avoidance of crypt epithelium in the deeper lamina propria. In total, at least five complete graticule areas were counted per section. The results were expressed as the median number of positive cells in the lamina propria per unit area (0·02 mm^2) in each patient group. Statistical analysis was by Mann-Whitney U test. Significance was set at $p < 0.05$.

The study was approved by the hospital ethics committee in accordance with the Declaration of Helsinki.

Results
Figures 1 and 2 show examples of IL-10 immunohistochemical staining and IL-4 messenger RNA expression respectively in the lamina propria.

CELL PHENOTYPE (TABLE I)
The lamina propria of the untreated coeliac group had a significantly increased number of CD45 staining cells representing the total inflammatory cell infiltrate (111·8/unit area) compared with both the treated coeliac group (81·6/unit area) and disease controls (43·5/unit area) (p<0·005 each). The total number of T lymphocytes (CD3) was also increased in the lamina propria of patients with untreated coeliac disease (96/unit area) compared with treated coeliac and control groups (71·0 and 42·5/unit area respectively) (p<0·05 each).

INTERLEUKIN-4 (TABLE II)
In the lamina propria of the untreated coeliac group the number of cells expressing product or message (5·4 and 8·8/unit area respectively) was not significantly different from the treated coeliac (8·6 and 7·2/unit area) and disease control (4·4 and 7·3/unit area) groups.

INTERLEUKIN-10 (TABLE II)
There were no significant differences in the number of cells expressing product or message between the untreated coeliac (4·3 and 8·8/unit area respectively), the treated coeliac (6·2 and 9·6/unit area), and the disease control (4·9 and 8·8/unit area) groups.

TABLE I   *Number of CD45 and CD3 staining cells in the lamina propria of untreated and treated coeliac patients and control patients*

Patient group	CD45	CD3
Coeliac disease:		
Untreated	111·8 (105·5–154·2)**	96 (66·5–104)*
Treated	81·6 (66·6–87·5)**	71 (51·5–78·8)*
Controls	43·5 (34·7–57·4)	42·5 (36–59·1)

Values are median/unit area (interquartile range).
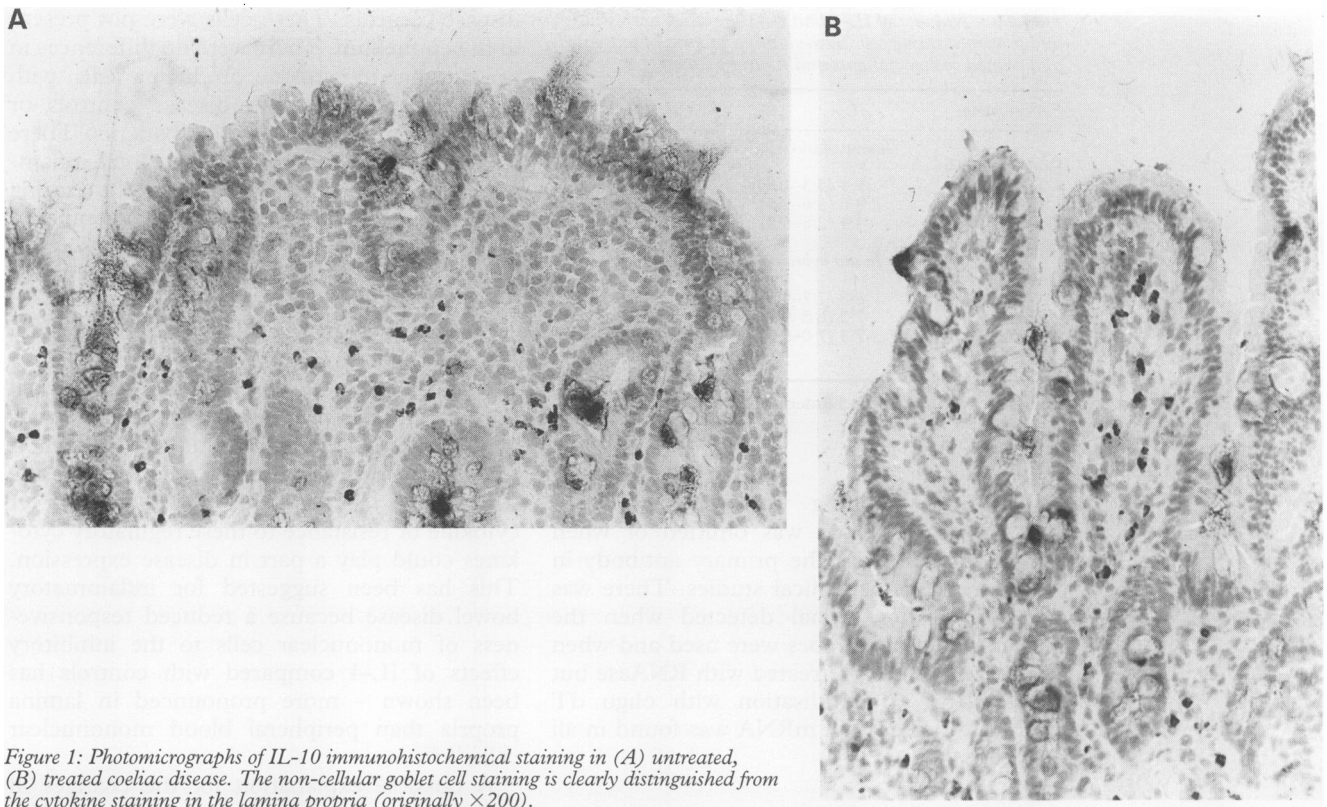*p<0·05; **p<0·005 v control group.

Figure 1: Photomicrographs of IL-10 immunohistochemical staining in (A) untreated, (B) treated coeliac disease. The non-cellular goblet cell staining is clearly distinguished from the cytokine staining in the lamina propria (originally ×200).
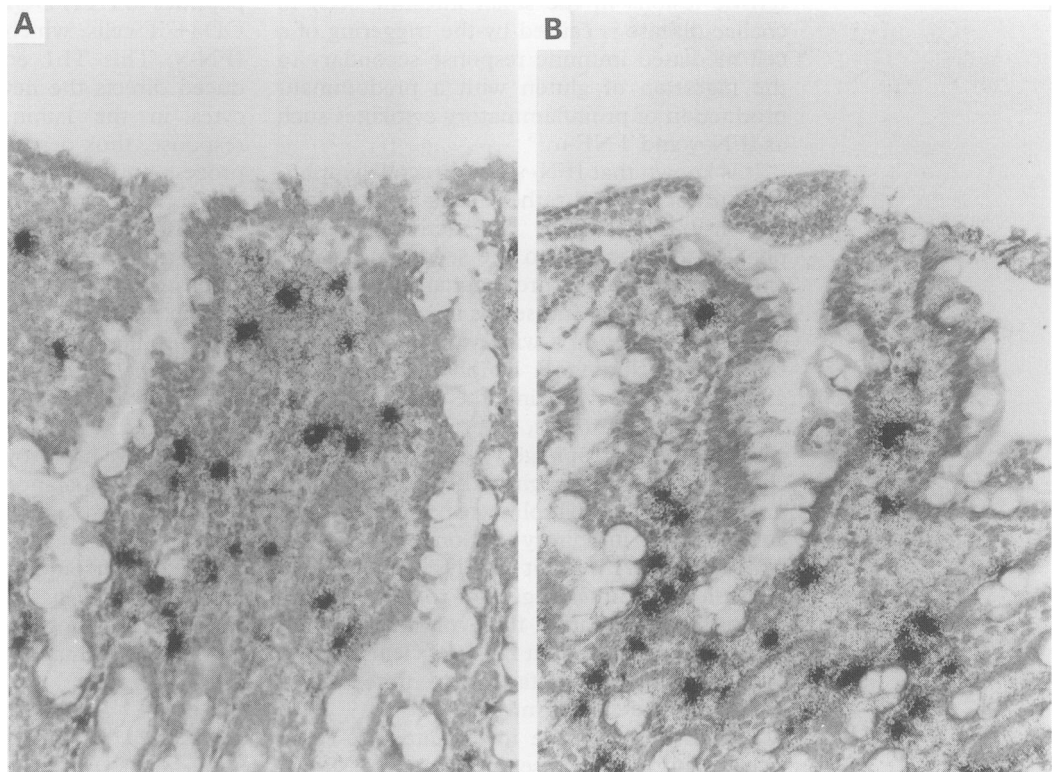

Figure 2: Photomicrographs of IL-4 messenger RNA expression in (A) untreated, (B) treated coeliac disease (originally ×200).

The study was focused on events in the lamina propria of patients with coeliac disease. There was staining of CD45 and CD3 cells in the epithelium (results not shown), however, there was virtually no cytokine staining in the epithelium with either method used and so it was not possible to submit epithelial cytokine staining to statistical analysis. There was some non-cellular goblet cell staining with the immunohistochemistry method which was clearly distinguishable from the specific staining.

TABLE II *Number of IL-4 and IL-10 mRNA expressing and protein staining cells in the lamina propria of untreated and treated coeliac patients and control patients*

Patient group	IL-4	IL-10
	Immunohistochemistry	
Coeliac disease:		
Untreated	5·4 (3·3–10·9)	4·3 (2·1–8·1)
Treated	8·6 (3·6–9·5)	6·2 (3·0–9·4)
Controls	4·4 (2·8–6·0)	4·9 (2·8–6·0)
	In situ hybridisation	
Coeliac disease:		
Untreated	8·8 (7·7–9·6)	8·8 (8·6–11·3)
Treated	7·2 (6·5–9·7)	9·6 (7·2–11·3)
Controls	7·3 (7·0–8·0)	8·8 (7·8–9·6)

Values are median/unit area (interquartile range).

CONTROLS

No positive staining was detected when the primary antibody was omitted or when WB6/8 was used as the primary antibody in the immunohistochemical studies. There was no hybridisation signal detected when the ^{35}S-labelled sense probes were used and when the sections were pretreated with RNAase but not DNAase. Hybridisation with oligo dT probes to detect total mRNA was found in all biopsies.

Discussion

There is mounting evidence that the characteristic lesions in the small intestine seen in coeliac disease is caused by the triggering of a cell mediated immune response secondary to the ingestion of gluten with a predominant production of proinflammatory cytokines such as IFN-γ and TNF-α.[13]

It is known that IFN-γ can direct T lymphocytes towards a Th1 rather than a Th2 cytokine response and IFN-γ can activate macrophages, thus leading to the secretion of IL-6 and TNF-α. IL-4 is predominantly produced by T lymphocytes and seems to have opposing effects to IFN-γ on a wide variety of immune functions including B lymphocyte immunoglobulin isotype switching, the development of T cell cytokine profiles, and several functional activities of macrophages.[14] IL-10 is another cytokine which is thought to be predominantly produced by T lymphocytes and to exert considerable inhibitory actions on macrophages and Th1 but not Th2 lymphocytes.[15]

Depletion studies suggest a role for IL-4 in the early proliferative form of murine graft versus host disease[16] but IL-4 deficient mice do not develop a spontaneous enteropathy,[17] unlike IL-10 deficient mice.[5]

Several studies have investigated IL-10 in inflammatory bowel disease. Some have shown raised concentrations of IL-10 in active disease compared with controls [18] [19]; others showed no changes[20] although IL-10 down regulated proinflammatory cytokine production from lamina propria mononuclear cells in vitro and in vivo after topical administration as well as an endoscopic improvement of refractory distal colitis.

In our study, we have shown the presence of IL-4 and IL-10 mRNA and protein producing cells in the lamina propria of patients with untreated and treated coeliac disease as well as

disease controls. These cells were not present in the epithelium. There were no differences in the number of cytokine producing cells, with either method used, in disease controls or untreated and treated coeliac patients. There was a significant increase in the total inflammatory cell infiltrate (CD45) in the lamina propria of untreated coeliac patients compared with treated coeliac patients and disease controls and most of these cells were ·T lymphocytes (CD3).

Our findings suggest that there is no primary deficiency in expression of IL-4 or IL-10 in coeliac disease; they are detected in normal mucosa, and they are probably involved in maintaining normal intestinal immunoregulation. It remains a possibility that as we are detecting cytokine producing cells, a relative lack of cytokine or resistance to these regulatory cytokines could play a part in disease expression. This has been suggested for inflammatory bowel disease because a reduced responsiveness of mononuclear cells to the inhibitory effects of IL-4 compared with controls has been shown – more pronounced in lamina propria than peripheral blood mononuclear cells.[21] [22]

A possible explanation for the changes in T lymphocytes in relation to cytokine profiles in coeliac disease is proposed. The ingestion of gluten results in the presentation of 'toxic' peptides to HLA DQ2 specific gliadin sensitive CD4+ T cells, which predominantly produce IFN-γ. This Th1 environment initially produced directs the newly recruited T lymphocytes in the lamina propria to the Th1 response, thus perpetuating the inflammatory process and the development of the mucosal lesion. This is supported by the fact that gliadin specific T cell clones derived from peripheral blood produce a Th0 cytokine profile whereas clones derived from the small intestine of coeliac patients produce more of a Th1 cytokine profile.[23] [24] There is evidence that HLA restriction[25] and the structure of the toxic epitope exert an influence on the T cell cytokine profile.[26]

The study has provided further information regarding the balance of proinflammatory and regulatory cytokines in the small intestine of patients with coeliac disease which will help in the understanding of how cytokines are involved in the pathogenesis of the lesions in the small intestine in coeliac disease and other chronic inflammatory conditions of the gastrointestinal tract.

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