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ORIGINAL ARTICLE

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Cytokine messenger RNA expression and proliferation status of intestinal mononuclear cells in noninflamed gut and Crohn's disease

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Abstract T-cell activation and local cytokine production probably contribute to the pathogenesis of Crohn's disease. This study investigates the proliferative status of intestinal mononuclear cells (MNC) and cytokine messenger RNA (mRNA) production in gut tissue sections from patients with Crohn's disease and noninflamed controls. mRNA in situ hybridization was performed using ^{33}P-labelled riboprobes for human interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, tumour necrosis factor-α and interferon-γ. The expression of the proliferation-associated antigen Ki-67 was analysed by immunohistochemical single and double staining. Compared with controls, where proliferation of MNC and cytokine expression was restricted to mucosal lymphoid follicles, inflamed gut tissue contained increased numbers of cells expressing cytokine mRNA, most prominently IL-1β and IL-6, but also interferon-γ and tumour necrosis factor-α. Proliferating T-cells were increased in number, and small amounts of IL-2-expressing cells were detected. IL-4 was expressed by a few cells exclusively in follicular germinal centres. IL-5 was negative. Proinflammatory cytokines are strongly expressed in situ in Crohn's disease and largely predominate over lymphokine mRNA. Our results provide in situ evidence of a local lymphocyte response in Crohn's disease with characteristics of a delayed-type hypersensitivity reaction.

Key words Crohn's disease · Cytokines · Ki-67 · Cell proliferation

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Introduction

Crohn's disease (CD) is a chronic intestinal inflammation of unknown and possibly heterogeneous aetiology. Because of their regulatory as well as potent proinflammatory activities, cytokines produced by inflammatory cells are likely to play a role in the initiation and perpetuation of the disease. Recent work has suggested that interleukin (IL)-1, mainly produced by activated macrophages, might be of importance in CD. Using bioassays, enzyme-linked immunoassays and polymerase chain reaction (PCR), increased levels of IL-1 activity, IL-1β or IL-1β mRNA have been found in mucosal biopsy specimens or their culture supernatants in CD patients when compared with controls [7, 12, 20, 26]. Additional macrophage products such as IL-6 and tumour necrosis factor-α (TNF-α) have been reported to be upregulated in CD using similar systems [12, 21, 25, 26]. However, conflicting data exist, for example concerning TNF-α, where both low [18] and increased production have been reported [21, 25].

Activated T-cells are also believed to play an important role in CD [35], but in the IL-2/IL-2 receptor (IL-2R) system, which is of central importance in T-cell activation and proliferation, both increased and decreased IL-2 activity have been described in CD [7, 19, 24, 27, 30] and conflicting data have also been published for interferon-γ (IFN-γ) [6, 14, 21, 34].

Most of these data have emerged from in vitro experiments on isolated intestinal or peripheral blood leucocytes. Cytokine production observed under such conditions does not necessarily reflect the functional status of these cells within their native tissue microenvironment. To gain more insight into in situ production, relative contribution and topographic distribution of T-cell and macrophage-derived cytokines in CD and noninflamed gut, we investigated tissue sections from surgically resected gut for the expression of cytokine mRNA by in situ hybridization (ISH) using ^{33}P-labelled RNA probes. To address the question of local T-cell activation, we also analysed the proliferation status of intestinal mononuclear cells

(MNC) by immunohistochemical single and double staining with the Ki-67 monoclonal antibody (mAb), which detects a nuclear antigen only in the proliferative phases of the cell cycle [15].

Materials and methods

Tissues

Intestinal tissue from 20 patients (13 female, 7 male) with active CD undergoing surgical resections of diseased gut was obtained. Five samples were derived from diseased colon, 15 from ileum. Diagnosis was established by conventional clinical and histopathological criteria. Disease duration ranged from 4 weeks to 27 years (median 6 years), the patient's age from 17 to 63 years. Fourteen patients received immunosuppressive therapy before the operation (corticosteroids, azathioprine in one case). Immediately after resection, small samples of grossly diseased tissue were dissected out, oriented on cork plates, snap-frozen in nitrogen-cooled isopentane (Merck, Darmstadt, Germany) and stored in liquid nitrogen. As control tissues, nonaffected ileum (2 cases) and colon (5 cases) from patients undergoing bowel resection for intestinal cancer was obtained. Sections 4 μm thick were cut at –20°C and used for immunohistochemistry and ISH. The inflammatory activity within the actually investigated tissue sections was scored as high in CD cases with a high degree of infiltration by MNC and or with demonstration of destructive sites such as fissures or larger ulcerations (n = 13). Cases displaying low to moderate increases of MNC without a prominent destructive activity were scored as low/intermediate (n = 7).

Immunohistochemistry

Double staining for proliferating T-cells was performed on acetone-fixed cryostat sections which were incubated with Ki-67 mAb (immunoglobulin G1) (IgG1; dilution 1:10) (Dako, Hamburg, Germany) in combination with anti-CD3 (OKT3, IgG2a, 20 μg/ml) (Ortho, Neckargemünd, Germany). As secondary antibodies, a combination of biotinylated goat anti-mouse IgG1 (1:100) and peroxidase-labelled goat anti-mouse IgG2a (1:200) (both from Southern Biotech USA; distributed by Dunn, Asbach, Germany) was used, followed by streptavidin (5 μg/ml) and biotinylated β-galactosidase (5 μg/ml, both from Boehringer, Mannheim, Germany). Negative primary mouse mAb of IgG1 and IgG2a isotype served as controls. The peroxidase reaction was developed using 3,3' diaminobenzidine (Sigma, Deisenhofen, Germany) (1 μg/ml in 0.05 M TRIS-HCl plus 0.01% H_2O_2) as substrate. The sections were then reacted with a solution containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal; Serva, Heidelberg, Germany) as a substrate for β-galactosidase (blue reaction product) [4] and mounted without further counterstaining. For comparative β-galactosidase single stainings, anti-CD3 and peroxidase-labelled reagents were omitted and *O*-aminoazotoluene-diazonium salt (Fast Garnet, 0.55 mM) with 6-bromo-2-naphthyl-β-D-galactopyranoside (BNG, 0.29 mM, both from Sigma) and 10 mM $MgCl_2$ in phosphate-buffered saline was used as the substrate, resulting in a red reaction product [16], which allowed counterstaining with haematoxylin. Ki-67 single staining was also done by the conventional alkaline phosphatase anti-alkaline phosphatase (APAAP) technique [10] in each case. In addition, staining for CD3 (T3-4B5, 1:200), CD68 (EBM11, 1:300) and CD20 (L-26, 1:250) was performed using a standard streptavidin-biotin complex method [17] or the APAAP procedure (reagents all from Dako). Here, naphthol AS-biphosphate (Sigma) and new fuchsin (Merck) was used as the substrate for alkaline phosphatase.

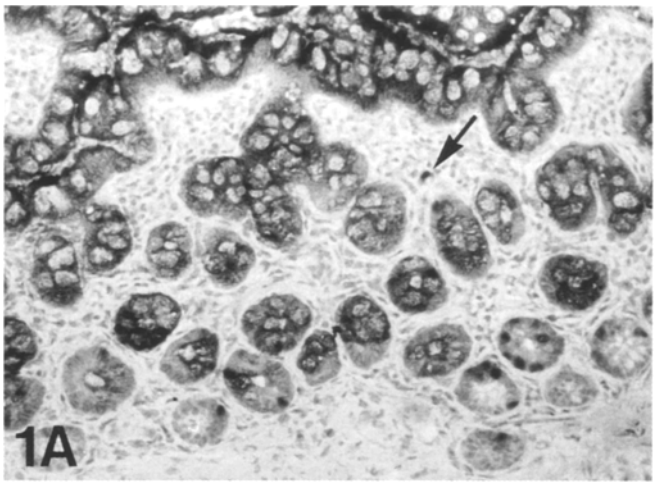
For enumeration of MNC expressing Ki-67 antigen, at least two fractions of 500 nucleated cells were counted in different areas in the lamina propria as well as in the submucosa and at follicular sites over adjacent high power fields (×400) using a grid and median values of the relative percentage of Ki-67-immunoreactive cells to the total number were calculated. Since nucleated cells were counted, the enumeration included endothelial cells, fibrocytes and other cell types, which cannot be distinguished with certainty from mononuclear leucocytes under the given conditions. Due to variable immunohistochemical background staining at the margins of ulcerations and fissures, counting was considered unreliable in these areas and they were excluded. For similar reasons, gut epithelial cells and intraepithelial lymphocytes were also excluded. Due to the heterogeneous distribution of Ki-67 antigen-expressing cells and in order to avoid false-negative results, sections were screened and positive areas demonstrating immunoreactive MNC were selected for enumeration in each case, if such areas appeared in the tissue sections. In the double stainings, fractions of 500 CD3 and/or Ki-67-immunoreactive cells (not nucleated cells) were counted and the relative percentage of double-positive cells to the total number of Ki-67-immunoreactive cells was calculated.

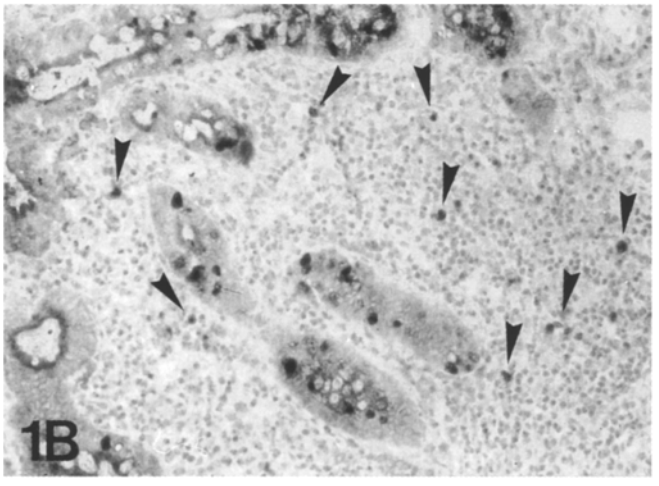
In situ hybridization

The following complementary DNAs (cDNAs), all subcloned into transcription vectors for synthesis of cRNA probes, were used: cDNAs of the coding regions (translated nature protein) of human IL-2, IL-4, IL-5, IL-6 and IFN-γ (Sau3A fragment) in pSPT 18 (Boehringer); a 744 basepair (bp) fragment of human TNF-α cDNA in pBluescript II SK (Stratagene, Heidelberg, Germany) (base 259-1002 of the sequence published by Wang et al. [42]); a 477 bp fragment of human IL-1β cDNA in pBluescript II SK (base 11–487 of the sequence published by Nishida et al. [32]; a full-length 1760 bp cDNA of human β-actin, subcloned into pGEM4 (Promega, Heidelberg, Germany). The identity of all constructs was confirmed by sequencing and the specificity of the cDNAs was tested in Northern-blot hybridizations prior to use in ISH (data not shown here).

Full-length antisense and sense (negative controls) cRNA probes of high specific activity (>10^8/μg RNA) were transcribed in vitro from linearized templates in the presence of ^{33}P-labelled uridine triphosphate (UTP; >1000 Ci/mmol) (Amersham, Braunschweig, Germany or DuPont NEN, Bad Homburg, Germany) with T7, SP6 and T3 RNA-polymerases using a RNA transcription kit (Stratagene). Limited alkaline hydrolysis of the resulting cRNA probes was performed to obtain fragments ranging in length from approximately 50 to 300 bp [1].

ISH was based on a protocol described by Bosch et al. [5] and performed under standard precautions to avoid RNAse contamination. Cryostat sections were placed onto 2% 3-aminopropyltriethoxysilane-treated slides (Merck) and fixed in 4% paraformaldehyde (PFA, Merck) buffered with 2×SSPE pH 7.4 (1×SSPE = 0.15 M NaCl, 0.01 M $NaH_2PO_4 \cdot H_2O$, 1 mM EDTA) plus 5 mM $MgCl_2$ for 15 min. Sections were then dehydrated (70%/100% ethanol), air dried and stored at –70°C until use or further processed for ISH. Rehydrated slides were digested with proteinase K (Boehringer) 0.5 μg/ml for 15 min at 37°C and then rinsed with 0.1 M glycine (Merck) in 2×SSC (1×SSC = 0.15 M NaCl, 0.015 M trisodium citrate). After postfixation (5 min at room temperature) with 4% PFA slides were acetylated in 0.1 M triethanolamine (Sigma) with 0.25% acetic anhydride (Merck) for 10 min and then equilibrated with 50% deionized formamide (FA) (Fluka, Neu-Ulm, Germany)/2×SSPE for at least 15 min. After shaking off excess solution, semidry sections were prehybridized with hybridization mix containing 50% FA/2×SSPE, 10 mM Tris-HCl, 0.1% sodium dodecyl sulfate (SDS, Serva), 10% dextran-sulphate (mol.wt. 500,000; Pharmacia), 1× Denhardt's solution (Ficoll, bovine serum albumin and polyvinylpyrollidone, each 0.02%, from Merck), 500 μg/ml tRNA from brewer's yeast (Boehringer) and 100 μg/ml denatured, sonicated herring-sperm DNA (Promega) at 42°C in a sealed chamber filled at the bottom with 50% FA/2×SSPE. After 1–3 h, 2×10^6 cpm ^{33}P-UTP-labelled RNA probe was added and hybridized ON at 42°C. For each specimen, two slides with two tissue sections per slide were hybridized with antisense probe and one similar slide was covered with the sense





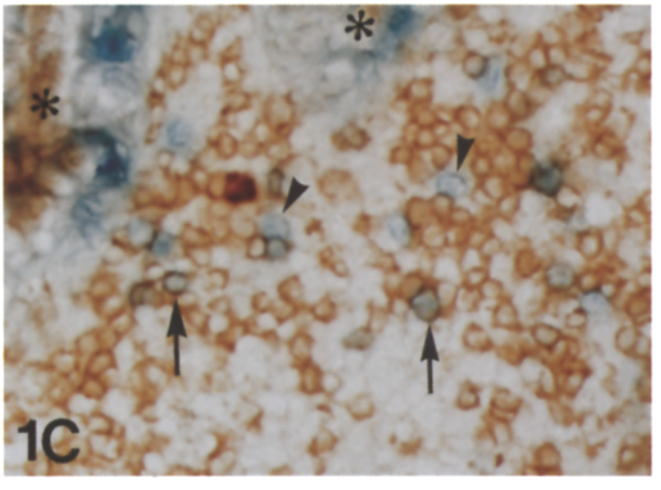
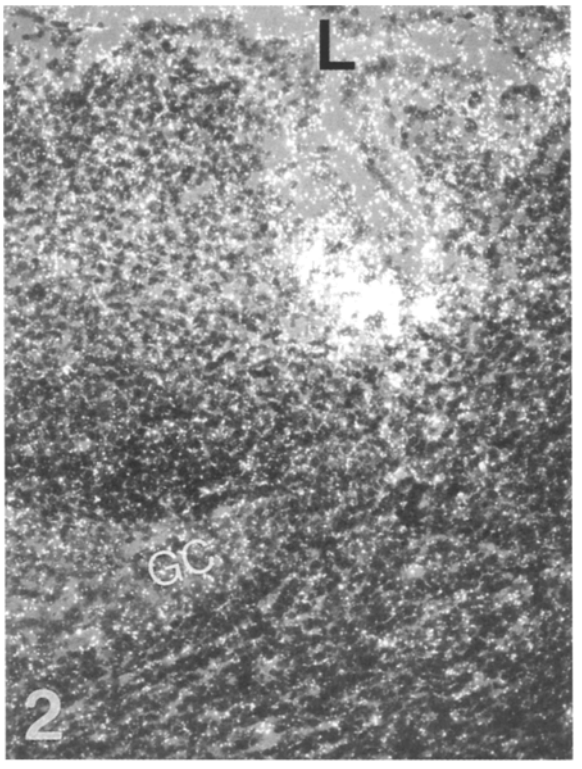


Fig. 1A–C Ki-67 antigen expression in noninflamed gut and Crohn's disease (CD). **A**, **B** Whereas Ki-67 antigen is negative or expressed by only few isolated mononuclear cells in the noninflamed extrafollicular diffuse lamina propria in control gut (**A**; positive cell indicated by *arrow*), the inflamed lamina propria in CD contains increased numbers of Ki-67-immunoreactive cells (*arrowheads* in **B**). Note that crypt epithelia also express Ki-67 antigen. (**A**, ileum, APAAP, ×200; **B**, colon, APAAP, ×310). **C** Ki-67/CD3 double staining of inflammatory cells in CD. Inflamed intact lamina propria with numerous CD3-positive inflammatory T-cells (brown surface staining) plus Ki-67/CD3 double positive T-cells (greenish with a brown rim, *arrows*) as well as some Ki-67 single-positive non-T-cells (bright blue nuclear staining, *arrowheads*). Two adjacent crypts are indicated by *asterisks*. Colon, double staining with peroxidase/β-galactosidase, ×600



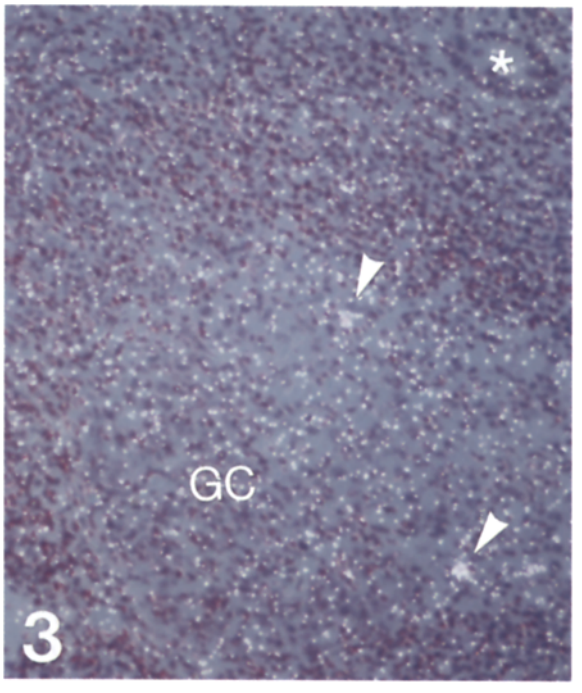


Fig. 2 Control gut with demonstration of IL-1β mRNA expressed by subepithelial cells in a Peyer's patch. Dark-field micrograph with positive cells displaying accumulations of white grains (*L* gut lumen, *GC* germinal centre). ISH, ileum, ×200

Fig. 3 Two cells express IL-4 mRNA within the germinal centre of a mucosal follicular lymphoid complex in control gut (*arrowheads*; *GC* germinal centre; *asterisk* crypt). ISH, dark field, ileum, ×200

probe. Postwashings were performed in a shaking water bath with 50% FA/2×SSPE for 30 min at 50°C; 2×SSC/0.1% SDS for 10 min at 37°C; 2×SSC/0.1% SDS plus 50 µg/ml RNAse A (Boehringer) for 30 min at 37°C; 50% FA/0.5×SSC/0.1% SDS for 30 min at 37°C; 0.1×SSC for 30 min at 37°C. Slides were then dehydrated in 50/70/95/100% ethanol containing 0.3 M ammonium acetate (Merck), air dried, dipped in Kodak NTB-2 emulsion (Technomara, Fernwald, Germany) for autoradiography and exposed at 4°C in absolute dark with desiccant for 5–6 weeks, followed by development in Kodak D-19, fixing in Kodak AL-4 (both from Technomara) and finally stained with haematoxylin and eosin. Autoradiographs were viewed with a Leitz orthoplan microscope (Wetzlar, Germany). Positive cells were identified in bright (black grains) and dark field illumination (white grains) and contained grain numbers of more than 3 times the background (about 20 grains/cell). Specific labelling was distinguished from nonspecific signals by consequently comparing sense and antisense slides.

To control the reactivity of the probes, each ISH of tissue sections was paralleled by incubation of cytospins (50,000–100,000 cells) of stimulated peripheral blood MNC (PBMNC) or T-cells with the same RNA probes used in the tissue. T-cells were isolated and stimulated with protein kinase C activators as described previously [37]. PBMNC were treated with phytohaemagglutinin (0.5 µg/ml) plus phorbol-12-myristate-13-acetate (10^{-8} M)(both from Calbiochem, Frankfurt, Germany) (stimulation times between 30 min and 25 h). Hybridizations for β-actin were used to check the principal availability of tissue RNA in our material (not shown).

Analogous to the evaluation of Ki-67-immunoreactive cells, the relative number of cytokine mRNA-expressing cells to fractions of 500 nucleated cells was analysed semiquantitatively and scored as follows: negative, 0; single positive or less than 2% positive cells, 1; 2–10 positive cells, 2; >10–50% positive cells, 3; >50% positive cells, 4. Due to relatively low background, all layers of the gut wall were analysed, including ulcerative sites. The individual score in each case was calculated according to the highest amount of positive cells found in the corresponding slides.

Statistical analysis

The Wilcoxon's two-tailed test for unpaired samples was used for statistical analysis. *P* values of <0.05 were considered to be significant.

Results

Proliferation status of intestinal MNC

In noninflamed control gut, MNC expressing the Ki-67 antigen were mainly confined to sites of organized lymphoid complexes such as Peyer's patches or solitary mucosal and submucosal follicles, in both ileum and colon. While the majority of germinal centre cells expressed Ki-67 (40–80%), a median of 1.8% (range 0.6–4.0%) of follicular cells outside germinal centres stained for this antigen. Among Ki-67-immunoreactive cells, a median of 40.5% (range 19–79%) were CD3/Ki-67 double-positive T-cells in these areas (not shown). When compared with such follicular sites, the noninflamed diffuse lamina propria contained significantly lower numbers (median 0.2%; range 0–1.0%; $P<0.001$) of Ki-67-immunoreactive cells (Fig. 1A), some of which were identified as T-cells in the double staining.

In CD, Ki-67-immunoreactive cells occurred in the majority of cases (13/20) within nonfollicular inflammatory infiltrates in the diseased lamina propria in significantly higher numbers (median 1.2%; range 0–3.4%) when compared with the lamina propria of controls (median 0.2%; range 0–1.0%; $P<0.003$) (Fig. 1B). Even more elevated numbers of Ki-67-immunoreactive cells were found in the submucosa (median 2.6%; range 0–11.6%), whereas such cells were absent in controls. Antigen expression was heterogeneous and positive cells were intermixed with nonproliferating cells in a focal pattern, particularly in the inflamed lamina propria. The number of positive cells tended to increase with inflammatory activity (median number 0.6% in the lamina propria and 2.2% in the submucosa in cases with low inflammatory activity versus 1.4% and 3.0% in those with high activity). No significant differences were found between tissues of patients receiving immunosuppressive therapy and those who did not. In the double staining (6 randomly selected CD cases counted in detail), a median

Table 1 Cytokine mRNA expression in control gut and Crohn's disease. The relative number of mRNA-expressing cells to fractions of 500 nucleated cells was semiquantitatively scored as follows: no positive cells = 0; single or <2% positive cells = 1; 2–10% positive cells = 2; >10–50% positive cells = 3; >50% positive cells = 4. The individual score in each case was calculated according to the highest number of positive cells found in the corresponding slides

Cytokine	Tissue	Number of positive cases/ investigated cases	Number of mRNA expressing cells	
			Median score value	Range
IL-1β	Control gut	5/7	1	0–1
	Crohn's disease	16/17	3	0–4
IL-6	Control gut	5/6	2	0–2
	Crohn's disease	17/17	3	1–3
TNF-α	Control gut	0/7	0	0
	Crohn's disease	9/17	1	0–2
IFN-γ	Control gut	2/6	0	0–1
	Crohn's disease	15/17	1	0–2
IL-2	Control gut	2/7	0	0–1
	Crohn's disease	5/17	0	0–1
IL-4	Control gut	3/7	0	0–1
	Crohn's disease	5/17	0	0–1
IL-5	Control gut	0/6	0	0
	Crohn's disease	0/17	0	0

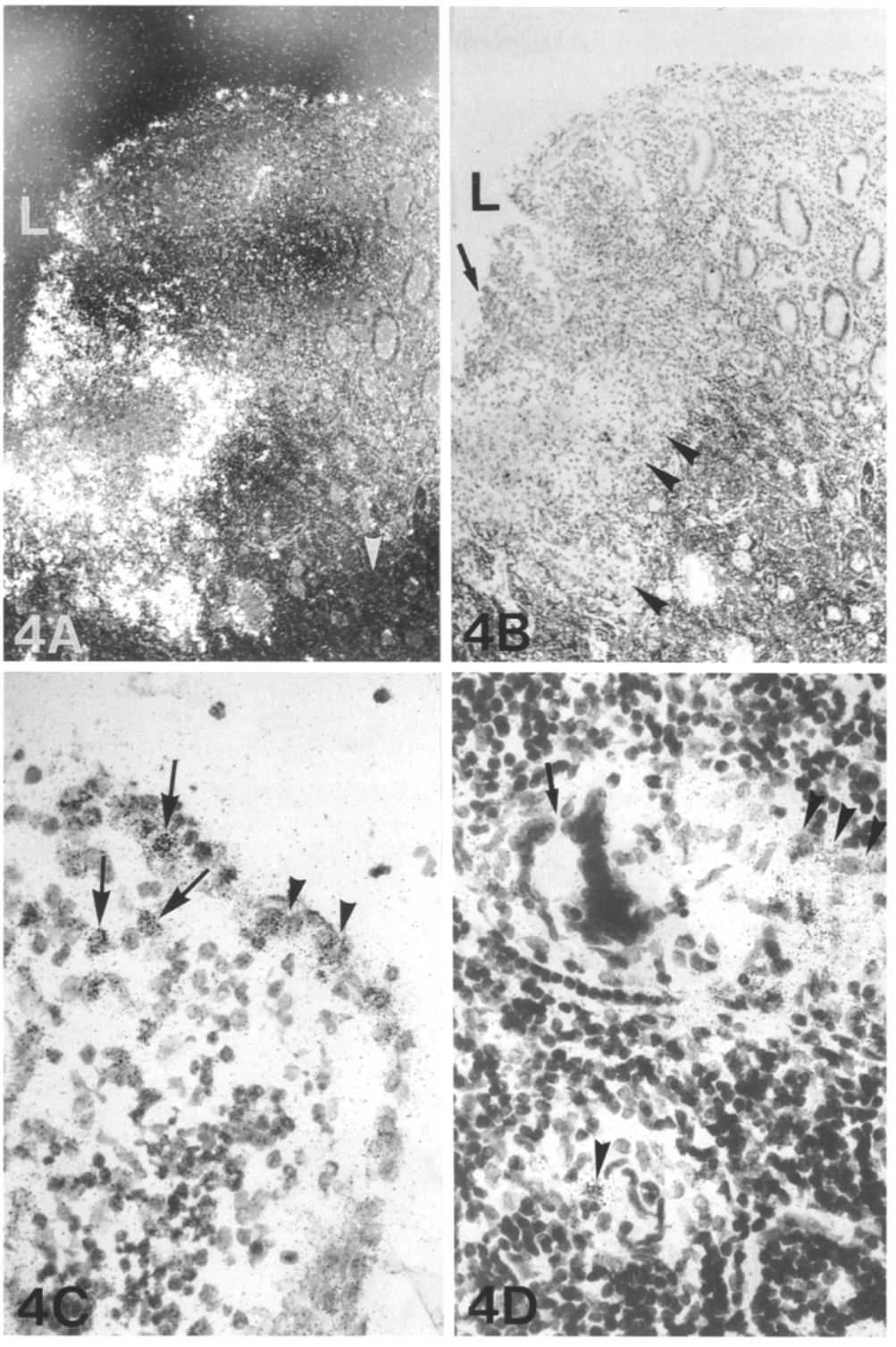


Fig. 4A–D IL-1β mRNA expression in Crohn's disease. **A**, **B** Mucosal ulceration in CD with decoration by bandlike accumulations of IL-1β mRNA-expressing cells. While signals are more easily visible in the dark field in this low magnification (**A**), the corresponding bright field (**B**) suggests that both endoluminal granulocytes (*arrow*) as well as adjacent mononuclear phagocyte-like cells with large pale nuclei (*arrowheads*) contribute to IL-1β production (*L* gut lumen). Lymphocytes are largely negative (*arrowhead* in **A**). ISH; **A**, dark field; **B**, bright field; colon, ×80. **C** Demonstration of IL-1β mRNA-positive cells (*arrows*) in the inflamed lamina propria in CD. Note that besides subepithelial cells some cells within the epithelial layer also contain a signal (*arrowheads*) with black grains. ISH, bright field, colon, ×400. **D** Submucosal granuloma in CD with IL-1β mRNA-expressing epithelioid- and macrophage-like cells (*arrowheads*) in the vicinity of a negative multinucleated giant cell (*arrow*). ISH, bright field; colon, ×400.

of 61.5% (range 37–66%) of Ki-67-immunoreactive cells in the inflamed lamina propria were CD3/Ki-67 double-positive T-cells (Fig. 1C).

Cytokine mRNA expression

Like Ki-67 expression, cytokine mRNA expression in noninflamed control gut was also found to be restricted to organized lymphoid follicles. The diffuse lamina propria outside such sites remained negative as did the deeper layers of the gut wall. We found low numbers (generally <2%) of cells containing mRNAs for IL-1β, IL-6, IL-2, IL-4 and IFN-γ, whereas IL-5 and TNF-α were negative in these cases. IL-1β was expressed focally by subepithelial cells of organized follicles, both in ileum and colon (Fig. 2). IL-6, IL-2, IL-4 and IFN-γ were the

product of single, randomly distributed follicular cells. Among these, IL-4 mRNA was expressed exclusively inside germinal centres (Fig. 3). IL-6 was an exception in that this message was expressed focally by elements of the myenteric plexus and isolated peri- and endovascular cells in the submucosa and deeper layers of the gut wall (not shown). These cells produce the somewhat higher median score for IL-6 in control cases when compared with other cytokines (Table 1).

In contrast to the controls, gut from CD patients contained significantly increased numbers of cytokine mRNA-producing cells, most prominently IL-1β and IL-6, and reduced numbers (<10%) of cells producing mRNA for IFN-γ and TNF-α ($P<0.05$). Expression of these messages was related to inflammatory cell infiltrates characteristic of the disease. Organized lymphoid complexes in CD showed a similar pattern of cytokine expression to the controls. Strong expression of IL-1β, IL-6, TNF-α and IFN-γ was found especially at sites of epithelial destruction such as aphthoid lesions and fissures or larger ulcerations, followed by submucosal diffuse or patchy inflammatory infiltrates in nonulcerated areas. In comparison with these sites, the inflamed intact lamina propria in CD contained reduced numbers of mRNA-producing cells (not TNF-α), with foci of positive cells intermingled with negative areas, thus again creating a pattern reminiscent to the expression of Ki-67 antigen. In IL-1β and TNF-α, mRNA expression tended to increase with disease activity (median score 1.0 and 0 in cases with low/intermediate activity versus 3.0 and 1.0 in cases with high activity, respectively).

At ulcerative sites, IL-1β mRNA was strongly expressed by bandlike accumulations of intensively labelled cells (Fig. 4A). Morphological analysis suggests that both endoluminal granulocytes and mononuclear phagocytes might contribute to IL-1β production (Fig. 4B). In the inflamed lamina propria, the message was focally expressed by subepithelial cells, most with large, pale nuclei (Fig. 4C). Very occasionally, some cells in the epithelial layer were also positive, probably representing intraepithelial lymphocytes or macrophages (Fig. 4C). The epithelia were negative for other cytokines. Epithelioid-like cells in granulomas regularly expressed IL-1β mRNA (multinucleated giant cells remained negative for this and other cytokines; Fig. 4D).

When compared with IL-1β, IL-6 mRNA was more widely distributed, especially within the inflamed submucosa. It did not show such a predominance for the subepithelial regions. Comparative staining with CD68 suggested that mononuclear phagocytes might produce IL-6 (Fig. 5A, B) whereas neutrophils were negative. Some submucosal vessels contained IL-6-expressing cells in the endothelial layer (not shown). TNF-α mRNA was found only in CD cases with high inflammatory activity. In contrast to IL-1β and IL-6, TNF-α was not found in the inflamed intact lamina propria. IFN-γ mRNA was present both at destructive and nondestructive sites, including the inflamed lamina propria (Fig. 6A, B).

Compared to these cytokines, IL-2 and IL-4 mRNAs were expressed by even lower numbers (IL-2<0.6%; IL-4<0.2%; median score 0) of MNC. IL-4 mRNA was again found exclusively within follicular germinal centres without a significant increase when compared with controls. In part, the same was true for IL-2 mRNA (Fig. 7A), but in addition to organized follicles IL-2 mRNA was also found to be produced by small numbers of nonfollicular inflammatory cells in the submucosa and/or diffuse lamina propria of CD tissues (Fig. 7B), which represents a striking difference to noninflamed gut tissue, where IL-2-positive cells were not found at such

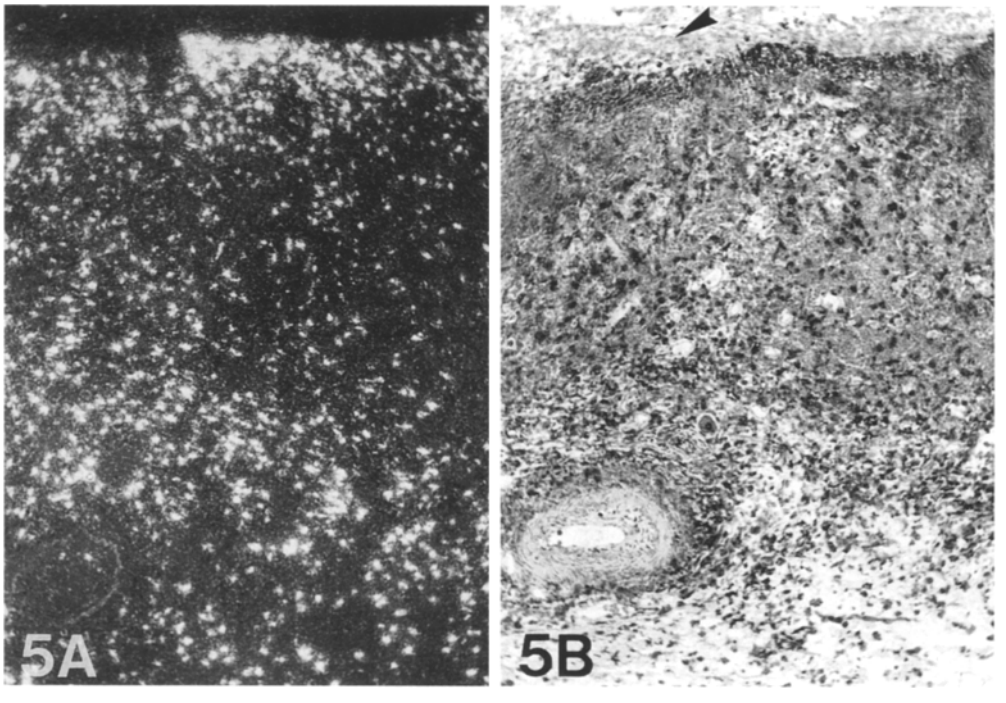


Fig. 5A, B IL-6 mRNA expression in CD. **A** Inflamed gut tissue adjacent to an ulceration in CD with demonstration of numerous IL-6 mRNA-positive cells distributed throughout all layers of the bowel wall. ISH; dark field; colon, ×80. **B** Immunohistochemical staining of CD68 on a parallel section reveals a similar staining pattern and comparison of **A** and **B** suggests that a high proportion of mononuclar phagocytes might contribute to IL-6 production. Note that neutrophils at the luminal border are negative for CD68 (*arrowhead* in **B**) and do not express IL-6 mRNA (not visible in **A**). Colon, APAAP, ×80

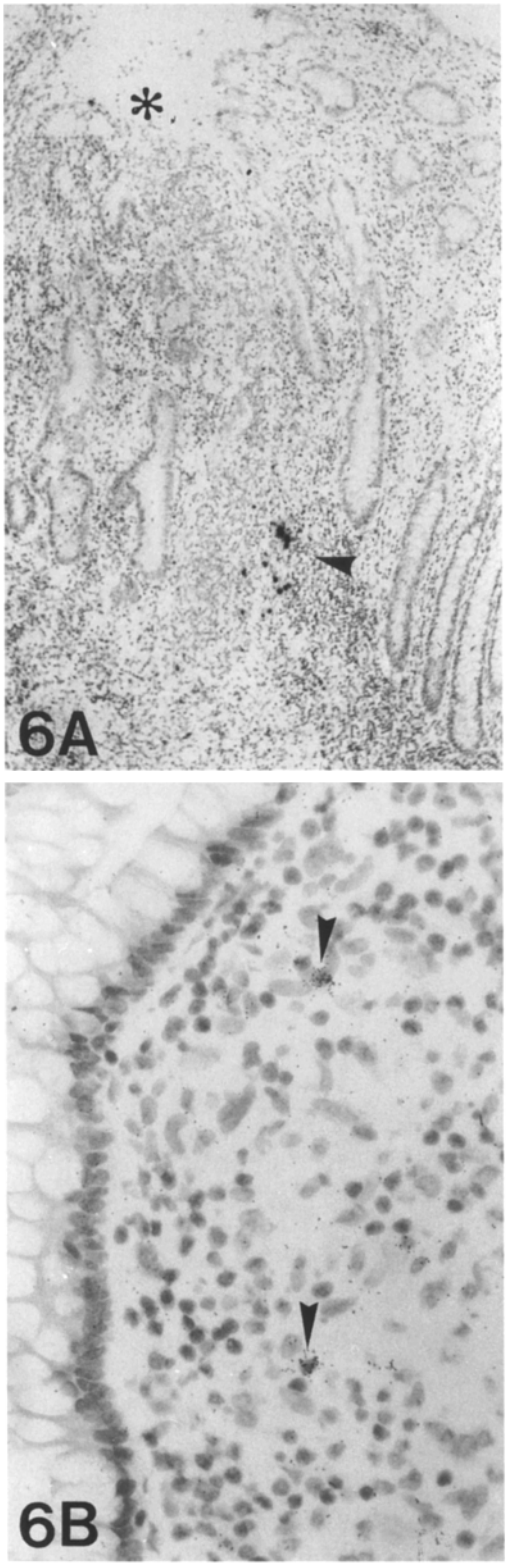


Fig. 6A, B IFN-γ mRNA expression in CD. **A** Inflamed lamina propria in CD with demonstration of IFN-γ mRNA-expressing cells at a site of fissural ulceration (*arrowhead*, position of the fissure indicated by an *asterisk*). ISH; bright field, colon, ×80. **B** Two IFN-γ-expressing cells within the intact inflamed lamina propria (*arrowheads*). ISH; bright field; colon, ×500

sites. If we exclude organized follicles from the quantitative analysis, this circumstance creates a significant difference between CD and control cases ($P<0.05$).

The morphological features of positive cells suggested that IL-2, IL-4 and IFN-γ might be produced mainly by lymphocytes and TNF-α by lymphocytes and mononuclear phagocytes. However, the need for sense control slides and the use of cryostat material with intermediate loss of sections during cutting limited the comparison of immunohistochemical stainings with ISH and definite immunophenotyping of mRNA-expressing cells could not be performed.

ISH for IL-5 mRNA yielded negative results in CD tissues, although we demonstrated cells expressing IL-5 and other cytokines in the cytospins constantly (not shown).

Discussion

Our results show that mRNAs for proinflammatory cytokines, especially IL-1β and IL-6, are strongly expressed in situ in CD and largely predominate over local production of mRNAs for lymphokines such as IL-2, IL-4 or IFN-γ. Our in situ data confirm previous studies reporting an enhanced production or activity of IL-1, IL-6 or TNF-α in CD [7, 12, 18, 20, 21, 22, 25, 26, 30, 43] and correspond to a recent study demonstrating lysozyme and TNF-α producing cells by mRNA-ISH in CD tissues [8]. TNF-α was recently also demonstrated in CD tissues by immunohistochemistry [29].

The different expression patterns of IL-1β and IL-6 mRNA observed by us indicate that heterogeneous subtypes of cells, presumably mononuclear phagocytes, might preferably produce these cytokines in vivo. IL-1β was the main message produced within granulomas characteristic of the disease. IL-1β, IL-6 and TNF-α can induce numerous inflammatory activities which are of relevance in the pathogenesis of CD, such as changes in vascular permeability, activation of inflammatory cells including T- and B-cells, production of inflammatory eicosanoids [9], activation of destructive enzymes and induction of the hepatic acute phase response, fever, anorexia and weight loss (reviewed in [39]). These cytokines induce the production of chemokines (IL-8, monocyte chemotactic factor and others) and, together with IFN-γ, can induce adhesion molecules like intercellular adhesion molecule-1, vascular cell adhesion molecule-1 or E-selectin, which might additionally amplify the local immune response [33, 40]. Recent work has shown an enhanced expression of these adhesion structures at inflammatory sites in CD, which provides a molecular basis for the longstanding accumulation of inflammatory cells in the bowel wall in chronic stages of the disease [2, 23]. Since the majority of MNC infiltrating the gut wall in CD do not express proliferation markers according to our results, it seems likely that the accumulation of these cells may, at least in part, be the result of an adhesion-mediated increased influx from the peripheral blood rather than the result of local cell proliferation.

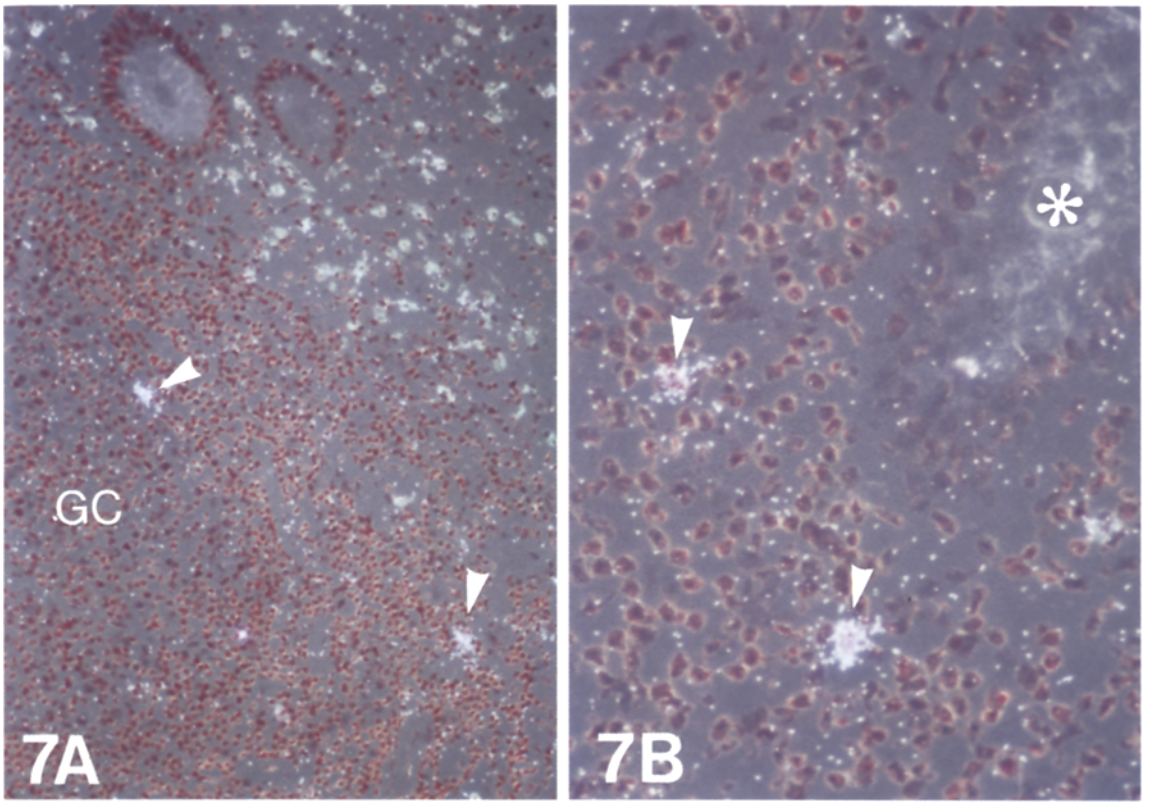


Fig. 7A, B IL-2 mRNA expression in CD. **A** Mucosal follicle in a case of CD with two IL-2 mRNA-expressing cells, one at the border of a germinal centre (*GC*) and one in the outer follicular area (*arrowheads*). Note the low background with mucosal eosinophils displaying green granules in the dark field, which should not be confused with autoradiographic signals (white grains on the two positive cells). ISH; dark field; ileum, ×200. **B** Two IL-2 mRNA-expressing cells are present in the inflamed intact lamina propria in CD (*arrowheads*, crypt indicated by an *asterisk*). ISH; dark field; ileum, ×500

Nevertheless, proliferation of MNC occurs in CD and includes local inflammatory T-cells, as we show in this study. Our finding of enhanced Ki-67 antigen expression on submucosal and lamina propria T-cells in combination with the demonstration of cells producing mRNA for IL-2 and IFN-γ in the same tissues are highly indicative of a T-cell-mediated immune response occurring in situ within diseased areas. In view of the fact that in our experiments IL-5 was negative and that we could detect only limited amounts of IL-4 mRNA-producing cells exclusively at follicular sites, it seems reasonable to speculate that a delayed-type hypersensitivity reaction possibly mediated by T-helper 1-like cells [41] is proceeding locally. This has also been suggested in a recent report demonstrating IFN-γ and IL-2-producing lymphocytes among MNC isolated from diseased tissue using a sensitive reverse haemolytic plaque assay [6]. Our results comply with findings of increased levels of IL-2 in sonicated gut biopsies [7] and are in agreement with findings of increased levels of IL-2 mRNA in gut tissue of CD patients using reverse PCR [27]. Concerning IL-4 and IL-5 mRNA expression, our data are supported by a recent preliminary PCR study which yielded similar results [28].

Our findings of increased levels of IFN-γ mRNA-producing cells in CD support recent work demonstrating spontaneous release of IFN-γ by intestinal MNC in CD, but not controls [14]. Because IFN-γ is a potent inducer of HLA class II antigens [3], this cytokine might be mainly responsible for the increased expression of these antigens on mucosal epithelium in CD [13]. Since IFN-γ is a very potent macrophage activator [31], it might also be involved in mediating the extensive in situ production of preinflammatory cytokines observed by us in CD.

In this context it should be taken into consideration that the tissues analysed by us derived from patients with active, often longstanding disease. Thus, we investigated the established, chronic inflammatory state in CD including its consequences (focal destruction with loss of intestinal barrier functions, secondary phenomena such as fibrosis) and our results might not necessarily also reflect the situation in the initial stages of the disease.

Our data on Ki-67 antigen expression in noninflamed gut support recent in vitro observations made by our laboratory and others [11, 36], that under normal conditions lamina propria T-cells have a low proliferative capacity. We have recently shown that the intestinal mucosa contains environmental, soluble nonprotein factors with oxidative properties which are likely to participate in mediating this downregulated functional status [37]. The physiological reason for this downregulation may be to prevent harmful effects on local tissue from proliferating MNC, which are under constant exposure to dietary anti-

gens, and to prevent unwanted cytokine production and cytolytic activities (which should be restricted to inductive follicular sites). Our findings support such a concept of compartmentalization into inductive and effector sites. One might speculate that the occurrence of proliferating T-cells within the inflamed lamina propria in CD represents a partial abrogation of this normal downregulating lamina propria activity, and recent results from our laboratory showing an enhanced proliferative response of lamina propria T-cells to triggering of the T-cell receptor/CD3 complex in CD would support such a view [38]. Perhaps impaired downregulating lamina propria activity, leading to enhanced T-cell responsiveness to antigens, results in increased IFN-γ production. Subsequent activation of MNC to secrete proinflammatory cytokines might contribute to chronic inflammation in CD.

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