Diversion of intestinal flow decreases the numbers of interleukin 4 secreting and interferon γ secreting T lymphocytes in small bowel mucosa

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Abstract

Background/Aims—The intestinal immune system faces large amounts of antigens, and its regulation is tightly balanced by cytokines. In this study, the effect of intestinal flow diversion on spontaneous secretion of interleukin (IL)-4 and interferon (IFN)- γ was analysed.

Methods—Eight patients (two with Crohn's disease, four with ulcerative colitis, and two with previous colon cancer) carrying a double lumen small bowel stoma after a total colectomy procedure were included in the study. For each patient, eight biopsy samples were taken endoscopically from both the diverted and non-diverted part of the small bowel. Intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) were isolated separately and assayed for numbers of cells spontaneously secreting IL-4 and/or IFN-γ by an ELISPOT technique.

Results—Compared with the non-diverted mucosa, a significant decrease in the number of spontaneously IFN-γ secreting CD3 lymphocytes was observed in the diverted small bowel mucosa among both IELs (p = 0.008) and LPLs (p = 0.007). The same results, although less significant, were obtained for IL-4, especially in LPLs (p = 0.01).

Conclusion—The intestinal content influences the spontaneous secretion of IFN-γ and IL-4 by intestinal lymphocytes. These results could help to elucidate the anti-inflammatory role of split ileostomy in patients suffering from inflammatory bowel diseases.

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Keywords: intestine; T lymphocytes; mucosa; interleukin-4; interferon-γ; ELISPOT

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The gut mucosa faces large amounts of alimentary and microbial antigens and the mucosal immune system has to cope efficiently with this considerable antigenic load. Locally secreted cytokines are of fundamental importance for the regulatory mechanisms. We have recently shown that, in the absence of in vitro stimulation, the healthy intestine contains significant numbers of interleukin (IL) 4 secreting and interferon (IFN) γ secreting lymphocytes.[1] This observation is in agreement with what has been called "physiological inflammation in the normal gut".[2–4] In fact, other observations are also consistent with the notion of a primed inflammatory immune response characterising the healthy intestinal mucosa. These include large numbers of CD45RO-expressing memory T cells and high levels of expression of CD69 and HLA-DR molecules.[5 6]

Remaining key questions include the identification of the triggers for this low grade inflammation of the disease-free intestine, the regulatory mechanisms that control this inflammation, and the factors involved in the pathogenesis of excessive inflammatory responses encountered in various bowel diseases. In coeliac disease, it is known that alimentary gliadin peptides induce abnormal immune responses of the gut mucosa. In inflammatory bowel disease, several observations and experiments suggest that the mucosal lymphoid tissue responds abnormally to the local antigenic load, although the specific antigens have not yet been identified. In Crohn's disease, it is well established that postoperative recurrence in patients who carry a split ileostomy affects the diverted part of the small bowel much less than the non-diverted part.[7] The possible role of some constituent of the luminal flow in the stimulation of mucosal immune cells has recently been suggested in a study showing the occurrence of early histological and ultrastructural changes after infusion of intestinal contents in the diverted ileum.[8]

As IFN-γ is a key immunomodulating cytokine in mucosal immune responses,[9 10] an understanding of the mechanisms involved in the regulation of its spontaneous secretion may be of particular importance. IL-4 has also been implicated in the pathogenesis of early inflammation in Crohn's disease.[11] To assess the possible role of intestinal flow in triggering spontaneous cytokine secretion by intestinal lymphocytes, we studied the numbers of cells spontaneously secreting IL-4 and/or IFN-γ in patients carrying a split ileostomy, within both the diverted and non-diverted mucosa.

Materials and methods

PATIENTS AND SAMPLES

Eight patients (five women, three men; median age 46 years (range 28–65)) underwent a total colectomy with a temporary split ileostomy. The indications for total colectomy were stenosing colon Crohn's disease (n = 2), severe

ulcerative colitis (n = 4), and right colon cancer with severe diverticulosis (n = 2). Split ileostomy is a rare surgical procedure chosen by the surgeon only when there are problems with healing of the ileoanal anastomosis. For this reason, the number of patients included in this study was rather low. However, even for patients with fairly divergent initial pathology, the surgical procedure applied was identical, allowing us to analyse the effect of the intestinal flow diversion on spontaneous cytokine secretion regardless of the initial pathology. It is noteworthy that none of these patients was under immunosuppressive drugs or antibiotics at the time of investigation. All gave informed consent, and the local ethical committee approved the study.

Eight biopsy specimens from both the diverted and non-diverted small bowel mucosa were obtained from each patient. In five patients, the specimens were taken during routine endoscopy before reconstructive surgery. The indication for endoscopy was to assess the endoscopic appearance of the small bowel mucosa before re-anastomosing the two parts of the stoma. For the other three patients, the specimens were taken during the operative reconstructive procedure. Biopsy samples were obtained six to eight weeks after intestinal resection in seven patients and four weeks after intestinal resection in one patient. The endoscope was successively passed through the two parts of the stoma and specimens were taken at 10–15 cm from the margin. One specimen from each part was used for histological staining. Histological inflammation was assessed according to the villus/crypt ratio, mucosal oedema, epithelial and lamina propria infiltration, and the presence of erosions or ulcers.

ISOLATION OF INTESTINAL LYMPHOCYTES

Intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) were isolated as previously described.[1] Briefly, the biopsy specimens were washed and incubated in Iscove's medium supplemented with 40 μg/ml gentamicin, 10% fetal calf serum, 50 mM 2-mercaptoethanol, and 1 mM EDTA (Sigma Chemical Co, Bornem, Belgium) for one hour at 37°C with continuous stirring. IELs and epithelial cells were collected in the supernatant. Histological examination of the remaining fragment disclosed that the villous and lamina propria structures were still preserved, whereas all the cells within the epithelium had disappeared using this procedure. In addition, the antigen defined by the HML-1 antibody (CD103), the best phenotypic marker of

IELs,[12] was expressed on more than 90% of the lymphocytes in the supernatant fraction.

The remaining fragments were then cut into small pieces and incubated with stirring for one hour at 37°C in Iscove's medium containing 1 mg/ml collagenase-dispase (Boehringer, Mannheim, Germany) for cytokine analysis (by an ELISPOT technique), and for 150 min at 37°C in collagenase type I (Sigma Chemical Co) only, for the phenotypic analysis of lymphocytes by flow cytometry. LPLs were collected in the supernatant. For both suspensions, the numbers of mononuclear cells were counted using a Bürker chamber, and the percentages of CD3 lymphocytes were estimated by flow cytometry.

PHENOTYPIC ANALYSIS OF INTESTINAL T LYMPHOCYTES

Phenotypic characterisation of the intestinal lymphocytes was performed by flow cytometry on isolated IELs and LPLs. Cell suspensions were directly resuspended in complete Iscove's medium at 1×10^6 cells/ml, and 100 μl of the cell suspension was incubated with 5 μl anti-CD3 peridinin chlorophyll *a* protein, anti-CD4 fluorescein isothiocyanate (FITC), anti-CD8 phycoerythrin, anti-CD25 FITC or anti-CD69 PE monoclonal antibodies (Becton Dickinson) at 4°C for 30 minutes in the dark. After staining, cells were washed, and at least 5000 cells were analysed by flow cytometry (FACScan; Becton Dickinson, San Jose, California, USA). Lymphocyte populations were gated by forward sideward scatter light and by gating for CD3 cells. Results are given as percentages of positive cells per CD3 T lymphocytes.

ENUMERATION OF CYTOKINE SECRETING CELLS

Cell suspensions were assayed for numbers of cells secreting IL-4 and/or IFN-γ by a two site reverse enzyme linked immunospot technique (ELISPOT) using as capture and developing reagents epitope specific mouse anti-human cytokine monoclonal antibodies, as previously described.[1] Briefly, individual wells of nitrocellulose bottomed 96-well millititre HA plates (MAHAN 4550; Millipore, Eschborn, Germany) were coated at 37°C for three hours with 100 μl of an adequate dilution of anti-cytokine monoclonal antibody (clone 1/D1K for IFN-γ (10 μg/ml; Chromogenix, Stockholm, Sweden) and clone 82-2 for IL-4 (10 μg/ml; Endogen, Woburn, MA, USA)). After the plates had been washed, blotted dry, blocked (10% fetal calf serum and 2% (w/v) milk powder in phosphate buffered saline), washed again and blotted dry

Table 1   CD3 T lymphocyte subsets in non-diverted and diverted intestinal mucosa

	IELs			LPLs		
	Non-diverted		Diverted	Non-diverted		Diverted
CD4	34.4 (16.7–63.1)	NS	43.4 (20.8–58.5)	60.5 (43.2–79.3)	NS	61.7 (61.7–76.0)
CD8	73.7 (54.8–84.4)	NS	74.9 (61.6–79.9)	52.0 (41.2–70.5)	NS	55.0 (42.1–70.9)
CD4CD8	15.5 (6.3–19.7)	NS	14.4 (4.7–21.7)	15.6 (9.8–24.2)	NS	15.7 (11.8–20.2)
CD4⁻CD8⁻	6.8 (2.3–9.5)	NS	6.5 (1.8–10.5)	2.3 (2.0–6.9)	NS	4.2 (2.2–6.2)

Results are expressed as median (range) of percentages of positive lymphocytes. The number of patients tested in each case was eight. IEL, intraepithelial lymphocyte; LPL, lamina propria lymphocyte.

Table 2   Lymphocyte activation markers in non-diverted and diverted intestinal mucosa

	IELs		LPLs	
	Non-diverted	Diverted	Non-diverted	Diverted
Colon cancer 1				
CD25	0.5	0.2	1.6	2.2
CD69	99.5	98.9	97	93.4
Colon cancer 2				
CD25	2.0	0	2.6	1.6
CD69	98.5	99.5	90.2	91.2
Ulcerative colitis				
CD25	1.3	0	0.7	0.4
CD69	99.2	99.3	98.4	97.8
Crohn's disease				
CD25	1.3	0.4	ND	ND
CD69	90.3	96.4	ND	ND

Results expressed as percentage of CD3 lymphocytes expressing the activation marker.
IEL, intraepithelial lymphocyte; LPL, lamina propria lymphocyte; ND, not determined.

again, 100 μl of different dilutions of cell suspensions was dispensed into the coated wells. The plates were incubated under 5% CO_2 for 20 hours at 37°C, followed by extensive washing. Biotinylated anti-cytokine monoclonal antibodies were then added (100 μl/well) for three hours at 20°C (anti-IFN-γ: clone 7/B6/1, 3 μg/ml, Chromogenix; anti-IL-4: clone 12–1, 1 μg/ml, Mabtech, Stockholm, Sweden). Spots were developed by the addition of extravidin-horseradish peroxidase (2 μg/ml; Sigma) followed by the substrate solution (3-amino-9-ethylcarbazole; Sigma). The reaction was stopped by a brief wash of the plates with tap water, and spots were counted under low magnification (× 50). Twofold serial dilutions were tested for each sample, and the

spots were counted in all wells that contained less than 200 spots. The spots in these wells were added up for each sample and then reported as numbers of cells secreting IL-4 or IFN-γ per 10^6 total number of cells or per CD3 cells. To avoid interobserver variations, all the plates were read by the same investigator.

STATISTICAL ANALYSIS
Non-parametric paired two tailed Wilcoxon U test was used to evaluate comparative statistical significance.

Results

ENDOSCOPIC APPEARANCE AND HISTOLOGICAL EVALUATION
The endoscopic appearance of the mucosa was normal in both the diverted and non-diverted parts of the small bowel in all the patients. Similarly, we did not find any major differences in the histological appearance between diverted and non-diverted small bowel mucosa.

T LYMPHOCYTE SUBTYPES
As shown in table 1, CD3 T lymphocyte subtypes were not significantly different between the diverted and non-diverted mucosa with regard to CD4, CD8, CD4CD8, CD4⁻CD8⁻ T cells. Analysis of the lymphocyte surface expression of these markers showed that the proportion of CD4 lymphocytes was higher among LPLs than among IELs (60.5% (43.2–79.3%) v 34.4% (16.7–63.1%) in the non-diverted mucosa (p = 0.03) and 61.7% (37.1–76.0%) v 43.4% (20.8–58.5%) in the diverted mucosa (p = 0.03)).
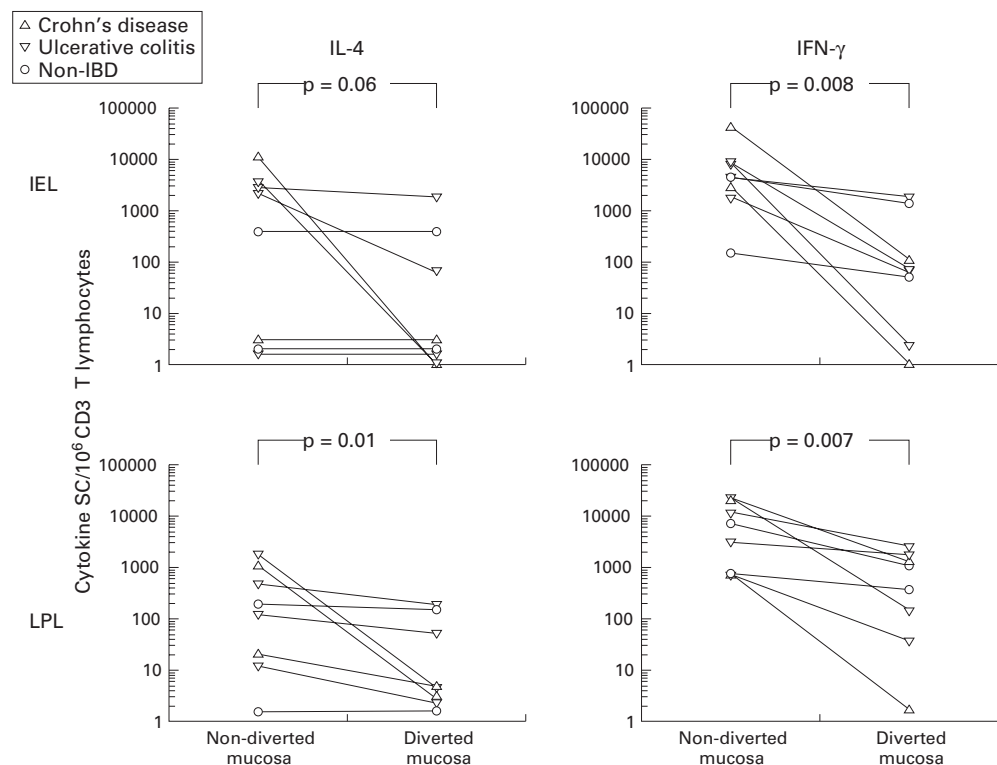

Figure 1   Numbers of cells spontaneously secreting interferon (IFN) γ or interleukin (IL) 4/10⁶ intraepithelial lymphocytes (IELs) or lamina propria lymphocytes (LPLs) in diverted and non-diverted small bowel mucosa of patients with Crohn's disease or ulcerative colitis, or patients without inflammatory bowel disease (IBD). Results from the same patients are linked. The degree of significance was evaluated with the paired Wilcoxon test. SC, secreting cells.
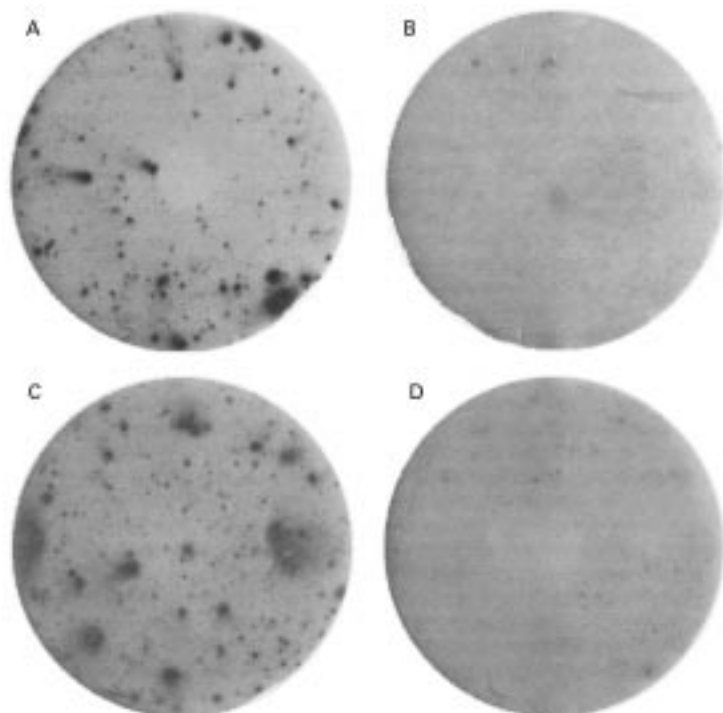
Figure 2   Interferon (IFN) γ ELISPOT for intestinal lymphocytes from a patient with ulcerative colitis carrying a split ileostomy. Upper part: intraepithelial lymphocytes from non-diverted mucosa (A) representing 10 000 IFN-γ secreting cells per 10⁶ CD3 T lymphocytes and from diverted mucosa (B) where no IFN-γ secreting cells could be observed. Lower part: lamina propria lymphocytes from non-diverted mucosa (C) representing 27 000 IFN-γ secreting cells per 10⁶ CD3 T lymphocytes and from diverted mucosa (D) representing 124 IFN-γ secreting cells per 10⁶ CD3 T lymphocytes.

Similarly, the proportion of lymphocytes expressing the early activation marker CD69 was the same on both normal and diverted mucosa and was high. In contrast, the proportion of lymphocytes expressing the IL-2 receptor (CD25) was low and it tended to be slightly lower in the diverted mucosa compared with the non-diverted mucosa (table 2). However, no statistical analysis of these results on lymphocytes expressing activation markers could be performed as a consequence of the low number of patients.

SPONTANEOUS CYTOKINE SECRETION
A significant decrease in the numbers of spontaneously IFN-γ secreting cells was observed in the diverted mucosa compared with the non-diverted mucosa, among both IELs and LPLs (fig 1). The median numbers of IFN-γ secreting cells among the IELs fell from 4018 per 10^6 CD3 cells (range 148–40259) to 60 per 10^6 CD3 cells (range 0–1603) (p = 0.008) and among the LPLs from 4435 per 10^6 CD3 cells (range 597–19360) to 641 per 10^6 CD3 cells (range 0–2066) (p = 0.007) (fig 1). A representative example is illustrated in fig 2 comparing the aspect of the ELISPOT wells for IFN-γ in non-diverted and diverted mucosa, for both IELs and LPLs.

With regard to spontaneous IL-4 secretion, the differences between diverted and non-diverted mucosa were less notable. For three patients, no IL-4 secreting cells were detected among the IELs and for one of them no IL-4 secreting cells were detected among the LPLs.

However, the number of spontaneously IL-4 secreting cells decreased in the diverted mucosa compared with the non-diverted mucosa in those patients that contained IL-4 secreting cells in the non-diverted mucosa (fig 1). Among the LPLs, the median number of IL-4 secreting cells fell from 149 per 10^6 CD3 cells (range 0–1626) in the non-diverted mucosa to 0 per 10^6 CD3 cells (range 0–182) in the diverted mucosa (p = 0.01).

Discussion
We have previously shown that, in the absence of any stimulation, the healthy intestine contains significant numbers of IFN-γ secreting and/or IL-4 secreting cells, with an IFN-γ/IL-4 ratio of about 3. These cells have been identified as T lymphocytes.[1] As many of the intestinal lymphocytes are memory T cells expressing the CD45RO molecule and as most of them also express the activation markers CD69 and class II molecules,[1 4 5] most resident human gut T lymphocytes seem to be terminally differentiated into activated Th1-type T cells primed to secrete IFN-γ. However, the reason why the normal intestine is constantly in a state of controlled inflammation remains unknown, as are the mechanisms that normally control these potentially inflammatory cells and which are altered in patients with inflammatory bowel diseases.[2–4]

In this study, we compared spontaneous IFN-γ and IL-4 secretion in intestinal lymphocytes from the diverted and non-diverted parts of the intestinal mucosa from patients carrying a split ileostomy. We found that the numbers of IFN-γ secreting cells, among both IELs and LPLs, are significantly decreased in the diverted mucosa compared with the non-diverted mucosa. Although the differences are less notable for IL-4, the numbers of IL-4 secreting cells also significantly fell in all those patients in whom a basal level of this cytokine could be detected. We therefore conclude that the numbers of both IFN-γ secreting cells and IL-4 secreting cells are significantly lower in the diverted mucosa than in the non-diverted mucosa, even though there was a wide range in the numbers of cytokine secreting cells. However, this range was comparable with that previously reported by us in the normal human colon.[1] The same results were obtained for patients with or without an inflammatory bowel disease. Therefore, even if the patients enrolled present with heterogeneous pathologies, with most of them suffering from an inflammatory bowel disease, our findings support the role of the luminal flow as a trigger for cytokine secretion by intestinal lymphocytes.

The decrease in cytokine secretion observed in the diverted bowel may be a more general phenomenon, as the numbers of IL-10 secreting cells and tumour necrosis factor (TNF)-α secreting cells also decrease as a consequence of diversion of the luminal flow (our unpublished observations). However, the results are less significant for IL-10 and TNF-α than for IFN-γ and IL-4, and they are more difficult to interpret, as monocytes are probably a major

source of IL-10 and TNF-α. In this paper we therefore focused on IFN-γ and IL-4 as we have previously shown that T lymphocytes represent the cellular source of these two cytokines when they are secreted spontaneously within the intestinal mucosa.[1]

In contrast with the cytokine secretion, expression of the activation marker CD69 was not different on intestinal lymphocytes from the diverted mucosa compared with those from the non-diverted mucosa, supporting the notion that cytokine secretion and CD69 expression at the cell surface feature qualitatively distinct properties of activated cells.[1] The expression of CD25 on T lymphocytes could be somewhat better correlated with their spontaneous cytokine secretion, as results obtained for some patients suggested a small decrease in CD25 expression on T lymphocytes within the diverted mucosa. We have already previously shown that the expression of CD25 on T lymphocytes is correlated with the numbers of cells spontaneously secreting IL-4 or IFN-γ in Crohn's disease when inflamed and non-inflamed mucosa are compared in the same patient.[13]

According to the results reported in this paper, we suggest that intestinal components contribute to the "physiological inflammation" of the normal gut, even if, for ethical reasons, the effect of diversion of the intestinal flow cannot be analysed in normal subjects. Previous studies have already incriminated both alimentary and microbial antigens in the triggering of pathological immune responses in the gut.[14–18] Evidence that intestinal components contribute to the inflammatory process in inflammatory bowel diseases has been provided by several clinical and histopathological studies. Split ileostomies have indeed been successfully used to treat inflammatory bowel diseases, and it has been shown that diversion of the faecal stream proximal to inflamed mucosa may diminish the inflammatory activity.[19–24] Rutgeerts and colleagues[25] demonstrated the role of the faecal stream in the pathogenesis of Crohn's disease by comparing the occurrence of new inflammatory lesions in patients with Crohn's disease carrying or not a split ileostomy. Furthermore, by instilling the effluent into the diverted intestinal part, Harper and colleagues[26] showed that factors greater than 0.22 μm in the faecal stream are responsible for the maintenance and exacerbation of inflammation in Crohn's disease. More recently, D'Haens and colleagues[8] demonstrated an increase in epithelial HLA-DR expression after infusion of the intestinal content into diverted ileum in patients with Crohn's disease. As HLA-DR expression by epithelial cells is upregulated by IFN-γ, this increased expression induced by the intestinal content could be a consequence of the induction of IFN-γ secretion by the intestinal content.

Our results may contribute to the understanding of why the diverted part of the small bowel is less affected than the non-diverted part in postoperative recurrence among patients carrying a split ileostomy.

Inflammatory bowel diseases are characterised by a breakdown of the controlled inflammation of the normal gut. This controlled inflammation is associated with a discrete balance in the secretion of inflammatory cytokines, namely IL-1, IL-2, TNF-α, and IFN-γ, and the secretion of anti-inflammatory cytokines, namely IL-4 and transforming growth factor-β.[27–32] However, the numbers of both IFN-γ secreting and IL-4 secreting cells were so dramatically reduced by the diversion of the intestinal flow that the residual levels of IFN-γ secreting cells are not expected to induce inflammatory effects. Therefore the absence of inflammation in the diverted part of the small bowel is probably directly related to the very low levels of IFN-γ secreting cells, rather than to an effect on the balance between inflammatory and anti-inflammatory cytokines.

In conclusion, the present study sheds light on the role of luminal flow as a trigger of physiological IFN-γ and IL-4 secretion by intestinal lymphocytes. Further studies are needed to investigate its effect on the secretion of other regulatory cytokines and to identify the key antigens associated with spontaneous cytokine secretion by intestinal lymphocytes.

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