Flow cytometric analysis of gut mucosal lymphocytes supports an impaired Th1 cytokine profile in spondyloarthropathy

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Abstract

***Objective*—To quantify the fraction of gut mucosal lymphocytes expressing the T helper type 1 (Th1) cytokines, interferon γ (IFNγ) and interleukin (IL)2, and the Th2 cytokines, IL4 and IL10, at the single cell level in patients with spondyloarthropathy (SpA) in comparison with healthy controls.**

***Methods*—An improved extraction protocol was used for the enrichment of intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) from colonic and ileal biopsy specimens obtained from patients with SpA (n=20) and healthy controls (n=13). After stimulation with phorbol ester/ionomycin, expression of the intracellular cytokines IFNγ, IL2, IL4, and IL10 was determined in CD3+, CD3+CD8+ and CD3+CD8− T cells by flow cytometry.**

***Results*—In colonic LPLs, a significant decrease in IFNγ-producing CD3+ cells was observed (p=0.02) in patients with SpA. In the CD3+CD8− subset, the proportion of cells producing IFNγ and IL2 was decreased in patients with SpA (p=0.021 and p=0.027 respectively). In ileal LPLs, the percentage of IL10-producing CD3+CD8− cells was significantly increased (p=0.046).**

***Conclusion*—An impaired Th1 cytokine profile is observed in gut mucosal lymphocytes from patients with SpA. This adds to the existing evidence that the gut mucosal immune apparatus is involved in the pathogenesis of SpA.**

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The concept of spondyloarthropathy (SpA) assembles a group of related chronic autoimmune diseases of the joint with common clinical, radiological, and genetic characteristics.[1] Entities belonging to this concept are ankylosing spondylitis, reactive arthritis, psoriatic arthritis, undifferentiated SpA, juvenile chronic arthritis, acute anterior uveitis, and arthritis with inflammatory bowel diseases such as Crohn's disease and ulcerative colitis.

The target organs in patients with SpA include not only the joint, but also the gut, eye, axial skeleton, enthesis, urogenital tract, and skin. Over recent years, attention has focused on the relation between subclinical gut inflammation in patients with SpA and joint disease. The prevalence of subclinical inflammatory gut lesions in patients with SpA indeed ranges from 25 to 75%.[2] A fraction of these patients go on to develop clinically overt Crohn's disease at the five year follow up.[3 4] Interestingly, a strong relation between gut and joint inflammation has been observed in SpA: remission of joint inflammation was found to be linked to the disappearance of gut inflammation, whereas persistence of articular inflammation was associated with persistence of gut inflammation.[3] The mechanisms that link gut and synovium in SpA are still not known, but recirculation of antigen specific memory T cells between gut and synovium could be the basis.

Both in vitro[5] and in vivo[6] investigations indicate that T lymphocytes and their cytokines play an important role in the regulation of gut immune responses and in the pathogenesis of intestinal inflammation. Human CD4+ T helper cells (Th) can be divided into two major subsets.[7] Th1 cells produce interferon γ (IFNγ) and interleukin 2 (IL2) and mediate cellular immune responses, whereas Th2 cells, which produce IL4 and IL10, are implicated in humoral responses and allergy.

Data on cytokine production in SpA are scarce. In the synovial membrane in patients with reactive arthritis more cells were positive for IL4 than for IFNγ.[8] In another study involving patients with reactive arthritis, T cells in synovial membrane secreted IFNγ but also IL4.[9] Braun *et al*[10] showed lower tumour necrosis factor α (TNFα) secretion by whole peripheral blood monocytes and T cells in patients with reactive arthritis compared with those with rheumatoid arthritis. For the cytokines IFNγ, IL10, and IL4, no differences were detected. Siegert *et al*[11] reported that CD3+ peripheral blood cells of patients with ankylosing spondylitis produced less IFNγ, similar amounts of IL4, and more IL10 than controls. Claudepierre *et al*[12] reported that patients with SpA with raised plasma levels of IL10 had more active disease. Our group has shown that the percentage of peripheral blood T cells positive for IFNγ and IL2 is decreased in SpA, whereas IL10 positive T cells are increased.[13] Treatment of these patients with monoclonal antibodies (Mabs) against TNFα induced a shift towards Th1 with restoration of the Th1/Th2 cytokine balance.[13 14] Together, these data suggest that the T cell involvement in the inflammation in SpA is more Th2-like than Th1. No data are available on cytokine expression by gut mucosal lymphocytes in SpA.

The aim of this study was to investigate the Th1/Th2 cytokine profile in isolated intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) from gut biopsy specimens from patients with SpA in relation to healthy controls.

Lymphocytes from mucosal gut biopsy samples are usually isolated with chemical agents and enzymes. We recently reported that chemical agents and enzymes affect the flow cytometric detection of T cell surface markers.[15] Therefore we used an improved extraction protocol for the isolation of IELs and LPLs from both the small and large intestine.

Methods

PATIENTS

Twenty patients with SpA (16 men, four women, median age 33 years, range 19–56 years) who fulfilled the European Spondyloarthropathy Study Group criteria for SpA[16] were included in the study. Of these, 14 had ankylosing spondylitis, three had psoriatic arthritis, two had arthritis with inflammatory bowel disease, and one had undifferentiated SpA. Histological examination showed that in 13 the bowel was normal, and in seven there was inflammation of the colon or ileum. Seven were receiving sulfasalazine and/or corticosteroids with or without non-steroidal anti-inflammatory drugs (NSAIDs), nine were receiving NSAID treatment only, and four had no treatment. Colonic and ileal biopsy specimens were obtained during ileocolonoscopy. Control samples were obtained from 13 patients (seven men, six women, median age 47 years, range 18–74 years) having an ileocolonoscopy for irritable bowel syndrome or for follow up of polyps. All ileocolonoscopic results were normal in these patients. The study was approved by the ethical committee of the local faculty of medicine.

ISOLATION OF IELS AND LPLS

To avoid artificial alterations in membrane marker expression,[15] we applied a more selective protocol for the isolation of IELs and LPLs. Eight to ten colonic biopsy samples and eight to ten ileal biopsy samples obtained by ileocolonoscopy were collected in 10 ml phosphate buffered saline (PBS; Gibco BRL, Grand Island, New York, USA), transferred to fresh PBS, and stirred for 20 minutes at 37°C to remove blood and debris (fraction a). Next, the samples were transferred to fresh PBS and stirred for another 60 minutes at 37°C to isolate IELs (fraction b). Subsequently, LPLs were obtained by cutting the remaining biopsy fragments into fragments of about 1–5 mm^3, which were then incubated at 37°C for three hours in collagenase type IV (50 U/ml; Sigma Chemical Co, St Louis, Missouri, USA) in RPMI 1640 medium (Gibco) (fraction c). Fraction b contains epithelial cells and IELs, and fraction c contains LPLs and some macrophages. No further purification of the cell populations was performed so as to retain the maximum number of cells. However, fluorescence activated cell sorting (FACS) analysis allowed CD3+ cells to be gated. IELs and LPLs were resuspended in 2 ml RPMI 1640 medium with 10% autologous serum and left overnight in a humidified chamber at 37°C.

PHENOTYPIC ANALYSIS OF IELS AND LPLS

Phenotypic analysis of the isolated lymphocytes was performed using the following Mabs directly conjugated to fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein, or allophycocyanin: anti-CD3 (Leu4, clone SK7; Becton Dickinson, San Jose, California, USA), anti-CD4 (Leu3a, clone SK3; Becton Dickinson), anti-CD8 (Leu2a, clone SK1; Becton Dickinson), anti-CD45 (HLe-1, clone 2D1; Becton Dickinson), anti-CD14 (Leu-M3, clone MϕP9; Becton Dickinson), anti-(αEβ7 integrin) (CD103, HML-1, clone 2G5; Immunotech, Marseille, France), and anti-(α4β7 integrin) (kindly provided by Dr Ringer, Leukosite, Cambridge, UK). Isotype matched immunoglobulins that did not react with human leucocytes were used as controls (Becton Dickinson).

Aliquots of cell suspensions (100 µl) were incubated with the appropriate amount of Mab in the dark for 30 minutes at 4°C. Cells were washed with 2 ml PBS and centrifuged at 1800 rpm for 10 minutes. After being stained, cells were fixed with 300 µl CellFIX (Becton Dickinson) and stored in the dark at 4°C until analysis.

DETECTION OF INTRACELLULAR CYTOKINES

Cells were stimulated as described by Morita *et al*.[17] After being left overnight in the humidified chamber at 37°C, IELs and LPLs were stimulated with a combination of 25 ng/ml phorbol 12-myristate 13-acetate (Sigma) and 1 µg/ml of the calcium ionophore ionomycin (Sigma) for five hours at 37°C in a humidified atmosphere containing 5% CO_2. After one hour, 10 µg/ml brefeldin A (Sigma) was added. Brefeldin A was used to enhance flow cytometric analysis of intracellular cytokine staining, through its inhibitory effect on protein secretion by interfering with the function of the Golgi apparatus.

The following human cytokine specific Mabs and isotype matched control Mabs conjugated to fluorescein isothiocyanate and phycoerythrin were obtained from Becton Dickinson: anti-(Hu IFNγ) (25723.11), anti-(Hu IL2) (5344.1 1), and anti-(Hu IL4) (3010.211). Anti-(Hu IL10) (JES3-9D7) was purchased from Biosource Europe (Nivelles, Belgium). The following Mabs against human surface antigens labelled with peridinin chlorophyll protein or allophycocyanin were also purchased from Becton Dickinson: anti-CD8 (Leu-2a, clone SK1) and anti-CD3 (Leu4, clone SK7).

After stimulation, the cells were washed and incubated with 10 µl anti-CD8 and 5 µl anti-CD3 for 30 minutes. Then 2 ml lysis buffer (Becton Dickinson) was added for 10 minutes. After centrifugation, 500 µl permeabilisation buffer (Becton Dickinson) was added for 10 minutes. After a wash with PBS, the cells were incubated with the Mabs anti-(Hu IFNγ), anti-(Hu IL2), anti-(Hu IL4), and anti-(Hu

Table 1 Phenotypic analysis of isolated intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) from colonic and ileal biopsy specimens

	Colon		*Ileum*	
	IELs	*LPLs*	*IELs*	*LPLs*
CD4	32 (20–48)	62 (51–74)	22 (11–33)	64 (47–66)
CD8	54 (35–71)	32 (20–50)	72 (51–85)	36 (26–52)
CD4/CD8	0.6 (0.3–1)	2.1 (1–3.4)	0.3 (0.2–0.7)	1.8 (0.9–2.2)
αEβ7	64 (31–82)	19 (5–34)	87 (64–95)	33 (23–45)
α4β7	1.4 (0.4–2.4)	17 (9–40)	0.3 (0.3–1.8)	27 (21–49)

Cell membrane marker expression was analysed in isolated colon and ileum lymphocytes in seven patients (two normal controls and five patients with spondyloarthropathy). Results are expressed as the median (range) percentage of CD3+ cells positive for the different markers.

IL10) for 30 minutes. Isotype matched control Mabs were used to assess aspecific binding. After a final washing step, the cells were resuspended in 300 µl CellFIX and kept at 4°C until analysis by flow cytometry. All incubations were carried out at room temperature and in the dark.

FLOW CYTOMETRIC ANALYSIS

Four colour flow cytometry was performed using a FACSort type apparatus (Becton Dickinson). A biparametric gate in the forward/side scatter dot plot was drawn around the lymphocyte population as defined by antigen expression (CD45+CD14−), and T lymphocytes were subsequently gated by their CD3 expression. Four colour flow cytometry allowed the percentages of cytokine-producing cells within the CD3+CD8+ and CD3+CD8− populations to be analysed. As CD4 expression is known to be downregulated after stimulation with phorbol esters,[18] the CD4+ population could only be analysed indirectly by gating of the CD3+CD8− population. Acquired data were analysed using Cellquest software (Becton Dickinson).

STATISTICAL ANALYSIS

Values were expressed as percentage positive cells, with median and range. Comparisons between the cytokine patterns of patients with SpA and healthy controls were analysed by the Mann-Whitney test. Because the analysis of each cytokine was considered as a separate and independent comparison, a correction for multiple testing was not applied. Spearman's test was used to calculate correlations. $p<0.05$ was considered to be significant.

Results

PHENOTYPIC ANALYSIS OF ISOLATED IELS AND LPLS

Table 1 summarises the distribution of lymphocyte cell surface markers in fraction b (IELs) and fraction c (LPLs). In the IEL fraction, the CD4/CD8 ratio was low (0.6 and 0.3 in colonic and ileal biopsy specimens). The cells were predominantly αEβ7 positive and α4β7 negative. In LPLs, the CD4/CD8 ratio was higher than 1 (2.1 and 1.8 in colonic and ileal biopsy specimens). LPLs showed substantially higher α4β7 expression and lower αEβ7

Table 2 Percentage of cytokine-producing CD3+, CD3+CD8+, and CD3+CD8− T cells in colon epithelium and lamina propria from healthy controls (C) and patients with spondyloarthropathy (SpA) after stimulation with PMA/ionomycin

	CD3+		*CD3+CD8+*		*CD3+CD8−*	
	C	*SpA*	*C*	*SpA*	*C*	*SpA*
IELs	n=10	n=19	n=8	n=17	n=8	n=17
IFNγ	29 (15–65)	26 (9–45)	42 (18–77)	40 (14–67)	19 (9–38)	20 (7–30)
IL2	43 (16–66)	43 (17–67)	37 (12–85)	44 (11–73)	47 (20–58)	41 (20–61)
IL4	1.1 (0.2–3.6)	0.9 (0.2–2.7)	0.9 (0.1–2.7)	0.9 (0.4–2.6)	1.1 (0–4.4)	1 (0.1–2.8)
IL10	2.9 (0.1–4.8)	3 (1.4–5.7)	2.2 (0.1–4.2)	2.1 (1.1–6.1)	4.2 (0.1–5.9)	3.5 (1.4–7.5)
LPLs	n=10	n=18	n=9	n=18	n=9	n=18
IFNγ	64 (42–72)	54 (19–73)*	78 (53–88)	67 (30–84)	57 (37–67)	47 (15–70)*
IL2	71 (49–85)	67 (21–74)	60 (38–87)	60 (15–81)	74 (47–84)	67 (21–78)*
IL4	1.8 (0.9–4.2)	1.7 (0.4–5.3)	3.4 (0.7–6.5)	2.9 (0.6–16)	1.4 (0.5–3.2)	1.3 (0–5.3)
IL10	3 (1.4–7.3)	3.7 (1.9–6.8)	3 (1.1–7.9)	4.2 (1.8–10.2)	2.6 (1.5–6.9)	3.3 (0.5–7)

Results are expressed as the median (range).
*p<0.05 compared with controls.
IFN = interferon; IL = interleukin; IEL = intraepithelial lymphocyte; LPL = lamina propria lymphocyte; PMA = phorbol 12-myristate 13-acetate.

Table 3 Percentage of cytokine-producing CD3+, CD3+CD8+ and CD3+CD8− T cells in ileum epithelium and lamina propria from healthy controls (C) and patients with spondyloarthropathy (SpA) after stimulation with PMA/ionomycin

	CD3+		*CD3+CD8+*		*CD3+CD8−*	
	C	*SpA*	*C*	*SpA*	*C*	*SpA*
IELs	n=9	n=16	n=9	n=16	n=9	n=16
IFNγ	33 (20–67)	33 (10–63)	52 (31–74)	43 (10–72)	15 (4–53)	21 (9–44)
IL2	50 (6.3–79)	54 (15–80)	64 (7–84)	63 (14–87)	42 (4–69)	43 (15–66)
IL4	1.1 (0.2–2)	0.7 (0.2–1.9)	0.7 (0.4–1.3)	0.6 (0.1–1.9)	1.2 (0.5–1.9)	0.7 (0–2)
IL10	3.9 (2.9–9.7)	4.9 (1.3–14)	3.8 (2.9–9.3)	4.7 (1.1–12)	3.1 (2.6–9.7)	6.1 (1.6–15)
LPLs	n=10	n=16	n=9	n=15	n=9	n=15
IFNγ	65 (13–82)	51 (17–72)	77 (35–89)	65 (23–85)	58 (5.4–74)	39 (12–66)
IL2	83 (26–89)	62 (19–83)	88 (30–88)	62 (18–92)	81 (8.8–89)	64 (21–85)
IL4	1.6 (0.6–3.8)	1.3 (0.2–5.4)	1.1 (0.4–3.8)	1.3 (0.2–11.9)	2 (0.4–3.8)	0.8 (0–3.3)
IL10	2.5 (1.1–4.1)	3.3 (1.1–13)	2.1 (0.6–4.8)	3 (0.9–14.3)	2.4 (1.1–4.5)	3.4 (1.3–19)*

Results are expressed as the median (range).
*p<0.05 compared with controls.
IFN = interferon; IL = interleukin; IEL = intraepithelial lymphocyte; LPL = lamina propria lymphocyte; PMA = phorbol 12-myristate 13-acetate.

expression than IELs. These phenotypes were concordant with previously reported data on IEL and LPL phenotypes.[19–22]

T cell cytokine profile in spondyloarthropathy: intracellular cytokine analysis in IELs and LPLs

Tables 2 and 3 summarise the cytokine profile in gut mucosal lymphocytes.

In colonic LPLs, the percentage of CD3+ cells positive for IFNγ was decreased in SpA compared with healthy controls (p=0.02) (table 2). Analysis of the T cell subsets showed that both CD3+CD8− and CD3+CD8+ had the potential to produce the cytokines under investigation. In the IELs, the CD3+CD8− cells were composed of 70–80% CD4+ cells and 43% were αEβ7+. In the LPLs, 95% were CD4+ and 12% were αEβ7+ (data not shown).

The decrease in IFNγ and IL2 was more pronounced in the CD3+CD8− subset (p=0.021 and p=0.027 respectively) (table 2).

In ileal LPLs, a tendency towards CD3+ cells producing less IFNγ and IL2 was observed (table 3). In the CD3+CD8− subset, the percentage of cells expressing IL10 was increased in SpA compared with healthy controls (p=0.046) (table 3).

There were no differences between cytokine producing cells in colonic and ileal IELs.

To explore the clinical relevance of the impaired Th1 profile in SpA, possible correlations were studied between T cell cytokines and a number of clinical features (C reactive protein levels, erythrocyte sedimentation rate, diarrhoea, subtype of SpA, duration of disease, axial and/or peripheral involvement) as well as age and sex. T cell cytokine expression did not correlate with gut inflammation or gut symptoms (diarrhoea) nor with axial and/or peripheral involvement, age, sex, and subtype of SpA. The number of IL2 positive cells appeared to correlate inversely with C reactive protein levels (r=−0.67; p=0.007) and erythrocyte sedimentation rate (r=−0.58; p=0.025).

Discussion

This study aimed to analyse the Th1/Th2 cytokine profile in gut mucosal lymphocytes from patients with SpA compared with healthy controls. Although techniques such as immunohistochemistry, enzyme linked immunosorbent assay, and reverse transcriptase polymerase chain reaction can all provide useful information on this subject, for several reasons we preferred a flow cytometric analysis of isolated gut lymphocytes. Firstly, flow cytometry allows exact quantification of intracellular cytokine production at the single cell level. Secondly, it allows phenotypically well defined cell populations to be identified therefore allowing the study to be focused on the cytokine profile of CD3+ T lymphocytes and CD3+CD8+ and CD3+CD8− T cell subsets. In addition, the use of a selective extraction protocol for IELs and LPLs meant that the two lymphocyte populations could be assessed separately. Although we cannot completely exclude contamination by LPLs or peripheral blood lymphocytes, the CD4/CD8 ratios and the expression of αEβ7 and α4β7 indicate that the fraction obtained by stirring the biopsy samples (fraction b) is indeed highly enriched in IELs. Moreover, the results obtained by this improved extraction protocol seem better than those obtained by enzymatic extraction.[23 24] It should be noted that the degree of αEβ7 expression was lower in IELs from colon than from ileum biopsy specimens, but this agrees with a report from Cerf-Bensussan *et al*[19] that the number of lymphocytes expressing αEβ7 decreased from the proximal to the distal part of the colon.

A first observation of the study is that, in both patients with SpA and healthy controls, there was a predominance of IELs and LPLs producing Th1 cytokines, with IFNγ mainly produced by CD3+CD8+ cells and IL2 by both CD3+CD8+ and CD3+CD8− cells. In contrast, the percentage of gut mucosal lymphocytes producing Th2 cytokines (IL4 and IL10) was low. These findings correlate well with previous reports on T cells in peripheral blood that produce IFNγ and IL2[17 25] and studies showing less than 5% Th2 cytokine-producing peripheral blood cells.[26 27]

The main finding of this study is that LPLs, but not IELs, from the gut mucosa of patients with SpA have an impaired Th1 cytokine profile: decreased IFNγ and IL2 and increased IL10 compared with healthy controls. This first report on gut lymphocytes in SpA correlates well with previous reports of low IFNγ and IL2 and high IL10 in peripheral blood as well as in synovium[8–13] and indicates that SpA is characterised by an impaired Th1 cytokine profile in peripheral blood, synovium, and gut. From the analysis of the T cell subsets, the impaired Th1 cytokine profile appears to be most pronounced in the CD3+CD8− T helper subset. Concerning the clinical relevance of the small but significant impairment of Th1 cytokines in SpA colon lymphocytes, it should be noticed that a similar trend was not only observed in ileum but also in peripheral blood lymphocytes.[13] Moreover, recent results indicate that similar changes occur in gut mucosal lymphocytes of patients with Crohn's disease.[28] Finally, anti-TNFα treatment, which has an impressive clinical effect in SpA,[14] restores the impaired Th1 cytokine profile in peripheral blood lymphocytes of patients with SpA.[13]

A point of interest is that, although the number of patients treated with corticosteroids or sulfasalazine was small, they seemed to have higher percentages of IFNγ and IL2 positive mucosal T lymphocytes than those treated with NSAIDs only (data not shown), suggesting that the former treatments may restore the Th1/Th2 balance, a phenomenon also observed in patients treated with Mabs against TNFα.[13] With regard to the therapeutic relevance of the restoration of the impaired Th1 cytokine profile by TNFα blockade, it seems important that low IL2 concentration correlates with higher disease activity, as evaluated by the inflammatory variables C reactive protein and erythrocyte sedimentation rate. This fits with the concept of an impaired Th1 profile in active disease because in peripheral blood also an

inverse correlation was found between IFNγ positive T cells and levels of C reactive protein.[13]

The present findings fit the hypothesis that a defective Th1 response plays a prominent role in SpA. Indeed, several forms of SpA, in particular gastrointestinal reactive arthritis, have been linked to the persistence of intracellular bacteria, which could trigger inflammation and/or autoimmunity.[29] As Th1 cytokines are known to be crucial in the defence against intracellular pathogens,[30 31] a shift towards a Th2 cytokine profile of not only peripheral blood but, more importantly, also gut mucosal lymphocytes could contribute to defective clearance of these bacterial antigens and thereby to the pathogenesis of the disease.

In summary, this study reports downregulated production of T cell IFNγ and IL2 and upregulated production of IL10 in gut LPLs of patients with SpA. Extending previous observations in peripheral blood and synovium, the decreased expression of IFNγ and IL2 in gut mucosal lymphocytes highlights a possible role of a defective mucosal Th1 response in the pathogenesis of SpA.

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