INTERFERON-γ IS INCREASED IN THE GUT OF PATIENTS WITH IRRITABLE BOWEL SYNDROME AND MODULATES SEROTONIN METABOLISM

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ABSTRACT

Mucosal immune activation and altered serotonin metabolism participate in the pathophysiology of irritable bowel syndrome (IBS). However, the reciprocal interplay between these two systems remains unknown. We evaluated the expression and release of interferon (IFN)-γ from the colonic mucosa of IBS patients and its impact on serotonin reuptake transporter (SERT) gene expression in Caco-2 cells. qPCR was used to evaluate IFN-γ gene expression in colonic mucosal biopsies, while IFN-γ protein amount was assessed by ELISA. Colonic T-bet and pSTAT4 protein amount were evaluated by western blot. The impact of colonic mucosal mediators on SERT gene expression was evaluated in Caco-2 cells using qPCR. IFN-γ receptor was silenced in Caco-2 cells to determine the effect of IFN-γ released by mucosal biopsies. Compared to asymptomatic control (AC), the expression of IFN-γ gene and its transcription factor T-bet were markedly increased in the colonic mucosa of IBS patients. Compared to AC, IFN-γ protein tissue levels and its release by mucosal biopsies were significantly increased in IBS. The exposure of Caco-2 cells to IBS supernatants induced a significant decrease in SERT gene expression, independently of IBS subtypes, in comparison with AC mucosal supernatants. In Caco-2 cells, IFN-γ receptor silencing reversed the reduction of SERT expression evoked by IBS supernatants vs non-silenced cell lines. IFN-γ gene, its transcription factor T-bet, IFN-γ protein expression and its release are increased in the colonic mucosa of IBS patients and down-regulates SERT gene expression in vitro. These results suggest that IFN-γ downregulates SERT expression, hence likely playing a role in altered serotonin metabolism of IBS patients.

Key words: gut mucosa; IBS; interferon-γ; SERT; T-bet.

Abbreviations: IBS, irritable bowel syndrome; IBS-D, diarrhoea predominant IBS; IBS-C, constipation predominant IBS; IBS-M, alternating predominant IBS; AC, asymptomatic controls; IFN-γ, interferon-γ; T-bet, T box expressed in T cells; pSTAT4, phosphorylated signal transducer and activator of transcription 4; SERT, serotonin transporter reuptake; 5-HT, serotonin.
INTRODUCTION

Irritable bowel syndrome (IBS) is one of the most common gastrointestinal disorders affecting 10-15% of the worldwide population. IBS is characterized by abdominal pain or discomfort and changes in bowel habit (39). The pathophysiology of IBS remains poorly understood. According to the current dogma, IBS is characterized by a brain-gut axis dysfunction, involving both central (e.g., stress, anxiety, depression) and peripheral factors (e.g., gut dysmotility and visceral hypersensitivity). With the application of quantitative cellular and molecular methods new mechanisms underlying gut dysfunction have been discovered. These include mucosal immune activation (5-6), changes in the metabolism of the monoamine serotonin (5-hydroxytryptamine, 5-HT) (1), increased epithelial permeability (44), abnormal gut microbiota (32), genetic polymorphisms in the serotonin reuptake transporter (SERT) and pro- and anti-inflammatory cytokines (10, 50) as well as enteric nervous system plasticity (22).

Up to 95% of the body’s 5-HT is produced and stored in the secretory granules of enterochromaffin cells in the gastrointestinal (GI) tract. Upon release, 5-HT exerts its action by binding to 15 different receptor subtypes (31). Activation of receptors located on enteric nerves, smooth muscle, epithelial cells and blood vessels modulates GI motility, sensitivity and secretion (29). 5-HT bioavailability for receptor binding is terminated by its reuptake from the interstitial space by a solute-carrier superfamily member, referred to as SERT. 5-HT reuptake is exerted through a mechanism associated with co-transport of Na+ and Cl− and counter-transport of K+ both in the GI tract and in the brain (30). Numerous fundamental studies have highlighted the importance of 5-HT in the pathophysiology of gut inflammation and sensorimotor dysfunction (23).

Abnormal SERT expression and 5-HT metabolism have been implicated in such diverse conditions as inflammatory bowel disease (IBD), celiac disease (20, 27) and IBS (46). We previously showed that compared with healthy controls, IBS patients had a higher amount of colonic 5-HT-positive cells. This was accompanied by greater 5-HT release, which correlated with abdominal pain severity, irrespective of bowel habit (17). In line with these adult data, children with IBS had increased 5-HT content in the rectal mucosa (25). Nonetheless, conflicting results have been reported concerning SERT expression. While compared to controls, colonic SERT expression was lower in both adults (16, 34) and children with IBS (25), others showed unchanged concentrations of the SERT protein in the colonic and rectal mucosa (11). The link between 5-HT and low-grade inflammation in patients with IBS has been firstly advanced by Foley et al, who showed that decreased levels of SERT were correlated with increased intraepithelial lymphocyte counts in the duodenum of patients with IBS with predominant diarrhoea (IBS-D) (27). These data are in line with the knowledge that inflammation down-regulates SERT expression, hence increasing tissue 5-HT availability (16, 37). While the exact mechanisms involved in inflammation-induced down-regulation of SERT remain unclear, some data suggest the participation of cytokines including tumor necrosis factor (TNF)-α and interferon (IFN)-γ (26).

The widely reported evidence of a low grade mucosal inflammatory component in IBS (42) has been more recently coupled with evidence that innate immunity can participate to symptom development. Accordingly, the vicinity of activated mast cells to nerve endings correlated to the severity and frequency of abdominal pain (5-6). Although the adaptive immune system is involved in IBS as shown by increased CD3+, CD4+ and CD8+ T cell infiltration (18), little is known about the T helper cell type (Th)1/Th2 polarity. Cytokines are effector molecules of the immune system acting via receptor-dependent mechanisms on numerous target cells. In the gut, they orchestrate immune responses and modulate
epithelial, endocrine and enteric nervous system function (28). In patients with IBS, several
studies have assessed intestinal and systemic levels of cytokine in serum and from
stimulated peripheral blood mononuclear cells (7, 12, 40). A recent meta-analysis
demonstrated that data are divergent (8). Among several cytokines investigated only
interleukin (IL)-10 was consistently reduced and this reduction was mainly driven by
inclusion of patients with post-infectious IBS. Most of these previous studies focused on
pro-inflammatory (e.g., TNF-α) and anti-inflammatory (e.g., IL-10) cytokines. IFN-γ is an
immunomodulatory cytokine principally synthesized and released by Th1 cells.
Based on its pleiotropic actions, IFN-γ could participate in different pathophysiological
abnormalities described in IBS, such as reduced mucosal barrier function and altered
serotonin metabolism. Accordingly, exposure of Caco-2 cells to IFN-γ induced a marked
increase in paracellular permeability (48). Moreover, the treatment of Caco-2 cells with
IFN-γ induced a decrease in SERT gene expression and 5-HT reuptake hence increasing
5-HT bioavailability, an effect further enhanced by the concomitant treatment with TNF-α
(26).
Based on this evidence, the aims of the present study were to assess IFN-γ gene and
protein expression in the colonic mucosa of IBS patients as well as the expression of Th1-
specific T box transcription factor (T-bet) and the activated form of STAT4 (i.e. pSTAT4) as
specific transcription factors of T cell polarization towards the Th1 phenotype. We
investigated also the expression of IFN-γ in the supernatants of cultured biopsies and the
effect of IBS supernatants on Caco-2 SERT expression.
MATERIALS AND METHODS

This study consists of three parts. Part 1 in which we analysed IFN-γ gene and protein expression in the colonic mucosa and the expression of IFN-γ transcription factors; Part 2 focused on the evaluation of IFN-γ release from colonic mucosa and the effect of cultured biopsy supernatants on SERT gene expression in Caco-2 cells; Part 3 investigated the involvement of IFN-γ on SERT gene expression.

Subjects

IBS patients were all seen at the Department of Medical and Surgical Sciences of the University of Bologna and they were diagnosed according to Rome III criteria (39). Asymptomatic controls (AC) were recruited among subjects undergoing colonoscopy for screening of colorectal carcinoma or polypectomy follow-up of and included in the study after the exclusion of gastrointestinal symptoms. Exclusion criteria were the following: celiac disease (based on negative anti-endomysial and anti-transglutaminase antibodies), allergic diseases, including asthma (family and personal history and specific anti-IgE antibodies), the use of corticosteroids, non-steroidal anti-inflammatory drugs and mast cell stabilizers, tricyclic antidepressant or serotonin selective reuptake inhibitors, serotoninergic agents, including 5-HT3 receptor antagonists (i.e. granisetron, ondansetron) and 5-HT4 receptor agonists (i.e. prucalopride), major abdominal surgery, and other organic or severe psychiatric disorders assessed by history taking, consultations and laboratory tests. Biopsies were also collected from inflamed colonic areas of six patients affected by Crohn’s disease (CD). Diagnosis of CD was ascertained according to the usual clinical criteria, and the site and extent of the disease were confirmed by endoscopy and histology. This study was approved by the Ethic Committee of St. Orsola-Malpighi Hospital of Bologna (approval identification no: 23/2012/U/TESS) and conducted in accordance with the Declaration of Helsinki. Both patients and AC gave written informed consent and each patient completed an Italian modified version of the Bowel Disease Questionnaire to evaluate symptoms (5). We obtained eight mucosal biopsies from the proximal descending colon during colonoscopy. Two biopsies were used for routine H&E histology and immunohistochemistry. Two biopsies were snap frozen and stored at −80°C for protein extraction; four biopsies were used for mucosal mediators release experiments.

Study Design

In Part 1 we assessed the expression of IFN-γ gene and protein, IFN-γ transcription factors T-bet and pSTAT4 and SERT gene expression in the colonic mucosa of IBS patients (IBS-D: n=25, IBS-C: n=16, IBS-A: n=10) compared to asymptomatic controls (n=23). These studies demonstrated an upregulation of IFN-γ in the colonic mucosa of IBS patients. In Part 2 we focused on cultured biopsy supernatants and evaluated IFN-γ amount and the effect on SERT gene expression in Caco-2 cells exposed to supernatants. Part 2 involved 12 AC and 46 IBS (IBS-D: n=20, IBS-C: n=14, IBS-A: n=12). The results of Part 2 showed an effect of cultured biopsy supernatants of IBS patients on SERT gene expression, therefore in Part 3 we investigated the role of IFN-γ in this effect by silencing its receptor. Part 3 involved 6 AC and 27 IBS (IBS-D: n=9, IBS-C: n=9, IBS-A: n=9).

Collection of Mucosal Mediators
Spontaneous release of mediators from colonic biopsies was obtained following a previously validated method (5) with few modifications. Briefly, after removal, biopsies were immediately immersed in plastic tubes containing 1 ml of Hepes-Krebs solution, weighed and volume adjusted to incubate 15 mg of biopsies in 1 ml of buffer. Incubation was carried out in continuous oxygenation at 37°C for 25 min. All samples were centrifuged at 200g for 10 min, and 200 μl of supernatant containing mediators (from now on called supernatants) aliquoted and stored at −20°C until the assay.

RNA Extraction and Reverse Transcription (RT)

Biopsies were fixed in 10% buffered formalin and paraffin embedded (FFPE); 20 μm slides were cut and RNA extracted using RecoverAll Ambion kit (Life Technologies, Milan, Italy) according to the manufacturer's instructions. RT was carried out using High Capacity cDNA Archive Kit (Life Technologies, Milan, Italy) in a total volume of 40 μl for 2 h 15 min (25°C for 10 min; 37°C for 2 h; 95°C for 5 min) in the Thermo Cycler 2720 (Life Technologies). Ancillary experiments were performed in order to evaluate gene expression using RNA extracted from fresh biopsies and from paraffin embedded ones. We split some biopsies in half: one-half was immediately used to extract RNA, while the other half was fixed and paraffin embedded before RNA extraction. We found the same level of gene expression (as ΔCt) independently of the starting material used to extract RNA.

Caco-2 cells were treated for 72 h with mucosal mediators obtained from cultured biopsies; RNA was extracted using RNeasy mini kit (Qiagen, Hilden, Germany) and quantified using NanoDrop 2000 spectrophotometer (Thermo Scientific, Milan, Italy). RT was performed on 1 μg of total RNA in a 20 μl total reaction volume using Quantitect reverse transcription kit (Qiagen, Hilden, Germany). Samples were incubated for 2 min at 42°C with gDNA Wipeout Buffer to avoid possible genomic DNA contamination. RT conditions were: 15 min 45°C, 3 min 95°C and 5 min 4°C. cDNA was stored at −20°C until the assay.

qPCR assay

Analyses of colonic SERT and IFN-γ gene expression were performed in a total volume of 20 μl combining 10 μl of TaqMan Master Mix (Life Technologies, Milan, Italy), 1.0 μl of probe/primers mix, 3 μl of cDNA and 6.0 μl of RNAsa/DNAsa free water (Life Technologies, Milan, Italy) using the HT 7900 Real Time PCR System (Life Technologies) in a 96 well plate. Thermal cycles began with a denaturation at 50°C for 2 min and then at 95°C for 10 min. Amplification conditions were: 15 sec at 95°C, 1 min at 60°C (for 40 cycles). SERT and IFN-γ mRNA expression were normalized to the reference gene β-glucuronidase (GUSB). The TaqMan assays used were the following: SERT, Hs00984349_m1; IFN-γ, Hs00174143_m1. The Applied Biosystem Human GUSB Endogenous Control was used. The AC group was used as calibrator. Data were analysed using Sequence Detection System software (Life Technologies, Milan, Italy).

SERT gene expression in Caco-2 cells was determined by using SYBR Green Quantitect PCR kit (Qiagen, Hilden, Germany) on a Real Time PCR ICycler (Bio-Rad Laboratories, Hercules, CA, USA) in a 96 well plate, in a final volume of 25 μl. SERT mRNA expression was normalized to the reference gene β-actin. The following primers were used: SERT: forward 5’-gcgctgtgctctccgct-3’; reverse: 5’-tgttgagctctggtcg-3’; β-actin: forward 5’-catgtgagacaccaac-3’; reverse 5’-ccaggaagggctgc-3’. Amplification conditions for SERT gene analyses were: 15 min at 95°C followed by 40 cycles of 15 sec at 95°C, 30 sec at 60°C, 30 sec at 72°C; for β-actin gene were: 15 min at 95°C followed by 40 cycles of 15 sec at 95°C, 30 sec at 53°C, 30 sec at 72°C. Melting
curve data were analysed at the end of each reaction. Caco-2 cells treated with AC supernatants were used as calibrator.
Caco-2 treated with Hepes-Krebs buffer were used as calibrator to compare SERT gene expression after the treatment with supernatants in the non-silenced and in the silenced Caco-2 cells.
A negative control for PCR reaction (1 µl of water instead of cDNA) and a no-reverse transcription control were added in each real-time PCR plate. Each sample was run in duplicate and the mean threshold cycle (Ct) was determined from the two runs. The specificity of all the PCR products was confirmed by agarose electrophoresis.
The relative gene expression was calculated as ΔΔCt (38). The levels of INF-γ and SERT gene expression were expressed as fold difference ($2^{-\Delta\Delta C_t}$).

**Protein extraction and ELISA assay**

Total proteins were extracted from biopsies using a tissue protein extraction reagent with the addition of a protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA) according to the manufacturer’s instructions. Protein amount was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Milan, Italy) and were stored at −80°C.

ELISA assay was performed using Human IFN-γ Mini ELISA Development Kit (Peprotech, London, UK) following the manufacturer’s instructions. Each sample was analysed in duplicate and IFN-γ amount was normalized to the total protein amount. To evaluate IFN-γ release from mucosal biopsies, supernatants were concentrated 10 fold using Amicon Ultra-0.5ml (Merck Millipore, Carrigtwohill, Co, Ireland) with a cut off of 10 KDa following the manufacturer’s instruction before IFN-γ ELISA assay.
Non concentrated supernatants showed a low IFN-γ amount under the detection limit of the ELISA kit. IFN-γ concentration in each sample was estimated from an IFN-γ standard curve. Each sample was assayed in duplicate.

**Caco-2 cell culture**

Caco-2 cells were obtained from American Type Culture Collection (ATCC), Manassas, VA. Cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) (Lonza, Verviers, Belgium) with the addition of 10% heat inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (Lonza, Verviers, Belgium). Cells were incubated at 37°C with 5.5% CO2 and grown for at least 5 days to achieve maturation (45).
Supernatants were filtered using 0.22 µm porous filter and added to Caco-2 cells together with complete DMEM (1.3:1, supernatant : DMEM). Incubation was carried out for 72 h; each supernatant was assayed in duplicate.

**RNA Interference Assay**

In order to evaluate the role of IFN-γ on SERT gene expression in Caco-2 cells after supernatant treatment, RNA interference assay was performed using specific short hairpin RNA (ShRNA) plasmids (purchased by Origene, Rockville, USA) used to silence IFN-γ receptor (IFN-γR) α chain.
Cells were seeded to have at least the 80% of confluence after the overnight growth, then medium was replaced with DMEM without serum. 100 ng of each ShRNA plasmid (Origene, Rockville, USA) was prepared in serum free DMEM in a final volume of 100 µl. For each well, 10 µl of Lipofectamine (Life Technologies, Milan, Italy) was diluted in 90 µl
of serum free DMEM, added to ShRNA plasmids, incubated for 45 min at room
temperature and used to transfect Caco-2 cells. After 4 h medium was replaced with
complete DMEM and cells were incubated at 37°C for 72 h until the treatment with
supernatants.
Four different ShRNA plasmids were supplied by the manufacturer for IFN-γRα gene plus
an empty vector (negative control plasmid).

**Western Blotting**

Western blotting for the Th1 specific transcription factor T-bet and the phosphorylated form
of signal transducer and activator of transcription (pSTAT)4 was performed according to
standard procedures (21). In brief, tissue samples were lysed in ice-cold lysis buffer (10
mM EDTA, 50 mM pH 7.4 Tris-HCl, 150 mM sodium chloride, 1% Triton-X-100, 2 mM
phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, 10 mg/ml leupeptin and 2
mg/ml aprotinin) and the amount of protein was determined by the Bio-Rad Protein assay
(Bio-Rad Laboratories, Hemel Hempstead, UK). Equivalent amounts of protein were
loaded in each lane and run on 10% sodium dodecyl sulphate-polyacrylamide gel
electrophoresis under reducing conditions. Proteins were transferred into nitrocellulose
membranes (Bio-Rad Laboratories, Hercules, CA), that were blocked with 5% non-fat
dried milk for 1 h, and then incubated overnight at 4°C with the following antibodies: rabbit
anti-human T-bet (1:600 dilution), rabbit anti-human phospho-STAT (pSTAT)4 (1:1000
dilution), and rabbit anti-human STAT4 (1:500 dilution), all from Abcam (Cambridge, UK).
Membranes were rinsed and incubated with the appropriate horseradish peroxidase-
conjugated secondary anti-rabbit antibody (diluted 1:2000; Dako, High Wycombe, UK) in
blocking solution. The reaction was developed with enhanced chemiluminescence (ECL
Prime Kit; Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Blots were then
stripped and analyzed for β-actin, as an internal loading control, using a rabbit anti-human
β-actin antibody (1:5000 dilution, Abcam). Bands were quantified by scanning
densitometry (LKB Ultrascan XL Laser Densitometer; Kodak, Hemel Hempstead, UK).
Protein expression levels were quantified by densitometric analysis, using the ImageJ
software after quantity normalization with β-actin.

Western blotting was also used to evaluate the silencing of IFN-γRα gene. After 72 h of
incubation with ShRNA and lipofectamine, cells were detached with trypsin and
centrifuged at 3000 rpm at 4°C for 5 min. Supernatant was discarded and the pellet was
resuspended using a home-made solubilisation buffer (50 mM Hepes, 1mM EDTA, 10%
Glycerol, 1% Triton-X-100, 150 mM sodium chloride, pH 7.4) containing phosphatase
(Sigma-Aldrich, Milan, Italy) and protease (Thermo Scientific, Rockford, IL, USA) inhibitors.
After an incubation of 15 min on ice, samples were frozen, thawed and passed through a
needle. Supernatants containing extracted proteins were collected after a centrifuge of 15
min at 4°C at 12000 rpm. Protein concentration was determined using NanoDrop 2000
spectrophotometer (Thermo Scientific, Milan, Italy). Proteins were separated by 7.5%
sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then proteins were
transferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK).
Membranes were blocked in 4% non-fat milk in T-PBS and then incubated overnight at
4°C with a rabbit anti-IFN-γRα antibody (1:200, Santa Cruz Biotechnology, Santa Cruz,
CA, USA). Membranes were washed three times before incubation with anti-rabbit
peroxidase conjugate antibody (1:2000, Chemicon-Millipore, Billerica, MA). Blots were
developed using enhanced chemiluminescence (Thermo Scientific, Rockford, IL, USA).
Membranes were stripped with Restore Plus Western Blot Stripping Buffer (Thermo
Scientific, Rockford, IL, USA) and then incubated overnight with a mouse anti-vinculin
antibody (1:5000, Sigma-Aldrich, Milan, Italy). Anti-mouse peroxidase conjugated
secondary antibody was used (1:2000, Chemicon-Millipore, Billerica, MA). Images were acquired by C-DiGit Blot Scanner (LI-COR Biosciences GmbH, Bad Homburg, Germany) and band intensity was quantified by Image Studio software (LI-COR Biosciences GmbH, Bad Homburg, Germany). Non-silenced Caco-2 cells were used as positive control of gene silencing. Band intensities were normalized to the intensity of vinculin band (reference protein).

**Statistical Analysis**

All results are expressed as means ± the standard error of the mean (SEM). Statistical analysis was carried out with the computer-assisted Prism GraphPad Program (Prism version 4.0, GraphPad Software, San Diego, CA). The significance of differences among groups was assessed by the Kruskal–Wallis test; paired comparisons were assessed using the Mann–Whitney U test; two-way ANOVA was used when indicated in the text; Spearman test was used for correlation analysis. $P$ values less than 0.05 were considered significant.
RESULTS

Subjects

Table 1 reports the demographic characteristics of the study population. We did not find significant difference between AC and IBS in the mean age any parts of the study. In Part 1 we detected a lower percentage of females in AC compared to IBS (26% vs 55%, P<0.05). To rule out a possible inference of unbalanced gender proportions, data from Part 1 were analysed by two-way ANOVA including gender as confounding factor and no significant differences were found.

IFN-γ gene expression and protein content from the colonic mucosa

IFN-γ gene expression in the colonic mucosa of IBS patients was evaluated using quantitative RT-PCR. Compared to AC, patients with IBS showed a significant increase in IFN-γ gene expression (0.7±0.2 vs 3.1±0.7, P<0.01). In comparison with AC, each of the IBS subgroups showed a significant increase in IFN-γ gene expression (IBS-D: 0.7±0.2 vs 2.9±1, P<0.01; IBS-C: 0.7±0.2 vs 4.5±1.2, P<0.05; IBS-M: 0.7±0.2 vs 1.6±0.2, P<0.05, Figure 1A), although there were no significant differences among IBS subtypes (P=0.31).

The amount of IFN-γ protein was assessed using ELISA assay and normalized to total protein amount (Figure 1B). Compared to AC, patients with IBS showed a significant increase in IFN-γ content (97.6±9.9 pg/mg vs 143.2±12.9 pg/mg of total proteins, P<0.05).

Regarding the IBS subgroups, compared to AC, IBS-D (97.6±9.9 pg/mg vs 152±22.7 pg/mg of total proteins, P<0.05) and IBS-M (97.6±9.9 pg/mg of vs 146±28.2 pg/mg of total proteins, P<0.05) patients showed a significant higher amount of IFN-γ; IBS-C patients showed a higher IFN-γ content, although not significant (97.6±9.9 pg/mg of total proteins vs 129.5±15.5 pg/mg of total proteins, P=0.17).

T-bet and pSTAT4 expression in the colonic mucosa

T-bet expression, normalized for β-actin, was significantly increased in inflamed CD mucosa in comparison to AC mucosa (6.8±0.5 vs 1.6±0.2; P<0.01) and IBS mucosa (2.6±0.2; P<0.05). Moreover, mucosal T-bet expression was significantly (P<0.05) higher in IBS patients in comparison to AC (Figure 2A). When IBS patients were split according to their predominant clinical phenotype, only IBS-D patients (2.9± 0.4) showed significantly (P<0.05) higher mucosal T-bet levels in comparison to AC. No significant difference was found between either IBS-C (2.4±0.2) or IBS-M patients (2.7±0.3) and AC. pSTAT4 expression, normalized for STAT4, was significantly increased in inflamed CD mucosa in comparison to AC mucosa (6.7±0.4 vs 2.8±0.4; P<0.01) and IBS mucosa (3.0±0.2; P<0.01). However, no significant difference was found in the expression of pSTAT4 between IBS patients and AC (Figure 2B). When IBS patients were split according to their predominant clinical phenotype, none of the three IBS subgroups, i.e. IBS-D patients (2.8±0.2), IBS-M patients (2.7±0.4) and IBS-C patients (3.4±0.3), showed significantly higher mucosal pSTAT4 levels in comparison to AC.

SERT gene expression in the colonic mucosa

SERT gene expression was evaluated in the colonic mucosa of AC and patients with IBS. As reported in Figure 3, compared to AC, patients with IBS showed a decrease in SERT gene expression (3±1.2 vs 1.6±1.1), although this difference didn’t reach the statistical significance (Figure 3A). Interestingly, in a subset of subjects a trend towards a negative
correlation was found out between colonic SERT and IFN-γ gene expression (Figure 3B, $P=0.08$, $r_s=-0.4$).

**SERT gene expression in Caco-2 cells exposed to cultured biopsy supernatants**

In order to test the effect of mucosal mediators on SERT gene expression in Caco-2 cells, supernatants from cultured biopsies were obtained. Supernatants were concentrated 10 times and loaded on the ELISA plate. Compared to AC, IFN-γ protein released by cultured biopsies was significantly increased in the IBS group ($22.1±6.7$ pg/ml vs $59.8±11.4$ pg/ml, $P<0.05$). Compared to AC, an increased amount of IFN-γ was detected in each IBS subtype (IBS-D: $22.1±6.7$ pg/ml vs $74.9±25.2$ pg/ml, $P=0.06$; IBS-C: $22.1±6.7$ pg/ml vs $45.9±9.1$ pg/ml, $P=0.08$; IBS-M: $22.1±6.7$ pg/ml vs $57.2±20.8$ pg/ml, $P=0.28$) (Figure 4A).

Caco-2 cells were incubated with AC or IBS supernatants for 72 h and SERT gene expression was evaluated using qPCR assay. Compared to Caco-2 cells exposed to AC supernatants, Caco-2 cells treated with IBS supernatants showed a significant decrease in SERT gene expression (1.2±0.2 vs 0.6±0.1, $P<0.01$, Figure 4B). A statistically significant difference was observed comparing AC and each IBS subtype (IBS-D: 1.2±0.2 vs 0.7±0.1, $P<0.05$; IBS-C: 1.2±0.2 vs 0.6±0.1, $P<0.05$; IBS-M: 1.2±0.2 vs 0.3±0.1, $P<0.05$).

**Role of INF-γ on SERT gene expression in Caco-2 cells exposed to biopsy supernatants**

In ancillary experiments, we confirmed previous data (26) showing a significant reduction of SERT expression in Caco-2 cells treated with 500 ng/ml of IFN-γ for 72 h (data not shown).

In order to evaluate the involvement of IFN-γ in the downregulation of SERT gene expression elicited by treatment of Caco-2 cells with IBS supernatants, we silenced the constitutive expression of IFN-γ receptor (IFN-γR) α-chain gene of Caco-2 cells. Densitometric analyses showed similar amounts of IFN-γRα in the negative (i.e., empty plasmid) and positive controls (non-silenced Caco-2 cells). In contrast, the ShRNA plasmids evoked a marked reduction in receptor expression (See Supplementary Figure 1). In ancillary experiments we identified the plasmid that gave the best (> 80%) reduction in protein expression and thus this plasmid was used to transfect Caco-2 cells (silenced Caco-2) in all subsequent experiments. Treatment with IBS or AC supernatants was performed as reported above.

Following exposure of Caco-2 cells with IBS supernatants, SERT gene expression in silenced IFN-γRα cells showed a substantial recovery over that of non-silenced Caco-2 cells (0.5±0.1 vs 0.2±0.1, $P<0.0001$, Figure 5). Compared to non-silenced Caco-2 cells treated with IBS-D, C and M supernatants, a significant recovery of SERT gene expression was obtained with each of the IBS subgroups (IBS-D: 0.3±0.1 vs 0.4±0.04, $P<0.05$; IBS-C: 0.1±0.02 vs 0.5±0.1, $P<0.001$; IBS-M: 0.1±0.1 vs 0.6±0.2, $P<0.05$).

No significant difference was observed treating the non-silenced or the silenced Caco-2 cells with control supernatants (0.8±0.3 vs 0.7±0.1, $P=0.32$).
DISCUSSION

In the present study, we showed increased levels of IFN-γ gene and protein expression in the colonic mucosa of patients with IBS compared to AC. In addition, we found that IBS mucosa produced soluble mediators that decreased SERT gene expression in epithelial cells, as shown by the effect of IBS mucosal supernatants on Caco-2 cells. IFN-γ exerted a key role in SERT down-regulation as demonstrated by the recovery of SERT expression following the silencing of IFN-γ receptor gene.

Our results showed a marked increase in IFN-γ gene expression and protein levels in the colonic mucosa of patients with IBS. These data are in line with a recent study by Darkoh et al., reporting increased serum and faecal levels of IFN-γ in IBS patients (19). The potential relevance of our findings is supported by the marked (4.7 fold) up-regulation of IFN-γ gene and by the concomitant increased gene translation leading to higher protein expression and release in the mucosal milieu. As the Th1-specific T box transcription factor T-bet and the activated form of STAT4 (i.e. pSTAT4) are essential mediators of naïve T cell polarization towards the Th1 phenotype (47), we explored their expression in the mucosa of IBS patients. We found that T-bet, but not pSTAT4, was significantly increased in the mucosa of IBS patients in comparison to AC mucosa, in particular in patients with IBS-D. This finding further supports the implication of IFN-γ in the pathophysiological mechanisms underlying IBS. The absence of a significant difference in mucosal pSTAT4 levels between IBS patients and AC might be interpreted as a consequence of the small number of samples analysed.

The evidence supporting the potential role of cytokines in IBS has been so far controversial (8, 12, 41). Although there are some data showing the presence of cytokine changes in the intestine of patients with IBS, most of these studies were small and could not be confirmed in subsequent experiments. Accordingly, a recent meta-analysis showed a limited evidence of the participation of IL-10 and TNF-α, two key prototypes respectively of anti-inflammatory and pro-inflammatory cytokines (8). Here we opted to study IFN-γ for its pleiotropic immunoregulatory properties (33) and for its role in increasing intestinal permeability in IBS. In line with this concept, IFN-γ has been shown to increase paracellular permeability in Caco-2 cells through the disruption of tight junctions (48). Our data showing increased IFN-γ gene expression in the IBS mucosa are in contrast with previous results showing increased IFN-γ mRNA only in patients with post-infectious IBS (14) no changes of IFN-γ after lymphocytes stimulation (35) or in the sigmoid colon of IBS (13). We advocate possible methodological differences to explain these divergent results. In addition, as declared by the authors of this previous study, the small number of patients and the inclusion of the IBS-D subset only, limited the value of their results. Finally, it should be considered that contrasting results are not unusual in a heterogeneous condition such as IBS and possibly related to regional, dietary, genetic, and experimental differences among studies.

The cause(s) of increased mucosal gene and protein expression of IFN-γ remain unknown, but may be linked to excessive immune stimulation by the intestinal microbiota, through a leaky mucosal barrier, and/or stress-induced activation of the immune system. Recent
research suggests also the participation of a genetic component. Two independent studies found an association of the risk gene associated to CD TNFSF15 gene (rs4263839G/A) with IBS (50). Indeed, TNFSF15 codifies for TL1A protein, a member of the TNF-α superfamily that activates Th17 cells to produce also IFN-γ. In CD the increased expression of TNFSF15 mRNA induces an increase in IFN-γ production by lamina propria mononuclear cells (2). Taken together, these data might suggest the hypothesis that subsets of IBS may represent an immunologically incomplete expression variant of CD (3). Our data showing increased IFN-γ levels, add further evidence of a participation of the immune system in the pathophysiology of IBS. In addition, these data suggest that this cytokine could participate to mast cell activation, which is a common feature described in patients with IBS (5). Interestingly, we have previously shown a strong significant correlation between mast cells, their mediators (i.e., histamine and tryptase) and the spontaneous release of 5-HT, suggesting that mast cell infiltration and activation could drive 5-HT release. IFN-γ is known to induce the expression and aggregation of the high-affinity human IgG receptor (Fc-γRI) by mast cells (43) resulting in their degranulation. These mechanisms have been shown to be candidate participants in the intestinal immune activation in CD patients in whom IFN-γ induces the expression of TLR-4 and Fc-γRI (36). Here we speculate that similar mechanisms could take part in the pathophysiology of IBS, although the magnitude of the immune response is certainly much less. The participation of the immune system in IBS pathophysiology has prompted the investigation of anti-inflammatory drugs in IBS. Previous studies reported a beneficial effect of the mast cell stabilizers disodium cromoglycate as well as ketotifen in IBS symptoms (4). Although a recent randomized placebo controlled study with mesalazine showed negative results in IBS, clearly a sustained response was identified in a subgroup of patients, suggesting that low-grade inflammation is a key participant factor, at least in some IBS patients (3).

Our data suggest that IFN-γ is involved in the reduction of SERT gene expression in IBS. Indeed, silencing the IFN-γ receptor in Caco-2 cells exposed to IBS supernatants abolished the reduction of SERT expression. These results are in line with the findings of Foley et al. who reported that IFN-γ reduced SERT expression in Caco-2 cells (26). In addition, we observed a reduction of SERT gene expression in the colonic mucosa of patients with IBS compared to AC (Figure 3A), although this difference didn’t reach the statistical significance, probably because of the small number of samples analysed. Interestingly, colonic SERT and IFN-γ gene expression seem to be inversely correlated (Figure 3B); although the correlation does not reach statistical significance (P=0.08) this preliminary data would confirm the involvement of IFN-γ in SERT reduction directly in the colonic mucosa of IBS patients.

The direct consequence of reduced SERT expression would be an increased tissue bioavailability of 5-HT (16). This is in line with evidence from our (17) and other laboratories (27) demonstrating increased mucosal levels of 5-HT in patients with IBS. 5-HT increased bioavailability could have relevant effects on gut motor function and sensation, respectively through activation of 5-HT4 receptors on enteric motor neurons and visceral sensitivity via 5-HT3 receptors on sensory neurons. In line with this concept, higher levels of mucosal 5-HT have been correlated with the severity of abdominal pain in IBS (17). SERT changes and consequently 5-HT availability did not correlate with bowel habits. Bowel function is the result of complex and not completely understood physiological event in which many different factors may be involved, including muscle contractility, enteric nervous system function, secretion, endocrine and immune factors. Although a mechanistic interpretation is currently lacking, the inflammatory hypothesis
leading to contrasting bowel habits is in line with evidence that both diarrhea and constipation can occur in patients with IBD. Interestingly, mice lacking SERT showed alternating diarrhoea and constipation and increased motility (15). In further support of the key role of 5-HT in IBS pathophysiology, there are data showing the effects of 5-HT on motor, secretory, and sensory functions, the impairment of 5-HT metabolism, including increased 5-HT release, in patients with IBS and the effectiveness of 5-HT receptor modulators, including 5-HT3 antagonists (e.g. alosetron, cilansetron) for IBS-D and 5-HT4 agonists (e.g., tegaserod, prucalopride) for IBS-C (29).

Reduced SERT expression and increased 5-HT levels could have also repercussions on the immune system activation described in IBS. 5-HT up-regulates mucosal inflammation, and SERT knock out mice exhibit enhanced inflammatory responses in the colon following antigen-based induced colitis (9). Recent evidence suggests that 5-HT degranulates mast cells via 5-HT1A receptors in both guinea pig and human intestine (49). In addition, the 5-HT3 antagonist granisetron reduced the severity of experimental colitis in rats (24).

In conclusion, our results show increased expression and release of IFN-γ, which may down-regulate SERT expression in the colonic mucosa of IBS patients, and provide the rationale for considering this immune response as a pathophysiological mechanism and a possible attractive therapeutic target at least in subsets of patients with IBS.
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Author contribution: M.R.B. and G.B. planned the study, designed the protocol, interpreted data and contributed to the writing of the paper. G.B., C.C., V.S., L.B., and A.D.S. contributed to patient’s recruitment. G.B. and C.C, performed the colonoscopies. M.R.B., P.G., M.F. and A.A, performed experiments. G.B. V.S. and A.D.S. supervised all steps of the study. All authors revised and approved the final draft of the paper.
REFERENCES


Table 1. Clinical characteristics of study subjects.

<table>
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<th>Patient Group</th>
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<th>Age years</th>
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<td></td>
<td></td>
<td>M/F</td>
<td>Mean±SEM</td>
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<td></td>
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<tr>
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Table 1. AC, asymptomatic control; IBS, irritable bowel syndrome obtained by averaging the results of the subgroups; IBS-C, constipation predominant IBS; IBS-D, diarrhoea predominant IBS; IBS-M, alternating predominant IBS.
FIGURE LEGENDS

Figure 1. IFN-γ gene and protein expression in the colonic mucosa.
1A: IFN-γ gene expression in the colonic mucosa of 10 AC and 19 patients with IBS (obtained by averaging the results of the subgroups). Compared to AC, there was a fourfold increase in IFN-γ gene expression in the colonic mucosa of patients with IBS (*P<0.01). Compared to AC, significant IFN-γ gene expression increase was found for all the three IBS subgroups (i.e., 11 IBS-D, #P<0.01; 5 IBS-C, §P<0.05; 3 IBS-M, @P<0.05).

Data are expressed as relative quantification using comparative Ct (2^-ΔΔCt). 1B: IFN-γ protein amount in the colonic mucosa of 15 AC and 38 patients with IBS. Compared to AC, a significant increase in IFN-γ amount was detected in IBS colonic mucosa (*P<0.05). Regarding the IBS subgroups, IBS-D and IBS-M reached the statistical significance (#P<0.05; @P<0.05) compared to AC; results are reported as mean ± SEM.

Figure 2. T-bet and pSTAT4 expression in the colonic mucosa.
2A: Expression of T-bet detected by immunoblotting in intestinal biopsies collected from 6 AC, 6 active Crohn’s disease (CD) patients, 6 patients with IBS-D, 6 patients with IBS-M and 6 patients with IBS-C. Compared to CD patients, both AC and IBS patients (obtained by averaging the results of the subgroups), independently from bowel habit, showed significant lower levels of T-bet expression (*P<0.01). Compared to AC, significant higher levels of T-bet were found in the colonic mucosa of IBS (**P<0.05) and IBS-D patients (#P<0.05). Blots were stripped and analysed for β-actin as an internal loading control. Each example shown in the upper panel is representative of experiments performed in all patients and controls. Results are mean ± SEM. 2B: Expression of phosphorylated form of signal transducer and activator of transcription (pSTAT)4 detected by immunoblotting in intestinal biopsies collected 6 AC, 6 active Crohn’s disease (CD) patients, 6 patients with IBS-D, 6 patients with IBS-M and 6 patients with IBS-C. Compared to CD patients, both AC and IBS patients, independently from bowel habit, showed significant lower levels of pSTAT4 expression (*P<0.01). Blots were stripped and analyzed for STAT4 as an internal loading control. Each example shown in the upper panel is representative of experiments performed in all patients and controls. Results are mean ± SEM.

Figure 3. SERT gene expression in the colonic mucosa and its correlation with IFN-γ gene expression.
3A: SERT gene expression in the colonic mucosa of 9 AC and patients with 10 IBS (obtained by averaging the results of the subgroups). Compared to AC, there was a decrease in SERT gene expression in the colonic mucosa of patients with IBS, even if this difference didn’t reach the statistical significance (P=0.2). Data are expressed as relative quantification using comparative Ct (2^-ΔΔCt). 3B: Colonic SERT and IFN-γ gene expression correlation. A trend towards a negative correlation was found between SERT and IFN-γ gene expression in the colonic mucosa of 19 subjects (P=0.08, rs: -0.41).

Figure 4. IFN-γ protein amount in the supernatants from cultured biopsies and the effect of supernatants on SERT gene expression in Caco-2 cells.
4A: IFN-γ protein amount in the supernatants from cultured biopsies of 5 AC and 22 patients with IBS (obtained by averaging the results of the subgroups). Supernatants from cultured biopsies include all mediators spontaneously released by cultured biopsies. Compared to AC, IBS supernatants contained a significant higher amount of IFN-γ (*P<0.05). 4B: Alteration of SERT gene expression induced by supernatants from 9 AC and 29 IBS supernatants in Caco-2 cells. Compared to AC, IBS supernatants induced a 45% significant decrease of SERT gene expression (*P<0.01). Compared to AC, each of
the three IBS subgroups induced a significant SERT gene expression reduction (12 IBS-D, #P<0.05; 9 IBS-C, §P<0.05; 8 IBS-M, @P<0.01). Data are expressed as relative quantification using comparative Ct (2^ΔΔCt). Results are reported as mean ± SEM.

Figure 5. IFN-γ receptor silencing reversed supernatant effect on SERT gene expression.

Comparison between SERT gene expression induced by 6 AC and 27 IBS supernatants, obtained by averaging the results of the subgroups, in silenced Caco-2 cells and non-silenced ones. Compared to AC, IBS supernatants induce 1.3 fold reduction in SERT gene expression in silenced Caco-2 cells without reaching the significance (P=0.13). We observed a significant recovery of SERT gene expression in silenced Caco-2 cells treated with IBS supernatants compared to non-silenced Caco-2 treated with IBS supernatants (*P<0.0001). A significant recovery of SERT gene expression in silenced Caco-2 cells was obtained for each of the three IBS subgroup compared to normal Caco-2 cells (9 IBS-D, #P<0.05; 9 IBS-C, §P<0.001; 9 IBS-M, @P<0.05). Data are expressed as relative quantification using comparative Ct (2^ΔΔCt). Results are reported as mean ± SEM.
Figure 1

A

![Bar graph showing IFN-γ mRNA levels (2^ΔΔCt) for different groups.](image)

B

![Bar graph showing IFN-γ pg/mg total protein for different groups.](image)

Legend:
- *: Significant difference compared to AC
- #: Significant difference compared to IBS
- §: Significant difference compared to IBS-D
- @: Significant difference compared to IBS-C

Groups: AC, IBS, IBS-D, IBS-C, IBS-M
Figure 2

A

[Bar graph showing the expression levels of T-bet and β-actin across different conditions (AC, CD, IBS-D, IBS-M, IBS-C, IBS).]

B

[Bar graph showing the expression levels of pSTAT4 and STAT4 across different conditions (AC, CD, IBS-D, IBS-M, IBS-C, IBS).]
Figure 3

A

SERT mRNA levels ($2^{-\Delta\Delta Ct}$)

IFN-γ mRNA levels ($2^{-\Delta\Delta Ct}$)

B

SERT mRNA levels ($2^{-\Delta\Delta Ct}$) vs. IFN-γ mRNA levels ($2^{-\Delta\Delta Ct}$)
Figure 4

A

B

SERT mRNA levels (2^{ΔΔCt})

IFN-γ pg/ml

AC IBS IBS-D IBS-C IBS-M

0.0 0.5 1.0 1.5 2.0

* # § @
Figure 5

SERT mRNA levels ($2^{-\Delta\Delta C_t}$)

- AC
- IBS
- IBS-D
- IBS-C
- IBS-M

Non-silenced Caco-2
Silenced Caco-2

Legend:
- Non-silenced Caco-2
- Silenced Caco-2

Signs:
- *
- #
- $\S$
- @