

Mucosal Immune Cell Numbers and Visceral Sensitivity in Patients With Irritable Bowel Syndrome: Is There Any Relationship?

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- OBJECTIVES:** Repeated exposure to stress leads to mast cell degranulation, microscopic inflammation, and subsequent visceral hypersensitivity in animal models. To what extent this pathophysiological pathway has a role in patients with the irritable bowel syndrome (IBS) has not been properly investigated. The objective of this study was to assess the relationship between visceral hypersensitivity, microscopic inflammation, and the stress response in IBS.
- METHODS:** Microscopic inflammation of the colonic mucosa was evaluated by immunohistochemistry in 66 IBS patients and 20 healthy volunteers (HV). Rectal sensitivity was assessed by a barostat study using an intermittent pressure-controlled distension protocol. Salivary cortisol to a psychological stress was measured to assess the stress response.
- RESULTS:** Compared with HV, mast cells, T cells, and macrophages were decreased in IBS patients. Similarly, λ -free light chain (FLC)-positive mast cells were decreased but not immunoglobulin E (IgE)- and IgG-positive mast cells. There were no differences between hypersensitive and normosensitive IBS patients. No relation was found between any of the immune cells studied and the thresholds of discomfort, urge, first sensation, or IBS symptoms (e.g., abdominal pain, stool-related complaints, bloating). Finally, stress-related symptoms and the hypothalamic–pituitary–adrenal-axis response to stress were not correlated with the number of mast cells or the presence of visceral hypersensitivity.
- CONCLUSIONS:** Although the number of mast cells, macrophages, T cells, and λ FLC-positive mast cells is decreased in IBS compared with HV, this is not associated with the presence of visceral hypersensitivity or abnormal stress response. Our data question the role of microscopic inflammation as an underlying mechanism of visceral hypersensitivity, but rather suggest dysregulation of the mucosal immune system in IBS.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/ajg>

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INTRODUCTION

The irritable bowel syndrome (IBS), consisting of chronic abdominal pain or discomfort and changes in bowel habit in the absence of an organic explanation, is the most common disorder seen in the out-patients practice of gastroenterologists (1). Although this syndrome is not life-threatening, it leads to considerable morbidity causing a clear restriction in daily life. Not only by medical consumption but also through indirect cost by sick leave, the

economic impact is enormous. In the last decades, several studies have shown that, compared with healthy volunteers (HV), IBS patients have an increased sensitivity to colonic distension (2–4). This so-called visceral hypersensitivity, now recognized as an important pathophysiological mechanism underlying symptom generation in IBS, is present in 33–65% of IBS patients (5–8).

One of the main factors involved in the initiation and exacerbation of IBS is stress (9). Animal studies have abundantly illustrated

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that stress is an important trigger leading to visceral hypersensitivity, a mechanism involving the release of corticotrophin releasing hormone (10–12) and subsequent mast cell activation (13–15). In addition to stress, mast cells can be activated by binding and subsequent antigen-specific crosslinking of IgE-, IgG antibodies, or free light chain immunoglobulins (IgFLC) (16,17). Activation of mast cells leads to increased mucosal permeability and increased exposure of the mucosal immune system to intraluminal antigens. Moreover, mast cell mediators are potent chemotactic agents contributing to increased influx of immune cells. Especially as inflammatory mediators released from immunocytes (tryptase, histamine, serotonin (5-HT), tumor necrosis factor- α , interleukin (IL)-1B, nerve growth factor (NGF), nitric oxide, prostanoids) sensitize visceral afferent nerves (18–23), microscopic inflammation is considered to have an important role in the development of visceral hypersensitivity in IBS. Several studies have indeed shown an increased number of mast cells and T cells in mucosal biopsies from IBS patients (24–31). Furthermore, in IBS patients, mast cells have been shown to be in close proximity to visceral afferent nerves (29) and exposure to the supernatant obtained from colonic biopsies of IBS patients, but not from healthy controls, increased the excitability of rat mesenteric sensory nerves and induced visceral hypersensitivity in mice (32,33).

Microscopic inflammation of the intestinal mucosa is abundantly reported to have an important role in the pathogenesis of IBS. Data on the number of immune cells in the intestinal mucosa are however inconsistent; some studies demonstrate an increased number of mast cells and T-cells, whereas others fail to confirm this finding (33–36). Moreover, the relationship between stress response, microscopic inflammation, and visceral sensitivity has not been studied in detail in IBS. In the present study, we hypothesized that visceral hypersensitivity was associated with microscopic inflammation and an increased response to an acute stressor. To this end, a large cohort of IBS patients and healthy controls underwent a rectal barostat and microscopic inflammation was assessed in descending and ascending colonic mucosal biopsies. In addition, the stress response to an acute stressor was evaluated in a subgroup of patients.

METHODS

Study subjects

IBS patients and HV were recruited between March 2004 and November 2008. IBS patients were identified using the Rome II criteria. Organic disease was excluded by a minimum work-up consisting of careful history taken, a normal physical examination, a normal sigmoidoscopy or colonoscopy, normal thyroid stimulating hormone levels and blood counts, and negative stool examinations. Furthermore, patients had to be free of concomitant diseases, including psychiatric disorders. Medication likely to interfere with gastrointestinal function and analgesics were discontinued at least 7 days before entering the study. HV were recruited by public advertisement. None of the HV had a history of gastrointestinal complaints or was taking any medication other than birth control. The study protocol was approved by the

Medical Ethics Committee of the Academic Medical Center. All participants gave their written and informed consent to participate in the study.

Symptom assessment

IBS symptoms (abdominal pain, diarrhea, constipation, abdominal bloating, flatulence, urgency, and incomplete evacuation) were scored by the Gastrointestinal Symptom Rating Scale, in which the intensities of the symptoms are scored on a 7-graded Likert scale, with descriptive anchors (0 = no symptoms at all; 1 = minimal symptoms; 2 = mild symptoms; 3 = moderate symptoms; 4 = rather serious symptoms; 5 = serious symptoms; and 6 = very severe symptoms) (37).

IBS subtyping into diarrhea predominant, constipation predominant, and alternating type of IBS was based on Rome II criteria of IBS. Additionally, patients were asked whether the onset of the IBS symptoms were acute (sudden onset) or gradual (insidious over time) and whether their IBS symptoms were triggered or exaggerated by stressful events.

Barostat studies

Rectal sensitivity to distension was assessed using an electronic barostat that automatically corrected for the compressibility of air (Distender series II, G&J Electronics, Toronto, Ontario, Canada). The barostat was connected to a 500-ml polyethylene bag (maximal diameter 9 cm), tightly wrapped on the distal end of a double lumen polyvinyl tube (Salem Sump tube 14 Ch.; Sherwood Medical, St Louis, MO) introduced in the rectum (6). The barostat protocol is described in detail elsewhere (5). Briefly, 60 min before rectal balloon placement, the subjects received a tap water enema. Participants were placed in the left lateral decubitus position and minimal distending pressure (MDP), defined as the minimum pressure at which the intrabag volume was >30 ml was determined. Subjects scored the perception of sensation evoked by rectal distension using a 6-point scale with verbal descriptors (0 = no sensation; 1 = first sensation; 2 = first urge to defecate; 3 = normal urge to defecate; 4 = severe urge to defecate; 5 = discomfort/pain). If a subject reported discomfort or pain, the bag was instantaneously deflated. For safety, the bag was automatically deflated at pressures above 50 mm Hg or volumes above 600 ml. Rectal distension series was performed according to a phasic, semi-randomly ascending isobaric distension protocol consisting of pressure increments of 3 mm Hg above MDP, each step lasting 2 min and separated by 1-min intervals at baseline (MDP). Sensations were scored during each distension step. The threshold for discomfort/pain was determined by the pressure at which an individual reported a sensation score of 5. Based on the 10th percentile of previously performed barostat studies in HV, patients were considered hypersensitive to rectal distension if the thresholds for discomfort was <24 mm Hg above intra-abdominal pressure (6). If a subject did not report discomfort/pain before the fixed pressure limit of 50 mm Hg was reached, we conservatively determined the threshold by the highest pressure reached plus one pressure step. Sensory thresholds for first sensation and urge to defecate were calculated for each individual by

the lowest pressures at which a certain sensation score (i.e., 1 and 3, respectively) was reported.

Immunohistochemistry

Materials. Participants underwent a total colonoscopy or sigmoidoscopy after cleansing the colon with KleanPrep or Moviprep (Norgine BV, Amsterdam, The Netherlands; **Supplementary Table S1** online). During endoscopy, biopsies were collected from the descending and ascending (only in patients undergoing colonoscopy) colon, fixed in formaldehyde immediately after collection, and subsequently embedded in paraffin. Immunohistochemical staining was performed on 4 μ m deparaffined sections.

Single staining protocol. For antigen retrieval, all sections of ascending and descending biopsies were heated during 10 min in TRIS-EDTA (pH 9). Sections were incubated for 1 h at room temperature (RT) with a polyclonal directed against human CD117 for mast cell staining, a primary monoclonal antibody directed against human CD68 and CD163 for macrophages, a primary monoclonal antibody directed against human CD3 and CD8 for T cells, or a primary monoclonal antibody directed against human FOXP3 (i.e., a master regulator in the development and function of regulatory T cells) for regulatory T cells (**Supplementary Table S1** online). The sections were reincubated with Powervision for 30 min (poly-horse radish peroxidase anti-mouse for CD68, CD163, CD8, and FOXP3; poly-horse radish peroxidase anti-rabbit for CD117 and CD3; Immunologic, Duiven, The Netherlands). Horse radish peroxidase activity was visualized with DAB (3,3'-diaminobenzidine, DAKO, Glostrup, Denmark) for 7 min. Only staining for CD117 was visualized with AEC (3-amino-9-ethylcarbazol; Sigma, St Louis, MO) for 10 min. All sections were counterstained with hematoxylin (Sigma) for 1 min. Negative controls were included and did not contain any staining. Positive cells were subsequently quantified in 18 high power fields of each section by computer-assisted image analysis (Qwin analysis system, Leica, Cambridge, UK) (38) and are expressed as mean number per square millimeter (/mm²) of mucosal tissue.

Double staining protocol. Sequential double alkaline phosphatase (AP) staining was performed to identify double-positive cells in sections from descending colon for CD117/IgE, CD117/IgG, and CD117/IgFLC (λ FLC and κ FLC; i.e., the two types of the small polypeptide subunit of an antibody (Ig)), respectively. After heat-induced epitope retrieval with TRIS-EDTA (pH 9.0, 10 min) for CD117/IgE and CD117/IgG or without heating for CD117/ λ FLC, sections were incubated with a serum-free protein block (Ultra V Block, Thermo Scientific/Labvision, Fremont, CA) for 10 min at RT. Next, the sections were incubated with CD117 for mast cells (1 h) or with λ FLC for free light chains (overnight at 4°C). The sections were reincubated with Powervision (poly-AP anti-mouse for λ FLC and anti-rabbit for CD117, Immunologic) for 30 min. AP activity was visualized with Vector Blue (SK-5300, Vector Labs, Burlingame, CA) for 10 min. Thereafter, all immuno-reagents used in the first staining sequence were removed by a second heat-induced epitope retrieval step with TRIS-EDTA

(pH 9.0, 10 min), leaving the blue reaction product intact (39). Subsequently, all steps from the first part were repeated and sections were incubated for 1 h at RT with polyclonal antibody directly against human IgE or IgG or CD117 (**Supplementary Table S1** online). The sections were reincubated with Powervision (poly-AP anti-rabbit) for 30 min. All second stainings were visualized with Vector Red (SK-5100, Vector Labs) for 20 min. As a recent study reported IgE-mediated activation of mast cells in eosinophilic esophagitis (IgE+ mast cells) (40), esophageal biopsies of these patients ($n=5$) were evaluated for CD117+IgE+ double staining as positive controls. Stainings were subsequently quantified by spectral imaging, using the NUANCE spectral imaging system (Caliper Life Sciences, Hopkinton, MA) (41). In short, the spectra of Vector Blue and Vector Red were determined using single staining. This spectral library was used to unmix the double staining images into two individual component images, and also allowing to visualize co-localization of two overlapping markers (41,42). Positive double- and single-stained cells were counted and expressed as mean number per square millimeter (/mm²) of mucosal tissue.

Stress test

Before performing the stress test, the degree of anxiety and depression was assessed using the *HADS* (*Hospital Anxiety and Depression Scale*; Dutch version) (43,44). The HADS scores 14 items, 7 related to anxiety and 7 related to depression. Each item is scored using a 4-point scale (0–3), yielding a score ranging between 0–21 for anxiety and depression.

The stress test consisted of three psychological stressful tasks: a Stroop test (color-word conflict test), a mirror-tracing task, and a speech task. The protocol started in the afternoon with a 20-min baseline period after which the tasks were performed. Each task took 5 min, with 6 min in between and 30 min of recovery after finishing the last task. Saliva samples were collected using Salivettes (Sarstedt, Rommelsdorf, Germany) at seven time points during the protocol: two samples during baseline, a sample after completion of each stress task, and two samples during the recovery period. The amount of free cortisol in saliva was determined using enzyme-immuno assay. Kits were purchased from Diagnostic System Laboratories (DSL, Veghel, The Netherlands). Sensitivity of cortisol was 1 ng/ml. Intra-assay variability of the cortisol was 2–10%. Baseline cortisol was defined as the mean of the first two samples collected during the baseline period. Delta cortisol was calculated by subtracting baseline values from the values obtained at every time point during the stress tasks. Delta peak (Δ peak) cortisol is the maximal increase from baseline in response to stress.

Statistical analysis

Statistical evaluations were performed using commercially available software (SPSS 16.0; SPSS, Chicago, IL). Continuous data were compared using the Student's *t*-test, nominal data were compared by the Mann-Whitney *U*-test, comparison of proportion by χ^2 test, and correlations by Spearman rank correlation test. Differences were considered significant at the 5% level ($P < 0.05$).

Table 1. Baseline characteristics

	HV	IBS		
		All	Hypersensitive	Normosensitive
<i>N</i>	20	66	33	30
Male:female	7:13 (65% F)	17:49 (74% F)	7:26 (79% F)	8:22 (73% F)
Age (years)	31±3	38±2	35±2	42±2
Range	20–61	19–65	19–61	20–65
IBS-D, <i>n</i> (%)	—	15 (23)	7 (21)	6 (20)
IBS-C, <i>n</i> (%)	—	15 (23)	9 (27)	6 (20)
IBS-A, <i>n</i> (%)	—	36 (54)	17 (52)	18 (60)
Acute onset, <i>n</i> (%)	—	19 (29)	9 (27)	10 (33)
After infection (PI-IBS), <i>n</i>	—	9	3	6
Duration of symptoms (years)	—	9±1	9±2	9±2
Range	—	0.3–50	0.3–50	0.3–41
Stress-related symptoms, <i>n</i> (%)	—	37 (60)	19 (60)	18 (64)

HV, healthy volunteers; IBS, irritable bowel syndrome; IBS-A, alternating constipation diarrhea IBS; IBS-C, constipation dominant IBS; IBS-D, diarrhea dominant IBS; PI-IBS, postinflammatory IBS.

Visceral sensory thresholds were determined in all but three IBS patients.

Data are shown as mean±s.e.m.

Data are presented as mean±s.e.m., or as median with range in case of a skewed distribution. All data are corrected for multiple testing by Holm–Bonferroni method.

To determine the differences in stress response over time between IBS and HV, linear mixed models were used, controlling for age, gender, body mass index, depression, anxiety, and smoking. Interaction effects between time and functional gastrointestinal disorder status (IBS or HV) and time and gender were also included in the model. An unstructured variance–covariance (UN) matrix was used for all the variables, as this was shown to produce the best fit based on the Akaike's Information Criterion in all models.

RESULTS

Study subjects

Seventy-one IBS patients were initially recruited (**Table 1**). Five patients were excluded due to withdrawal of consent ($n=2$) or problems during endoscopy and biopsy sampling ($n=3$), leaving 66 IBS patients (age: 38±2 years, 77% female) for further analysis. Of these 66 IBS patients, 29 participated afterwards in a clinical trial evaluating the effect of ketotifen (45).

Forty-eight IBS patients and 20 HV underwent a colonoscopy and biopsies were taken in descending and ascending colon. Eighteen IBS patients underwent a sigmoidoscopy yielding biopsies of the descending but not ascending colon. Visceral sensory thresholds were determined in all but three patients. Baseline characteristics are shown in **Table 1**. Data from IBS patients were compared with that of 20 HV (age: 31±3 years, 65% female), free of any gastrointestinal symptoms. The subtypes of IBS was not different among IBS patients with an acute onset ($n=19$; diarrhea $n=4$, constipa-

tion $n=3$, alternating $n=12$) compared with those with a gradual onset ($n=42$; diarrhea $n=10$, constipation $n=10$, alternating type $n=22$).

Additionally, the response to stress was assessed in 22 IBS patients and 18 HV.

Sensory thresholds

Thirty-three IBS patients (52%) were considered visceral hypersensitive to rectal distension (i.e., threshold of discomfort < 24 mm Hg above MDP). The MDP was comparable between visceral hypersensitive patients (8±1 mm Hg), normosensitive (8±1 mm Hg), and HV (7±1 mm Hg). The threshold of first sensation was not different between groups, whereas the threshold of urge and discomfort were significantly lower in hypersensitive IBS patients (urge: 6±1, discomfort: 13±1 mm Hg above MDP) compared with both normosensitive IBS patients (urge: 9±1, discomfort: 33±2 mm Hg above MDP, $P=0.002$ and $P<0.001$, respectively) and HV (urge: 10±1, discomfort: 38±3 mm Hg above MDP, $P<0.001$). No significant differences in threshold of urge and discomfort were observed between normosensitive IBS patients and HV. Finally, there were no gender differences in MDP, discomfort level, first sensation, or urge between IBS female and HV female or IBS male and HV male (data not shown).

Numbers of colonic mucosal immune cells

IBS vs. HV. Biopsies taken from the descending colon of 66 IBS patients contained a lower number of CD117-positive mast cells ($P<0.001$), CD68-positive macrophages ($P=0.001$), and CD8-positive T cells ($P=0.02$) compared with HV (**Table 3**), whereas CD3-positive T cells, FOXP3-positive regular T cells,

Table 2. Number of immunocytes in descending (desc) and ascending (asc) colonic mucosa in HV and IBS patients

	HV (n=20 desc, 19 asc)	All (n=66 desc, 48 asc)	IBS Hypersens. (n=33 desc, 25 asc)	Normosens. (n=30 desc, 20 asc)	P value After H.-B. correct.
<i>Colon descendens (number/mm²)</i>					
CD3	587±66	493±34	408±38	521±43	0.03 ^a
CD8	526±50	388±28	373±30	391±45	0.02 ^b , 0.03 ^a
CD117	370±39	186±10	170±12	200±17	<0.001 ^{a,b,c}
CD68	1,261±146	729±64	710±91	690±97	<0.003 ^{a,b,c}
CD163	678±104	821±21	708±71	948±92	NS
FOXP3	107±8	87±6	80±8	94±9	NS
<i>Colon ascendens (number /mm²)</i>					
CD3	1,033±103	908±58	871±75	988±102	NS
CD8	277±32	207±16	224±22	189±27	NS
CD117	268±26	229±11	227±12	227±20	NS
CD68	1,412±99	1,106±82	1,088±112	1,100±134	NS
CD163	1,040±66	958±45	963±59	943±78	NS
FOXP3	149±27	88±15	80±15	100±31	NS

After H.-B. correct, after Holm–Bonferroni correction for multiple testing; ANOVA, analysis of variance; HV, healthy volunteers; Hypersens., visceral hypersensitive IBS patients; IBS, irritable bowel syndrome; Normosens., visceral normosensitive IBS patients.
Data are presented as mean±s.e.m. Student's *t*-test (b) and one-way ANOVA (a + c) were used for statistical analysis and *P* values were corrected for multiple testing according to Holm–Bonferroni method:
^aHV vs. IBS hypersensitive.
^bHV vs. IBS.
^cHV vs. IBS normosensitive.

and CD163-positive macrophages were not significantly different between IBS and HV (Table 2).

According to gender, CD117-positive mast cells and CD68-positive macrophages were decreased in IBS female and male compared with HV female ($P<0.001$ and $P=0.006$, respectively) and male ($P=0.003$ and $P=0.03$, respectively). CD8-positive T cells were only decreased in IBS female ($P=0.005$). There were no differences in CD3-positive T cells, FOXP3-positive regular T cells, and CD163-positive macrophages (Table 3). Independent of the IBS diagnosis, there were no differences in colonic mucosal immune cells between female and male (data not shown).

Similar findings were obtained in biopsies taken from the ascending colon of 48 IBS patients, but after correction for multiple testing, the differences were not statistically significant (Table 2; gender data not shown).

Hypersensitive vs. normosensitive IBS patients. In the descending colon, mast cells ($P<0.001$) and CD68-positive macrophages ($P<0.003$) were decreased in hypersensitive and normosensitive IBS patients, whereas CD3- and CD8-positive T cells were only decreased in visceral hypersensitive IBS patients ($P=0.02$ and $P=0.03$, respectively; Table 2) compared with HV. The number of FOXP3-positive regulatory T cells and CD163-positive macrophages was comparable between all groups. There were no

significant differences between visceral hypersensitive and normosensitive IBS patients (Table 2). Compared with HV female, CD117-positive mast cells were decreased in IBS hypersensitive as normosensitive female ($P<0.001$). CD68-positive macrophages and CD3-positive T cells were decreased in IBS hypersensitive female ($P=0.007$ and $P=0.008$, respectively), whereas CD8-positive T cells were decreased in IBS normosensitive female ($P=0.03$) (Table 3). Compared with HV male, the number of CD117-positive mast cells were decreased in both IBS hypersensitive as normosensitive male ($P=0.04$ and $P=0.02$, respectively). CD68-positive macrophages were only decreased in IBS normosensitive male ($P=0.02$) (Table 3). There were no differences in other colonic mucosal immune cells (Table 3).

In the ascending colon, the numbers of immunocytes did not differ significantly between hypersensitive, normosensitive IBS patients and HV after correction for multiple testing (Table 2; gender data not shown).

IBS clinical subtypes. In the descending colon, but not the ascending colon, the number of mast cells was significantly lower in patients with IBS vs. HV, independently of the bowel habit subtype ($P\leq 0.002$; Figure 1). CD68-positive macrophages were significantly lower in diarrhea and alternating predominant IBS ($P<0.01$; Figure 1). The number of T cells (CD3 and CD8), FOXP3-positive

Table 3. Number of immunocytes in descending colonic mucosa according to gender

	HV	All	IBS		P value
	(fem., n=13; male, n=7)	(fem., n=49; male, n=17)	Hypersens. (fem., n=26; male, n=7)	Normosens. (fem., n=22; male, n=8)	After H.-B. correct.
<i>Female (number/mm²)</i>					
CD3	634±93	463±34	382±29	522±54	0.008 ^a
CD8	535±68	351±27	367±36	340±43	0.005 ^b , 0.03 ^c
CD117	355±40	183±12	166±14	206±22	<0.001 ^{a,b,c}
CD68	1,190±152	713±78	621±96	780±123	0.006 ^b , 0.007 ^a
CD163	816±138	799±57	720±65	905±102	NS
FOXP3	103±11	84±6	79±9	90±8	NS
<i>Male (number/mm²)</i>					
CD3	500±76	634±93	496±141	520±62	NS
CD8	509±76	491±69	383±30	524±108	NS
CD117	394±83	194±17	181±18	183±26	0.003 ^b , 0.04 ^a , 0.02 ^c
CD68	1,394±321	773±114	1,094±168	465±124	0.03 ^b , 0.02 ^c
CD163	424±104	883±137	676±232	1,054±201	NS
FOXP3	116±14	96±14	88±18	104±25	NS

After H.-B. correct, after Holm–Bonferroni correction for multiple testing; ANOVA, analysis of variance; fem., female; HV, healthy volunteers; Hypersens., visceral hypersensitive IBS patients; IBS, irritable bowel syndrome; Normosens., visceral normosensitive IBS patients.

Data are presented as mean±s.e.m. Student's *t*-test (b) and one-way ANOVA (a+c) were used for statistical analysis and *P* value were corrected for multiple testing according to Holm–Bonferroni method:

^aHV vs. IBS hypersensitive.

^bHV vs. IBS.

^cHV vs. IBS normosensitive.

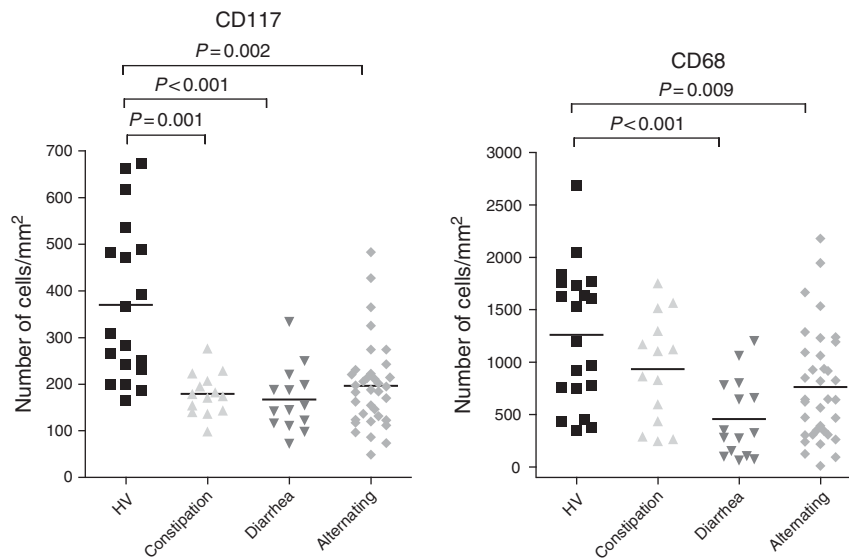


Figure 1. Number of immune cells (CD117+ mast cells, CD68+ macrophages) in the descending colonic mucosa of healthy volunteers (HV) (black) and irritable bowel syndrome (IBS) patients with constipation predominant (blue), diarrhea predominant (red), and alternating type (green) of IBS. One-way analysis of variance (ANOVA) was used for statistical analysis and *P* values were corrected for multiple testing according to Holm–Bonferroni method. Horizontal lines represent the mean. The color reproduction of this figure is available on the *American Journal of Gastroenterology* journal online.

Table 4. Numbers of mast cells and number of λ FLC-, IgE-, and IgG-positive mast cells in HV, IBS patients, and IBS patients subdivided for visceral hypersensitive (hypersens.) and normosensitive (normosens.)

	HV	IBS			<i>P</i> value
	(<i>n</i> =20 desc)	All (<i>n</i> =66 desc)	Hypersens. (<i>n</i> =33 desc)	Normosens. (<i>n</i> =30 desc)	After H.-B. correction
CD117	161 (82–253)	129 (30–408)	125 (30–304)	133 (34–409)	NS
λFLC+ CD117+	7 (0–33)^{a,b,c}	1 (0–34)^a	2 (0–34)^b	0 (0–24)^c	<i>P</i> =0.004 ^a <i>P</i> =0.01 ^{b,c}
CD117	176 (91–415)	163 (37–370)	160 (111–268)	163 (37–370)	NS
IgE+ CD117+	25 (0–130)	16 (0–148)	23 (0–97)	13 (0–148)	NS
CD117	101 (48–235)	91 (24–523)	91 (39–213)	95 (24–523)	NS
IgG+ CD117+	0.7 (0–9)	2 (0–16)	2 (0–16)	2 (0–16)	NS

After H.-B. correct, after Holm–Bonferroni correction; HV, healthy volunteers; IBS, irritable bowel syndrome.

Data are shown as median (range). Mann–Whitney *U*-test (a) or Kruskal–Wallis test (b+c) were used for statistical analysis and *P* values were corrected for multiple testing according to Holm–Bonferroni method.

^aHV vs. IBS.

^bHV vs. IBS hypersensitive.

^cHV vs. IBS normosensitive.

regulatory T-cells, and CD163-positive macrophages in both the ascending and descending colon was comparable between all IBS subtypes and compared with HV (data not shown). On the other hand, IBS patients with an acute onset had a lower number of colonic CD68-positive macrophages compared with those with a gradual onset of IBS (552 ± 139 vs. $784 \pm 73/\text{mm}^2$, $P=0.02$).

The number of mast cells, T cells (CD3 and CD8), FOXP3-positive regulatory T cells, and CD163-positive macrophages or the number of these immunocytes observed in the ascending colon was comparable between acute and gradual onset of IBS symptoms (data not shown).

Ig antigen-specific mast cell binding

IBS vs. HV. As mast cell activation has been proposed to have an important role in IBS, we evaluated the hypothesis that mast cells activation might be part of an adaptive immune response targeted against foreign antigens, that is, commensal bacteria and/or food antigens. As crosslinking of mast cell IgE or IgG receptors or binding of IgFLC (λ and κ) to mast cells are known to be involved in mast cell activation, mast cells were double stained with antibodies targeted against IgE, IgG, λ FLC, and κ FLC. In IBS patients, there was a significant decrease of λ FLC-positive mast cells compared with HV ($P<0.01$). κ FLC-positive mast cell staining was also performed, but no clear positive staining could be detected at different dilutions for κ FLC (max 1:25), probably due to Ab use in combination with CD117 staining. There were no differences in IgE- and IgG-positive mast cells between both groups (Table 4). The control staining of IgE-positive mast cells in patients with eosinophilic esophagitis was positive in esophageal biopsies of four out of five patients ($38.8 (7.7\text{--}78.4)/\text{mm}^2$). When stratified for gender, no differences in Ig-specific mast cell binding were observed between IBS and HV (data not shown).

Hypersensitive vs. normosensitive IBS patients. In hypersensitive and normosensitive IBS patients, there was a small but statistically different decrease in λ FLC-positive mast cells compared with HV ($P \leq 0.01$; Table 4). The number of IgG- and IgE-positive mast cells was however not statistically different (Table 4). When stratified for gender, no differences were seen in IBS sensitivity and Ig-specific mast cell binding between IBS and HV (data not shown).

IBS clinical subtypes. Diarrhea and alternating predominant IBS, but not constipation predominant IBS, had significantly decreased numbers of λ FLC-positive mast cells ($0 (0\text{--}17)/\text{mm}^2$, $P=0.01$; $1.6 (0\text{--}24)/\text{mm}^2$, $P<0.01$; $2.2 (0\text{--}34)/\text{mm}^2$, $P=0.2$, respectively) compared with HV ($6.6 (0\text{--}33)/\text{mm}^2$). The number of IgE- or IgG-positive mast cells was comparable between all IBS subtypes or compared with HV (data not shown).

In the acute onset IBS patients, the number of IgE+ mast cells was significantly lower compared with gradual onset of IBS ($3 (0\text{--}82)$ vs. $29 (0\text{--}148)/\text{mm}^2$, $P=0.001$). There were no differences between an acute or gradual onset in λ FLC- and IgG-positive mast cells staining (data not shown).

Correlation between sensory thresholds and IBS symptoms with microscopic inflammation and Ig-positive mast cells

In IBS patients and HV, there was no correlation between the sensory thresholds of first sensation, urge or discomfort and the number of immunocytes of mast cells, CD68-positive macrophages, T cells (CD3 and CD8), regulatory T cells (FOXP3), and CD163-positive macrophages, respectively, in descending and ascending colon or with λ FLC-, IgE-, and IgG-positive mast cells in the descending colon (data not shown).

Similarly, the number of immune cells in descending and ascending colon and λ FLC-, IgE-, and IgG-positive mast cells in descending colon did not correlate with IBS symptoms, like

Table 5. Stress response of HV, IBS patients, and IBS patients subdivided for visceral hypersensitive (Hypersens.) and normosensitive (Normosens.) (hyper, <24 mm Hg above MDP) and normosensitivity (normo, ≥24 mm Hg above MDP) to rectal distension)

	HV	IBS			P value
		All	Hypersens.	Normosens.	After H.-B. correction
N	18	22	11	11	—
HADS					
Anxiety total score	4.4±0.7	6.6±0.8	6.8±1.4	6.4±1.0	NS
Depression total score	2.1±0.6	3.5±0.6	3.6±0.8	3.5±0.8	NS
Baseline cortisol in nmol/l (range)	6.8 (19.7)	5.3 (22.1)	5.3 (22.1)	5.9 (6.8)	NS
Δpeak cortisol in nmol/l (range)	1.3 (29.6)	1.3 (18.3)	0.8 (14.9)	1.3 (13.7)	NS
Correlations r_s (P)					
Basel. cortisol—mast cell	0.10 (P=0.7)	−0.04 (P=0.9)	−0.03 (P=0.9)	−0.24 (P=0.5)	—
Δpeak cortisol—mast cell	−0.26 (P=0.3)	0.41 (P=0.1)	0.55 (P=0.1)	0.28 (P=0.5)	—
Basel. cortisol—CD68	0.29 (P=0.3)	−0.14 (P=0.2)	0.18 (P=0.6)	−0.58 (P=0.1)	—
Δpeak cortisol—CD68	−0.15 (P=0.6)	−0.27 (P=0.3)	−0.29 (P=0.4)	−0.48 (P=0.2)	—

HADS, Hospital Anxiety and Depression Scale; HV, healthy volunteers; IBS, irritable bowel syndrome; MDP, minimal distending pressure.

Baseline cortisol (basel.cortisol) and Δpeak cortisol (the peak response, expressed as the maximal increase from baseline in response to stress), are shown as median (range). Mann–Whitney *U*-test (HV vs. IBS) or Kruskal–Wallis test (HV vs. IBS hyper–normo) were used for statistical analysis and *P* values were corrected for multiple testing according to Holm–Bonferroni method. Correlations between cortisol levels and the number of CD117+ mast cells and CD68-positive macrophages were analyzed by the Spearman rank correlation test.

abdominal pain, bloating, urgency, incomplete evacuation, hard stools, loose stools, frequent and decreased bowel moments and flatulence (data not shown).

Stress-related symptoms

Of all IBS patients, 60% ($n=37$) scored their symptoms to be stress related, without any difference between the hypersensitive (60%) and the normosensitive group (64%). Whether symptoms were perceived as stress related did not influence the number of mast cells (stress: 182 ± 13 vs. no stress: $192\pm18/\text{mm}^2$, NS), the number of the other immunocytes in descending and ascending colon or the number of $\lambda\text{FLC-}$, IgE-, or IgG-positive mast cells (data not shown).

Stress response

IBS patients ($n=22$) and HV ($n=18$) had comparable scores for depression ($P=0.09$) and anxiety ($P=0.06$) measured by HADS questionnaire (Table 5).

Baseline cortisol concentrations and Δpeak cortisol levels, expressed as the maximal increase from baseline, were not significantly different between IBS and HV or between hypersensitive and normosensitive IBS patients (Table 5). Similar results were obtained after correction for depression or anxiety (data not shown). Mixed model analysis for delta cortisol levels (time $F_{4,24}$: 1.2; $P=0.3$) showed no differences in IBS diagnosis (IBS or HV) or interaction between time and IBS diagnosis. There were no differences in delta cortisol stress response according to gender or interaction time and gender (Figure 2). Smoking, depression, anxiety, and body mass index did not affect the outcome (data not shown).

There was no correlation between baseline cortisol or peak cortisol levels and number of mast cells or CD68-positive macrophages (Table 5) or between cortisol concentrations and number of T cells (data not shown).

DISCUSSION

Visceral hypersensitivity is a consistent finding in IBS patients and is considered an important pathophysiological factor in the generation of IBS symptoms. Both central (alterations in descending pain facilitatory and inhibitory pathways (46)) and peripheral mechanisms have been reported to underlie this increased perception. Although an abnormal response to stress seems to have an important role in the development of IBS symptoms, the mechanisms leading to increased visceral perception are not fully understood. Based on animal data, increased exposure to intraluminal antigens following stress-induced impaired mucosal barrier function and subsequent microscopic inflammation has been proposed as a potential underlying mechanism. Mediators and cytokines released by immune cells are indeed capable to activate visceral afferents, thereby increasing visceral perception (18,19,21). Moreover, mucosal immune activation induced by repetitive stress triggered the onset of visceral hypersensitivity (47). In line with these data, an increase in mucosal inflammatory cells has been repeatedly reported in mucosal biopsies taken from IBS patients, suggesting immune activation (24–31). In addition, the supernatant of biopsies from IBS patients, but not from healthy controls, increased the excitability of rat mesenteric sensory nerves and induced visceral hypersensitivity in mice (25,32,33). To what extent this microscopic inflammation is also

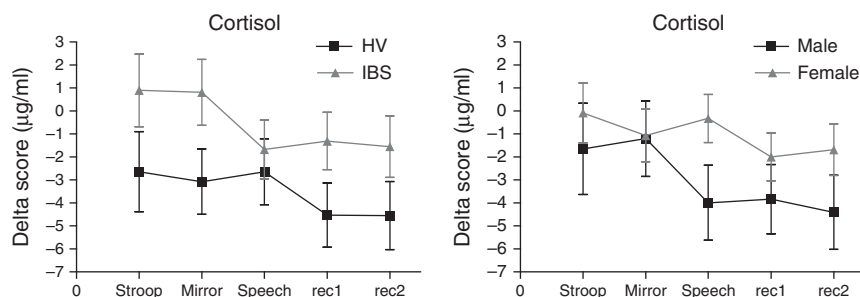


Figure 2. Delta score (every point is corrected for baseline value) for cortisol stress response. Data are shown as mean $\pm$ s.e.m. Delta scores are analyzed using mixed models analyses, with Bonferroni as *post hoc* comparison and corrected for age, body mass index (BMI), smoking, anxiety, and depression (HADS). Left: Interaction between irritable bowel syndrome/healthy volunteers (IBS/HV) and time ($F_{4,22}$: 1.57, $P=0.22$). Right: Interaction gender and time ($F_{4,24}$: 1.64, $P=0.20$). rec1, recovery 1; rec2, recovery 2.

associated with visceral hypersensitivity, however, has not been investigated in great detail.

Of all immune cells, mast cells have been frequently proposed to have a pivotal role in the development of visceral hypersensitivity in IBS patients (25,32,33). The relationship between mast cells and sensitivity to rectal distension, however, has hardly been studied. One earlier study by Park *et al.* (28) demonstrated a reduced mast cell number in patients with visceral hypersensitivity compared with normosensitive patients. Similarly, we failed to demonstrate an increased number of mast cells in hypersensitive patients, indicating that the number of mast cells may not be a key factor. Instead, the activation status of mast cells or even their interaction with nerves may be more relevant. For example, the close proximity of mast cells to neuronal endings has been correlated with abdominal pain (25). Moreover, we recently found that the mast cell stabilizer ketotifen effectively decreased visceral hypersensitivity and IBS symptoms (45) even though the number of mast cells was decreased. Similar findings were observed in animal models of stress-induced visceral hypersensitivity. In maternal separated rats, stress-induced visceral hypersensitivity did not coincide with an increased number of mucosal mast cells (15); nevertheless, treatment with a mast cell stabilizer (doxantrazole) did reverse visceral hypersensitivity. Taken together, these data suggest that the activity of mast cells rather than an increased number is essential in the pathophysiology of visceral hypersensitivity.

Mast cells activation can occur upon crosslinking of surface-bound IgG or IgFLC but is mostly triggered by crosslinking of IgE. Interestingly, increased numbers of IgE-positive mast cells in esophageal biopsies, independently of the presence of atopy (40), have been reported in patients with eosinophilic esophagitis, suggesting that mast cell activation via IgE crosslinking can also be relevant in the pathophysiology of other disorders like IBS. However, although we confirmed the presence of IgE-positive mast cells in esophageal biopsies of patients with eosinophilic esophagitis, we failed to show increased numbers of IgE-positive mast cells in our IBS cohort. In addition, no differences in IgG and IgFLC-positive mast cells were detected between IBS and HV. In inflammatory disorders like inflammatory bowel disease and rheumatoid arthritis,

increased mast cell numbers are associated with increased levels of IgFLC, suggesting that IgFLCs are important in the activation of mast cells (17,48,49). However, we rather observed a decreased number of λ FLC-positive mast cells and normal levels of IgG-positive mast cells, suggesting that antigen-specific activation of mast cells may not predominantly be involved in IBS.

Immune activation is currently considered as an important pathophysiological mechanism of IBS. Increased baseline serum levels of IL-6 and IL-8 and increased release of proinflammatory cytokines like tumor necrosis factor- α , IL-1 β , and IL-6 by peripheral blood mononuclear cells have indeed been reported in IBS (50,51). This hypothesis is further supported by the alterations in immune cells observed in mucosal tissue (24,25,27,29–31). Several studies have reported increased numbers of inflammatory cells, that is, T cells (CD3, CD4, CD8) and macrophages, enhanced release of mast cell mediators (histamine and tryptase) and proteases activity (25–27,33,34,52). However, in contrast to these reports, we observed a decreased number of macrophages (CD68) and T cells (CD3 and CD8) and normal counts of FOXP3-positive regulatory T cells in IBS patients compared with HV. Interestingly, the decrease in T cells (CD3 positive) was only observed in hypersensitive IBS patients (Table 2). From somatic pain studies, it has become clear that T cells may have anti-nociceptive properties especially under stressful conditions (53,54). One could therefore hypothesize that the reduction in T cells observed in the hypersensitive IBS subgroup may contribute to increased visceral perception. Animal data indeed suggest that T cells can induce an opioid-mediated anti-nociceptive effect (55,56). Moreover, mice lacking T cells (severe combined immune deficiency or SCID mice) have lower pain thresholds to colorectal distension, a finding that is normalized by transfer of T cells. Conversely, rectal barostat studies performed in inflammatory bowel disease patients with mild or quiescent disease demonstrated normal or even decreased sensitivity to visceral stimulation by barostat (57,58). All these findings corroborate to the hypothesis that T cells may provide an anti-nociceptive input. Nevertheless, the decrease in the number of T cells in our study did not correlate with the threshold of discomfort and no association was found

with any of the IBS symptoms recorded. It should be stressed though that a barostat study is a rough method in which subtle differences may be missed. Furthermore, to what extent the threshold of discomfort represents the activation status of visceral afferents remains questionable, especially in view of the increasing insight that this parameter is susceptible to habituation and anticipation (59). Whether a reduced number of T cells contribute to the pathogenesis of visceral hypersensitivity therefore clearly requires further study.

Recently, more data in line with our observation have been reported. Indeed, Chang *et al.* (60) demonstrated reduced expression of proinflammatory cytokines in the sigmoid colon of IBS patients. Similarly, microarray studies of colonic mucosal gene expression revealed compromised mucosal immunity in IBS (61,62), in particular downregulation of genes related to T cells and macrophages (61). In addition, a preliminary report showed a decreased number of CD68-positive macrophages in colonic biopsies of 14 IBS patients (63). Taken together, these data would rather suggest mucosal immune dysregulation than immune activation. Although the underlying mechanisms remain unclear, repetitive stress or even a bacterial infection could theoretically be involved (35,64). Reduction in the number of mucosal CD68-positive macrophages has indeed been described following gastrointestinal infection with *Campylobacter pylori*, a pathogen associated with the development of post-infectious IBS (35). Interestingly, we also observed a more pronounced reduction in CD68-positive mucosal macrophages in patients with diarrhea predominant IBS and acute onset IBS, both suggested to be associated with post-infectious IBS.

The discrepancy in literature on microscopic inflammation in IBS is difficult to explain but could be attributed to differences in study population, study design, or the immunohistochemical staining protocol used. Moreover, differences in methodology to quantify the different cell types could potentially represent an important source of variation. We used two validated methods of spectral imaging (41) and a computer-assisted image analysis system, a sensitive, standardized, and accurate method for the quantification of the number of cells, minimizing variance, and increasing reliability (65). Differences in patient population is another important potential factor. In previous studies, the majority or even all IBS patients were diarrhea predominant, whereas our patient population consisted mainly of alternating and constipation predominant IBS. Moreover, our control group consisted of healthy individuals without gastrointestinal complaints and without any indication to undergo a colonoscopy, whereas in other studies, healthy controls consisted of patients undergoing colonoscopy for polyps, intestinal bleeding, or cancer screening. One could also argue that biopsies of the rectum should have been studied rather than ascending and descending colon biopsies as visceral sensitivity was assessed in the rectum. It remains questionable though whether the number of immune cells in the rectum is a reliable read-out to evaluate the role of immune activation in IBS. Nevertheless, in a previous study we did study the number of mast cells in rectal biopsies and like in the present study failed to demonstrate a relationship with rectal visceral sensitivity (45).

Finally, to evaluate the relationship between stress and microscopic inflammation and visceral hypersensitivity, IBS patients were exposed to a standardized stress protocol. Although the majority of our IBS patients reported stress-related exacerbation of their symptoms, the HPA (hypothalamic–pituitary–adrenal)-axis response to a psychological stressor was not different between IBS patients and HV. Similar findings were reported in a larger IBS cohort ($n=44$). No differences in cortisol response to a visceral stressor (sigmoidoscopy) were observed in IBS patients compared with HV. Interestingly, only participants with a history of early life events had higher serum levels of cortisol, independent of IBS diagnose (66). However, mixed models analysis in our study showed no differences in cortisol response in time, suggesting that the psychological stressor was not sufficient enough. In one of our previous studies, with a different cohort, the same stress tasks did induce a cortisol stress response (67) arguing against this possibility. Moreover, in a larger similar cohort, this stress protocol did induce an increased response of cortisol in HV during the speech task ($n=52$; unpublished data). Therefore, due to the lower number of participants (IBS $n=22$, HV $n=18$) that underwent the stress response test in this study, the lack of changes in HPA-axis activation may be explained by a type II error. Furthermore, it should be noticed that female participants were allowed to take birth control medication and we did not take the menstrual cycle into account (68). Finally, in the present study, stress-related symptoms and the stress response were not significantly correlated to the number of immune cells or the presence of visceral hypersensitivity. It should be emphasized though that immune dysregulation and pain sensitivity can be affected by stress response systems by non-HPA CRF pathways (69). Taken together, our current findings seem to argue against an abnormal stress response as underlying mechanism responsible for microscopic inflammation, but larger studies are needed to be conclusive.

In conclusion, in the present study we demonstrated that the number of mast cells, macrophages (CD68), T cells (CD3 and CD8), and λ FLC-positive mast cells is decreased in IBS patients compared with HV, and is not associated with the presence of visceral hypersensitivity or abnormal stress response. Our data therefore question the role of microscopic inflammation (defined as an increased number of inflammatory cells) as underlying pathophysiological mechanism of visceral hypersensitivity and rather suggest that immune dysregulation, as evidenced by a decreased number of immune cells, may be involved in IBS.

CONFLICT OF INTEREST

Guarantor of the article: Guy E. Boeckxstaens, MD, PhD.

Specific author contributions: Design of the study, data collection, analysis, interpretation, and writing of the manuscript: Breg Braak; design of the study, data collection, analysis, interpretation, writing of the manuscript, and approving the final version: Tamira K. Klooker; interpretation of the data and approving the final version: Mira M. Wouters, Chris M. van der Loos, and Rene M. van den Wijngaard; data collection and approving the final version: Olaf Welting, Oana I. Stanisor, and Sophie van Diest; design of the study,

interpretation of the data, writing of the manuscript, and approving the final version: Guy E. Boeckstaens.

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Potential competing interests: None.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE

- ✓ Irritable bowel syndrome (IBS) is a prevalent functional bowel disorder.
- ✓ Visceral hypersensitivity is an important underlying pathophysiological mechanism.
- ✓ Microscopic inflammation of the intestinal mucosa is abundantly reported to have an important role in the pathogenesis of IBS, and has been suggested to contribute to the development of visceral hypersensitivity.

WHAT IS NEW HERE

- ✓ In a cohort of irritable bowel syndrome (IBS) patients, a decreased number of mast cells, macrophages (CD68), and T cells (CD3 and CD8) was detected in normosensitive and hypersensitive patients compared with healthy controls.
- ✓ Although CD3+ T cells were only decreased in hypersensitive IBS patients, no correlation was found between visceral hypersensitivity, IBS symptoms, or the number of immune cells in IBS patients.
- ✓ The stress response of IBS patients was comparable to that of healthy controls and did not correlate with the number of immune cells.
- ✓ These data question the role of microscopic inflammation in visceral hypersensitivity but rather suggest immune dysregulation as potential pathophysiological mechanism of IBS.

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Antibody (anti-human)	Source	Major specificity in tissue	Concentration
<i>Single staining</i>			
CD117 (Ckit)	Dako A4502, Glostrup, Danmark	Mast cells	1:100
CD68	Dako M0876	Monocytes/Macrophages	1:800
CD163	Serotec MCA1853T, Dusseldorf, Germany	Activated monocytes/macrophages (scavenger receptor)	1:400
CD3 (Clone SP7)	Thermo Scientific, Waltham, USA	T-cells	1:200
CD8	Dako M7103,	(cytotoxic) T-cells	1:200
FOXP3	Abcam, ab20034, Cambridge, UK,	Regulatory T-cells	1:50
<i>Double staining</i>			
CD117 (Ckit)	Dako A4502, Glostrup, Danmark	Mast cells	1:7000
λ FLC	FL-G9, obtained from Dr A Solomon, University of Tennessee, Knoxville, Tennessee, USA,	Immunoglobulin free light chains, lambda	1:1000
IgE	Dako A0094	IgE immunoglobulins	1:3000
IgG	Dako A0423	IgG immunoglobulins	1:10000

Supplemental Table 1: Antibodies used in this study.