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Pro-inflammatory chemokine C-C motif ligand 16 (CCL-16) dysregulation in irritable bowel syndrome (IBS): a pilot study

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Abstract

Background—Irritable bowel syndrome (IBS) is a serious health problem that affects an estimated 10–15% of people worldwide and has economic consequences in the United States of over $30 billion annually. In the US, IBS affects all races and both sexes, with more females than males (2 : 1) reporting symptoms consistent with IBS. Although the etiology of this functional gastrointestinal disorder is unknown, literature suggests that a subclinical inflammatory component has a role in the etiologic mechanisms underlying IBS. The aim of this study was to evaluate the gene expression of inflammatory biomarkers in patients with and without IBS and among different IBS phenotypes.

Methods—Irritable bowel syndrome patients (n = 12) that met Rome III Criteria for IBS longer than 6 months were compared with healthy matched controls (n = 12). Peripheral whole blood from fasting participants was collected and RNA was extracted. The expression of 96 inflammatory genes was then analyzed using a custom quantitative real-time PCR array.

Key Results—CCL-16 gene expression was upregulated by 7.46-fold in IBS patients when compared with controls. CCL-16 was overexpressed by over 130-fold in IBS-constipation patients when compared with both controls and IBS-diarrhea patients.

Conclusions & Inferences—These results further suggest a subclinical inflammatory component underlying IBS. To better understand the phenotypic differences in IBS it is important to broaden the study of these inflammatory and other biomarkers.

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APPROVALS

The protocol (09-NR-0064) was approved by the Institutional Review Board at the National Institutes of Health. Clinicaltrial.gov # NCT00824941.

CONFLICT OF INTEREST

All of the authors have no potential conflicts of interest to disclose. The opinions expressed herein and the interpretation and reporting of these data are the responsibility of the author(s) and in no way should be seen as an official recommendation, interpretation, or policy of the National Institutes of Health or the US Government.

GUARANTOR OF THE ARTICLE

WAH accepts full responsibility for the conduct of the study.

AUTHOR CONTRIBUTIONS

All of the authors have approved the final draft of the manuscript submitted; All authors contributed to the analysis and writing of the manuscript; WAH and MH devised the study and wrote the protocol.

Keywords

constipation; functional bowel disorders; hemofiltrate CC chemokine 4; inflammation; liver-expressed chemokine

INTRODUCTION

Irritable bowel syndrome (IBS) is a functional gastrointestinal (GI) disorder of unknown etiology. Irritable bowel syndrome is a serious health problem with limited clinical treatment options that affects an estimated 10–15% of people worldwide. Economic costs of IBS in the US are more than $30 billion annually, including direct medical care costs and time lost from work.[1–3] In the US, IBS afflicts persons of all races and both sexes, with more women than men (2 : 1) reporting symptoms consistent with IBS.[4]

Irritable bowel syndrome is defined by Rome III as chronic abdominal pain and changes in stool frequency and appearance for a minimum of 3 days every month for at least 3 months. Irritable bowel syndrome is classified into four different subtypes; IBS with constipation (IBS-C), IBS with diarrhea (IBS-D), IBS mixed (IBS-M), and unsubtyped-IBS.[5]

Evidence suggests that neuroimmune dysregulation and an underlying subclinical inflammatory component are part of the molecular mechanisms in IBS. Low-grade inflammation and mast cells are found in mucosal biopsies of IBS patients.[6–12]

Patients with IBS have mucosal infiltration of immunocytes.[13] Microarray data also confirm alterations in colonic mucosal immunity in IBS.[14] Moreover, a subset of IBS patients have increased intestinal permeability.[10,15–17] These findings together support an inflammatory component in this pathology. The aim of this study was to evaluate the gene expression of inflammatory biomarkers in patients with and without IBS and among different IBS phenotypes.

MATERIALS AND METHODS

Design and setting

This pilot study was composed of adult patients who met Rome III criteria for IBS for longer than 6 months and matched healthy controls. Participants were recruited under a natural history protocol (09-NR-0064, Clinicaltrials.gov # NCT00824941) conducted at the National Institutes of Health (NIH) Hatfield Clinical Research Center (CRC). Biological samples were collected during a span of two outpatient visits at the CRC from January 2009 to June 2010. All participants gave written consent and the study was approved by the Institutional Review Board and the Office of Human Subjects Research at the NIH.

Sample

A cohort of 24 participants (45.83% male) included IBS patients ($n = 12$) and matched healthy controls ($n = 12$). Participants were matched for race (100% caucasian), age (mean 28.00 ± 7.54 years, range 22–45 years), and body mass index (BMI, mean 25.43 ± 5.92 kg m^{-2}, range 18.82–43.22 kg m^{-2}) (Table 1). Participants with a history of organic GI disease (e.g., inflammatory bowel disease, celiac disease) or currently taking medications daily for GI symptoms (e.g., antispasmodics, laxatives) were excluded.

RNA extraction

Peripheral whole blood (2.5 mL) was collected in PAXgene® RNA tubes from fasting participants via venipuncture. RNA was extracted using the PAXgene® Blood miRNA Kit

(Qiagen, Franklin Lakes, NJ, USA). RNA quality was verified with the Experion ™ RNA Analysis Kit (Bio-Rad, Hercules, CA, USA).

Gene expression

RNA (500 ng) was reverse transcribed using the RT^2 First Strand Kit (SA Biosciences by Qiagen, Frederick, MD, USA). RT^2 Profiler™ PCR Array Human Inflammatory Response and Autoimmunity (Customized PAHS-077, SA Biosciences by Qiagen, Frederick, MD, USA) was used to analyze the expression of 96 key genes, including 84 genes involved in inflammatory immune responses, five housekeeping genes and controls to check for genomic DNA contamination, RNA quality, and general PCR performance (Table 2). Any gene with dysregulated expression was analyzed further at the protein level using ELISA assays.

Protein levels: ELISA

Based on results from aim 1, plasma from fasting patients and healthy controls was used to determine CCL-16 protein levels using a Human CCL16/HCC-4 DuoSet® ELISA Development System (R & D Systems, Minneapolis, MN, USA). ELISA assays were performed according to manufacturer directions. Optical density was determined for each well using amicroplate reader set to 450 nm.

Data and statistical analysis

The PCR data were uploaded to SA Biosciences Web Analysis (http://www.sabiosciences.com) for analysis. As recommended from literature, threshold cycle (Ct) cut-off was set at 35 cycles and boundary to 4-fold.[18] Fold-change [2^(− Delta Delta Ct)] was defined as the normalized gene expression [2^(− Delta Ct)] in the test group (IBS patients) divided by the normalized gene expression [2^(− Delta Ct)] in the control group. Fold-change values greater than one indicates an up-regulation, and the fold-regulation is equal to the fold-change. Fold-change values less than one indicate a down-regulation, and the fold-regulation is the negative inverse of the fold-change. Results were reported as fold-regulation because it represents fold-change values in a biologically meaningful way.[19] Clinical data (Table 1) were analyzed with unpaired *t*-tests using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA) and independent sample *t*-test using SPSS 15 (SPSS Inc., Chicago, IL, USA). *P*-values <0.05 were considered significant.

RESULTS

The cohort ($N = 24$) composed of IBS patients ($n = 12$) and matched healthy controls ($n = 12$) were not significantly different in terms of gender (45.83% males), race (100% caucasian), age ($P = 0.40$), body mass index (BMI, $P = 0.87$), hemoglobin (HGB, $P = 0.41$), albumin ($P = 0.53$), C-reactive protein (CRP, $P = 0.61$), erythrocyte sedimentation rate (ESR, $P = 0.77$) or liver transaminases; alanine aminotransferase (ALT, $P = 0.26$), aspartate aminotransferase (AST, $P = 0.29$) and gammaglutamyl transpeptidase (GGTP, $P = 0.22$) (Table 1).

Inflammatory Gene Expression in Irritable Bowel Syndrome (IBS)

Chemokine (C-C motif) ligand 16 (CCL-16) gene, a liver expressed chemokine,[20,21] was upregulated by 7.46-fold in IBS patients ($n = 12$, Avg Ct = 30.67 ± 4.92) when compared with healthy controls ($n = 12$, Avg Ct = 33.08 ± 1.39) using a custom quantitative realtime PCR array (Fig. 1). Although not statistically significant, CCL-16 protein levels in plasma were also higher in IBS patients (9.45 ± 3.25 ng mL^{-1}) when compared with healthy

controls (7.80 ± 4.20 ng mL^{-1}; $P = 0.34$) (Human CCL16/HCC-4 DuoSet® ELISA Development System, R & D Systems, Minneapolis, MN, USA).

Inflammatory Gene Expression in Different IBS Phenotypes

The CCL-16 gene expression was significantly upregulated (fold regulation = 199.78) in IBS-C patients ($n = 4$, Avg Ct = 25.59 ± 5.88) when compared with healthy controls ($n = 12$, Avg Ct = 33.08 ± 1.39) (Fig. 2A). IBS-C patients' CCL-16 expression was also upregulated (fold regulation = 138.47) when compared with IBS-D patients ($n = 8$, Avg Ct = 33.16 ± 1.39) (Fig. 2C). In contrast, IBS-D patients showed no difference in gene expression when compared with healthy controls (Fig. 2B). The CCL-16 protein levels in plasma were significantly elevated in IBS-C (10.91 ± 2.58 ng mL^{-1}) patients when compared with IBS-D (6.06 ± 1.71 ng mL^{-1}, $P = 0.018$) in CCL-16 ELISA assay.

There were no statistically significant differences between IBS groups and control groups with regard to other inflammatory genes in the array. The CCL-16 gene expression levels were validated in all 24 samples using a CCL-16-targeted RT-PCR Primer Assay (SA Biosciences).

DISCUSSION

The upregulation of chemokine (C-C motif) ligand 16 (CCL-16) gene expression in patients with IBS, particularly in patients with IBS-constipation, when compared with healthy controls and IBS-diarrhea patients suggests differences in inflammatory genes expression dependent on phenotype (i.e., constipation). Elevated gene expression of this pro-inflammatory liver chemokine seems to be related to GI pathophysiology and not evident liver damage as liver transaminases (e.g., ALT, AST, GGTP) were within normal clinical range and not different between IBS patients and healthy controls (Table 1).

The CCL-16, also known as Hemofiltrate CC chemokine 4 (HCC-4), liver-expressed chemokine (LEC) and monotactin-1 (MTN-1), is a small cytokine part of the CC chemokine family. Mature CCL-16 protein is 97 amino acids long and shows 19–38% identity to other human CC chemokines with the highest identity to HCC-1/CCL14.[20] The CCL-16 protein is constitutively expressed in liver parenchymal cells, thymus and spleen and present at high levels in normal human plasma.[20,22,23] The CCL-16 is a low-affinity ligand for several C-C chemokine receptors (CCR) including CCR1, CCR2, CCR5, and CCR8.[22] The CCL-16 was shown to be inducible in monocytes by IL-10,[24] and chemotactic for monocytes and lymphocytes. [23] In addition, this chemokine was shown to have potent myelosuppressive activity comparable to that of macrophage inflammatory protein (MIP)-1α/CCL3 [23] and to induce tumor rejection.[25] Moreover, CCL-16 is a strong pro-inflammatory chemokine which is upregulated in active ulcerative colitis patients.[26] In the case of the patients in this study, CCL-16 elevated gene expression in IBS, suggests and supports a more subclinical inflammatory component as there were no differences in the expression of clinical inflammatory markers (e.g., CRP, ESR) between IBS patients and healthy controls (Table 1).

The CCL-16 protein was present at high concentrations (8.6 ng mL^{-1}) in the plasma of both healthy volunteers and patients with IBS, with slightly higher levels in IBS patients, though not statistically significant. These values are similar to previous studies (~11 ng mL^{-1}) in healthy volunteers by Nomiyama *et al.*[22]

The CCL-16 acts by interacting with cell surface receptors (C-C chemokine receptor) CCR1, CCR2, CCR5 and CCR8.[22] In addition, CCL-16 is strongly induced by interleukin 10 (IL-10).[20,21,24] Gene expression of these and other receptors and cytokines (Table 2) were

also studied here without any significant dysregulation. Future studies should look at the expression of these upstream and downstream mediators at the protein level in order to better elucidate the mechanism of action of CCL-16.

This study has several limitations, including the use of blood as peripheral surrogate for colonic gene expression. It is critical that prospective studies look at the expression of the genes in colonic tissue biopsies. Future studies should also include a larger sample and other ethnic groups to further corroborate the findings of this pilot study. Finally, this study is limited to a targeted inflammatory pathway that only includes the commonly expected chemokines and cytokines, it is important to broaden our search to other inflammatory and non-inflammatory biomarkers not included in this analysis. The paucity of efficacious treatments for IBS makes elucidating the underlying inflammatory etiology critical to allow testing of targeted novel agents for this syndrome and related symptoms.

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Abbreviations

ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
Avg	Ct Average Threshold Cycle
BMI	Body Mass Index
CCL-16	Chemokine (C-C motif) ligand 16
Ct	Threshold cycle
CCR	C-C Chemokine Receptor
CRP	C-reactive Protein
ESR	Erythrocyte Sedimentation Rate
GGTP	Gamma-glutamyl Transpeptidase
GI	Gastrointestinal
HCC4	Hemofiltrate CC Chemokine 4
HGB	Hemoglobin
IBS	Irritable Bowel Syndrome
IBS-C	Irritable Bowel Syndrome-Constipation
IBS-D	Irritable Bowel Syndrome-Diarrhea
IL-10	Interleukin 10
LEC	Liver-Expressed Chemokine
MTN-1	Monotactin-1
PCR	Polymerase Chain Reaction

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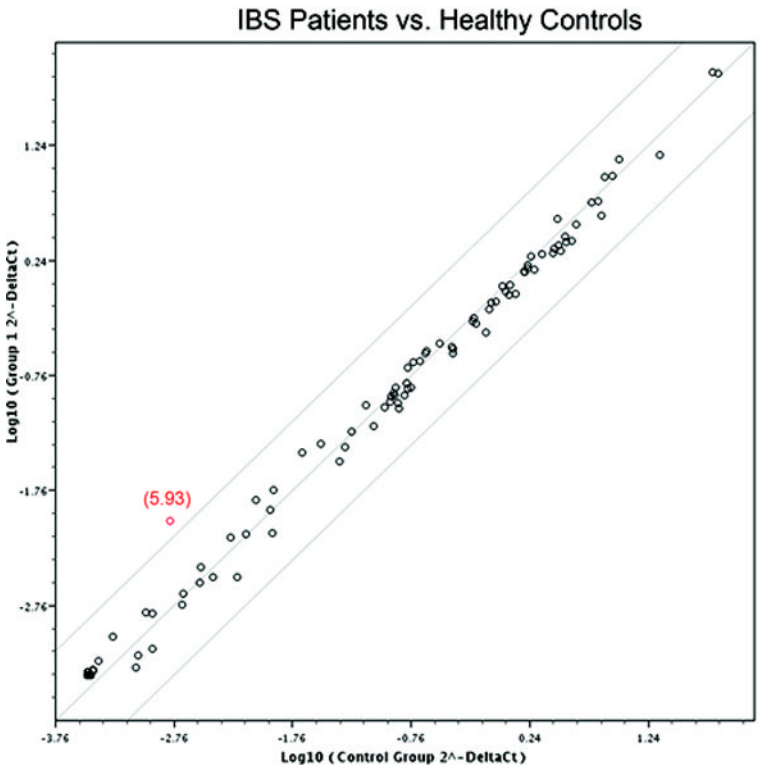

Figure 1.

Inflammatory Gene Expression in IBS patients *vs* healthy controls. Compares the expression of all 96 genes and PCR controls for IBS patients ($n = 12$) *vs* healthy participants ($n = 12$). CCL-16 fold regulation = 7.46. Boundary = 4.

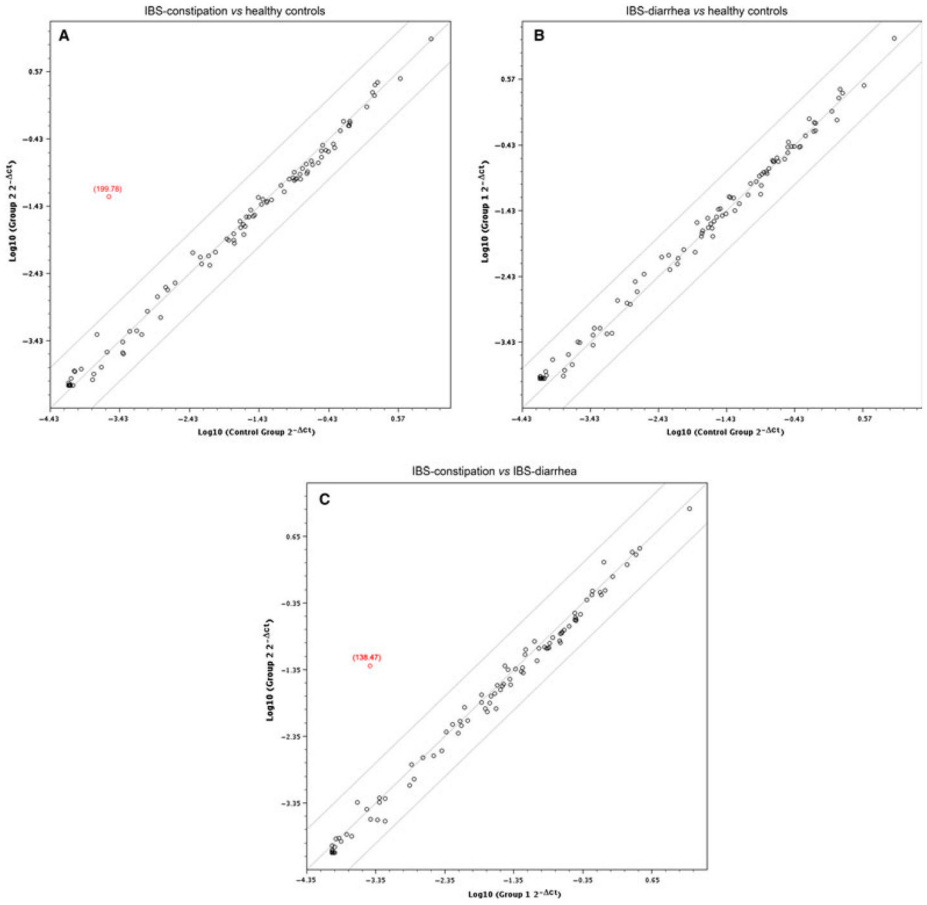

Figure 2.

Inflammatory Gene Expression in constipation-IBS (IBS-C) patients, diarrhea-IBS (IBS-D) patients and healthy controls. Shows the genetic expression for (A) IBS-C patients ($n = 4$) *vs* healthy controls ($n = 12$) (CCL-16 fold regulation = 199.78); (B) IBS-D patients ($n = 8$) *vs* healthy controls ($n = 12$) and, (C) IBS-C *vs* IBS-D (CCL-16-fold regulation = 138.47). Boundary = 4.

Table 1

Sample characteristics and clinical

Variable	Overall (*N* = 24)	IBS patients (*n* = 12)	Healthy controls (*n* = 12)	*P* value
Gender (*n*)				
Male	11	5	6	–
Female	13	7	6	–
Race (*n*)				
Caucasian	24	12	12	–
Age	28.00 ± 7.54	26.67 ± 7.29	29.33 ± 7.87	0.40
Range (years)	(22.00–45.00)	(14.00–44.00)	(23.00–45.00)	
BMI	25.43 ± 5.92	25.23 ± 4.18	25.64 ± 7.46	0.87
Range (kg m^{-2})	(18.82–43.22)	(20.19–35.07)	(18.82–43.22)	
Hemoglobin	13.99 ± 1.39	14.23 ± 1.72	13.75 ± 0.97	0.41
Range (g dL^{-1})	(10.90–16.70)	(10.90–16.70)	(12.20–15.00)	
Albumin	4.13 ± 0.35	4.18 ± 0.45	4.08 ± 0.23	0.53
Range (g dL^{-1})	(3.70–4.90)	(3.70–4.90)	(3.70–4.40)	
CRP	2.78 ± 4.30	3.24 ± 4.70	2.32 ± 4.01	0.61
Range (mg L^{-1})	(0.04–17.50)	(0.04–17.50)	(0.04–13.60)	
ESR	7.00 ± 5.49	7.33 ± 7.08	6.67 ± 3.55	0.77
Range (mm)	(2.00–25.00)	(2.00–25.00)	(2.00–13.00)	
Liver Transaminases				
ALT	32.88 ± 20.20	37.58 ± 26.84	28.17 ± 9.19	0.26
Range (U L^{-1})	(18.00–107.00)	(18.00–107.00)	(18.00–44.00)	
AST	19.50 ± 9.52	21.58 ± 11.57	17.42 ± 6.79	0.29
Range (U L^{-1})	(9.00–52.00)	(11.00–52.00)	(9.00–30.00)	
GGTP	30.42 ± 20.49	35.58 ± 26.51	25.25 ± 10.81	0.22
Range (U L^{-1})	(15.00–103.00)	(15.00–103.00)	(15.00–51.00)	

All values expressed as mean ± standard deviation (M ± SD). BMI, Body mass index. Normal Clinical Range: Hemoglobin (12–18 g dL^{-1}), Albumin (3.5–5 g dL^{-1}), C-reactive protein (CRP, <10 mg L^{-1}), Erythrocyte sedimentation rate (ESR, 12–23 mm), Alanine aminotransferase (ALT, 6–41 U L^{-1}), Aspartate aminotransferase (AST, 9–34 U L^{-1}), Gamma-glutamyl transpeptidase (GGTP, 5–85 U L^{-1}).

Table 2

Human inflammatory response and autoimmunity gene array

	1	2	3	4	5	6	7	8	9	10	11	12
A	BCL6	C3	C3AR1	C4A	CCL11	CCL13	CCL16	CCL17	CCL19	CCL2	CCL21	CCL22
B	CCL23	CCL24	CCL3	CCL4	CCL5	CCL7	CCL8	CCR1	CCR2	CCR3	CCR4	CCR7
C	CD40	CD40LG	CEBPB	CRP	CSF1	CXCL1	CXCL10	CXCL2	CXCL3	CXCL5	CXCL6	CXCL9
D	CXCR4	FASLG	FLT3LG	FOS	HDAC4	IFNG	IL10	IL10RB	IL18	IL18RAP	IL1A	IL1B
E	IL1F10	IL1R1	IL1RAP	IL1RN	IL22	IL22RA2	IL23A	IL23R	IL6	IL6R	IL8	IL8RA
F	IL8RB	IL9	ITGB2	KNG1	LTA	LTB	LY96	MYD88	NFATC3	NFKB1	NOS2	NR3C1
G	RIPK2	TIRAP	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TNF	TNFSF14	TOLLIP
H	B2M	HPRT1	RPL13A	GAPDH	ACTB	HGDC	RTC	VIP	S100A8	S100A9	S100A10	PPC

BCL6, B-cell CLL/lymphoma 6; C3, complement component 3; C3AR1, complement component 3a receptor 1; C4A, complement component 4A; CCL11, chemokine (C-C motif) ligand 11; CCL13, chemokine (C-C motif) ligand 13; CCL16, chemokine (C-C motif) ligand 16; CCL17, chemokine (C-C motif) ligand 17; CCL19, chemokine (C-C motif) ligand 19; CCL2, chemokine (C-C motif) ligand 2; CCL21 chemokine (C-C motif) ligand 21; CCL22, chemokine (C-C motif) ligand 22; CCL23, chemokine (C-C motif) ligand 23; CCL24, chemokine (C-C motif) ligand 24; CCL3, chemokine (C-C motif) ligand 3; CCL4, chemokine (C-C motif) ligand 4; CCL5, chemokine (C-C motif) ligand 5; CCL7, chemokine (C-C motif) ligand 7; CCL8, chemokine (C-C motif) ligand 8; CCR1, chemokine (C-C motif) receptor 1; CCR2, chemokine (C-C motif) receptor 2; CCR3, chemokine (C-C motif) receptor 3; CCR4, chemokine (C-C motif) receptor 4; CCR7, chemokine (C-C motif) receptor 7; CD40, CD40 molecule; TNF receptor superfamily member 5; CD40LG, CD40 ligand; CEBPB, CCAAT/enhancer binding protein (C/EBP) beta; CRP, C-reactive protein; CSF1, colony stimulating factor 1; CXCL1, chemokine (C-X-C motif) ligand 1; CXCL10, chemokine (C-X-C motif) ligand 10; CXCL2, chemokine (C-X-C motif) ligand 2; CXCL3, chemokine (C-X-C motif) ligand 3; CXCL5, chemokine (C-X-C motif) ligand 5; CXCL6, chemokine (C-X-C motif) ligand 6; CXCL9, chemokine (C-X-C motif) ligand 9; CXCR4, chemokine (C-X-C motif) receptor 4; FASLG, Fas ligand; FLT3LG, Fms-related tyrosine kinase 3 ligand; FOS, V-fos FBJ murine osteosarcoma viral oncogene homolog; HDAC4, histone deacetylase 4; IFNG, interferon; gamma; IL10, interleukin 10; IL10RB, interleukin 10 receptor beta; IL18, interleukin 18; IL18RAP, interleukin 18 receptor accessory protein; IL1A, interleukin 1 alpha; IL1B, interleukin 1 beta; IL1F10, interleukin 1 family; member 10 (theta); IL1R1, interleukin 1 receptor; type I; IL1RAP, interleukin 1 receptor accessory protein; IL1RN, interleukin 1 receptor antagonist; IL22, interleukin 22; IL22RA2, interleukin 22 receptor alpha 2; IL23A, interleukin 23; alpha subunit p19; IL23R, interleukin 23 receptor; IL6, interleukin 6; IL6R, interleukin 6 receptor; IL8, interleukin 8; IL8RA, interleukin 8 receptor; alpha; IL8RB, interleukin 8 receptor; beta; IL9, interleukin 9; ITGB2, integrin; beta 2 (complement component 3 receptor 3 and 4 subunit); KNG1, kininogen 1; LTA, lymphotoxin alpha; LTB, lymphotoxin beta; LY96, lymphocyte antigen 96; MYD88, myeloid differentiation primary response gene (88); NFATC3, nuclear factor of activated T-cells; cytoplasmic; calcineurin-dependent 3; NFKB1, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1; NOS2, nitric oxide synthase 2; inducible; NR3C1, nuclear receptor subfamily 3; group C; member 1 (glucocorticoid receptor); RIPK2, receptor-interacting serine-threonine kinase 2; TIRAP, toll-interleukin 1 receptor (TIR) domain containing adaptor protein; TLR1, toll-like receptor 1; TLR2, toll-like receptor 2; TLR3, Toll-like receptor 3; TLR4, toll-like receptor 4; TLR5, toll-like receptor 5; TLR6, toll-like receptor 6; TLR7, toll-like receptor 7; TNF, tumor necrosis factor (TNF superfamily; member 2); TNFSF14, tumor necrosis factor (ligand) superfamily, member 14; TOLLIP, toll interacting protein; B2M, beta-2-microglobulin; HPRT1, hypoxanthine phosphoribosyltransferase 1; RPL13A, ribosomal protein L13a; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ACTB, actin beta; HGDC, human genomic DNA contamination; RTC, reverse transcription control; VIP, vasoactive intestinal peptide; S100A8, S100 calcium binding protein A8; S100A9, S100 calcium binding protein A9; S100A10, S100 calcium binding protein A10; PPC, positive PCR control.