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SHORT COMMUNICATION

Activation of the JAK/STAT pathway in Behcet's disease

This article has been corrected since Advance Online Publication and an erratum is also printed in this issue.

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Th1/Th17-type T-cell responses are upregulated in Behcet's disease (BD). However, signaling pathways associated with this aberrant immune response are not clarified. Whole-genome microarray profiling was performed with human U133 (Plus 2.0) chips using messenger RNA of isolated $CD14^+$ monocytes and $CD4^+$ T cells from peripheral blood mononucleated cell (PBMC) in patients with BD ($n = 9$) and healthy controls (HCs) ($n = 9$). Flow cytometric analysis of unstimulated (US) and stimulated (phytohaemagglutinin) signal transducer and activator of transcription (STAT3) and pSTAT3 expressions of PBMCs were also analyzed (BD and HC, both $n = 26$). Janus family of kinase (JAK1) was observed to be upregulated in both $CD14^+$ monocytes (1.95-fold) and $CD4^+$ T lymphocytes (1.40-fold) of BD patients. Using canonical pathway enrichment analysis, JAK/STAT signaling was identified as activated in both $CD14^+$ monocytes ($P = 9.55E-03$) and in $CD4^+$ lymphocytes ($P = 8.13E-04$) in BD. Interferon signaling was also prominent among upregulated genes in $CD14^+$ monocytes ($P = 5.62E-05$). Glucocorticoid receptor signaling and interleukin (IL-6) signaling were among the most enriched pathways in differentially expressed genes in CD14+ monocytes ($P = 2.45E-09$ and $1.00E-06$, respectively). Basal US total STAT3 expression was significantly higher in BD (1.2 vs 3.45, $P < 0.05$). The JAK1/STAT3 signaling pathway is activated in BD, possibly through the activation of Th1/Th17-type cytokines such as IL-2, interferon (IFN-γ), IL-6, IL-17 and IL-23.

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INTRODUCTION

Behcet's disease (BD) is a multisystemic, chronic inflammatory disorder with a complex genetic background, leading to a proinflammatory activation of the innate and adaptive immune systems.[1,2] Both $CD4^+$ and $CD8^+$ T cells producing Th1-type proinflammatory cytokines interleukin (IL-2), IL-12 and interferon (IFN-γ) are increased in the peripheral blood (PB) and inflammatory tissues in BD.[3,4] Th17 cells represent a relatively new subset of T-helper cells which mainly produce IL-17A, IL-17F, IL-22 and tumor necrosis factor (TNF-α),[5] IL-6, IL-23 and transforming growth factor-β, and induce the differentiation and maturation of Th17 cells from naive T cells. Th17 cells are suggested to be involved in various disorders such as spondyloarthropathies, psoriasis and inflammatory bowel disease, which have strong clinical and genetical similarities with BD.[6,7] Recent data also implicate the participation of IL-17 and Th17-type responses in BD pathogenesis.[8,9] Active BD is characterized by high serum levels of IL-6, IL-17, IL-23 and activated Th17 cells.[10–12] Therapies such as IFN-α and anti-TNF-α agents, which are effective in ocular BD, are also shown to suppress Th17 responses.[13,14]

Various cytokines such as IFNs, ILs and colony-stimulating factors bind to the similar type of I/II cytokine superfamily receptors on cell membranes,[15] and associate with Janus family of kinase (JAK1-3) membrane proximal domains, leading to their phosphorylation. Recruitment of signal transducer and activator of transcription (STAT) family of transcription factors then modulates gene transcription.[16] IFN-γ, granulocyte-macrophage colony-stimulating factor, IL-2, IL-6, IL-12, IL-15, IL-17, IL-21, IL-22 and IL-23 activate through various JAK/STAT combinations in the cell surface and are implicated in the pathogenesis of infections, malignancies and autoimmune/inflammatory disorders such as rheumatoid arthritis, inflammatory bowel disease and psoriasis.[16,17] Although proinflammatory and most Th1/Th17-associated cytokines are also implicated in BD pathogenesis, the role of intracellular signaling pathways, such as JAK/STAT system, has not been characterized.

In this study, we demonstrated the role of the JAK/STAT pathway in PB $CD14^+$ monocyte and $CD4^+$ T cells with whole-genome gene expression analysis and confirmed our results with flow cytometric STAT3 analysis in PB of patients with BD.

RESULTS AND DISCUSSION

When genes with 1.5-fold differences were selected, upregulation of the signals of 1035 probes (738 genes) and downregulation of 1431 probes (1004 genes) were detected in $CD14^+$ monocytes of BD patients, whereas signals from 335 probes (230 genes) upregulated and 68 probes (51 genes) downregulated in $CD4^+$ T cells. JAK1 (probe ID: 1552611) was upregulated in both $CD14^+$ monocytes (1.95-fold) and $CD4^+$ T lymphocytes (1.40-fold) of BD patients compared with the controls (Supplementary Table 2).

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Table 1. Canonical pathways overrepresented by upregulated genes in CD14+ monocytes in BD patients

Ingenuity canonical pathways	*Molecules*	P-*value*
Interferon signaling	IFIT3, IFIT1, IFITM3, OAS1, JAK1, MX1, BAX, IFITM1	5.62E – 05
Mouse embryonic stem cell pluripotency	IL6ST, TCF4, JAK1, SOS1, SOS2, PIK3R5, BMPR2, PIK3CB, SMAD5, FZD1, STAT3, TCF3, FZD2	1.00E – 04
TGF-β signaling	TGFBR2, IRF7, JUN, CDC42, SOS2, SOS1, SMAD7, BMPR2, SMURF2, VDR, SMAD5, ACVR1B	2.29E – 04
Systemic lupus erythematosus signaling	LSM14B, CD79B, NFATC3, SOS2, PIK3R5, FCGR1A, PTPRC, SNRNP48, CD3G, RNPC3, JUN, CBL, NFAT5, LSM12, SOS1, PRPF6, PIK3CB, FCGR1B	4.17E – 04
EGF signaling	CSNK2A2, JAK1, JUN, SOS2, MAP3K1, SOS1, PIK3R5, PIK3CB, STAT3	4.37E – 04
B-cell receptor signaling	CD79B, POU2F2, NFATC3, SOS2, MAP3K1, PIK3R5, TCF3, PTPRC, EBF1, CAMK2D, NFAT5, JUN, DAPP1, CDC42, SOS1, PIK3CB, MAP3K3	5.50E – 04
Role of NANOG in mammalian embryonic stem cell pluripotency	IL6ST, RIF1, JAK1, SOS2, SOS1, PIK3R5, BMPR2, PIK3CB, STAT3, FZD1, SMAD5, FZD2	1.51E – 03
T-cell receptor signaling	PTPRC, CD3G, NFAT5, JUN, CBL, NFATC3, SOS2, MAP3K1, SOS1, PIK3R5, PIK3CB	1.70E – 03
Glucocorticoid receptor signaling	JAK1, PRKAB2, POU2F2, NFATC3, HSPA1A/HSPA1B, MAP3K1, SOS2, PIK3R5, HSPA6, STAT3, FCGR1A, TGFBR2, CD3G, HSP90B1, JUN, NFAT5, TAF5, SMARCA2, SOS1, PIK3CB, TAF15	2.24E – 03
Activation of IRF by cytosolic pattern recognition receptors	IFIH1, IRF7, JUN, DDX58, ZBP1, SIKE1, IFIT2, ISG15	3.09E – 03
SAPK/JNK signaling	JUN, NFATC3, CDC42, SOS2, MAP3K1, SOS1, PIK3R5, PIK3CB, MAP4K5, MAP3K3	3.31E – 03
Wnt/β-catenin signaling	TCF4, CSNK1G1, FRAT1, GNAQ, BMPR2, FZD1, TCF3, ACVR1B, TGFBR2, CSNK2A2, JUN, NLK, RARA, TLE4, FZD2	3.47E – 03
PDGF signaling	CSNK2A2, JAK1, JUN, SOS2, MAP3K1, SOS1, PIK3R5, PIK3CB, STAT3	4.37E – 03
PKCθ signaling in T lymphocytes	CD3G, NFAT5, JUN, CAMK2D, NFATC3, SOS2, MAP3K1, SOS1, PIK3R5, PIK3CB, MAP3K3	4.68E – 03
IL-2 signaling	CSNK2A2, JAK1, JUN, SOS2, SOS1, PIK3R5, PIK3CB	5.89E – 03
Molecular mechanisms of cancer	TCF4, JAK1, SOS2, PIK3R5, BMPR2, SMAD5, FZD1, CDKN2B, TGFBR2, CAMK2D, NLK, JUN, SOS1, HIPK2, FZD2, SMAD7, GNAQ, BAX, AURKA, TCF3, NBN, CBL, CDC42, RBPJ, PIK3CB	7.24E – 03
ErbB2-ErbB3 signaling	NRG1, JUN, SOS2, SOS1, PIK3R5, PIK3CB, STAT3	7.94E – 03
PPARα/RXRα activation	PRKAB2, CPT1B, CD36, SOS2, GNAQ, BMPR2, NR2C2, ACVR1B, TGFBR2, HSP90B1, JUN, GPD2, SOS1, INSR	8.51E – 03
PTEN signaling	TGFBR2, CSNK2A2, CBL, CDC42, SOS2, SOS1, PIK3R5, BMPR2, PIK3CB, INSR, IGF2R	9.12E – 03
JAK/Stat signaling	JAK1, JUN, SOS2, SOS1, PIK3R5, GNAQ, PIK3CB, STAT3	9.55E – 03
HGF signaling	JUN, CDC42, SOS2, MAP3K1, SOS1, PIK3R5, PIK3CB, STAT3, MAP3K3, ELF1	1.07E – 02
Acute myeloid leukemia signaling	TCF4, RARA, SOS2, SOS1, PIK3R5, PIK3CB, STAT3, TCF3	1.12E – 02
Regulation of IL-2 expression in activated and anergic T lymphocytes	TGFBR2, CD3G, NFAT5, JUN, NFATC3, SOS2, MAP3K1, SOS1	1.29E – 02
CD28 signaling in T-helper cells	PTPRC, CD3G, NFAT5, JUN, ARPC5L, NFATC3, CDC42, MAP3K1, PIK3R5, PIK3CB	1.29E – 02
CNTF signaling	IL6ST, JAK1, SOS1, PIK3R5, PIK3CB, STAT3	1.35E – 02
Epithelial adherens junction signaling	TGFBR2, TCF4, ACTA2, ARPC5L, CDC42, FER, WASL, BMPR2, TCF3, IQGAP1, CLIP1, ACVR1B	1.38E – 02
Role of NFAT in regulation of the immune response	CD3G, JUN, NFAT5, CD79B, CSNK1G1, NFATC3, SOS1, SOS2, GNAQ, PIK3R5, PIK3CB, FCGR1A, FCGR1B	1.41E – 02
Lysine degradation II	AASDH, AASDHPPT	1.55E – 02
Lysine degradation V	AASDH, AASDHPPT	1.55E – 02
April-mediated signaling	NFAT5, JUN, NFATC3, TNFSF13, MAP3K1	1.91E – 02
Renal cell carcinoma signaling	JUN, CDC42, SOS2, SOS1, PIK3R5, PIK3CB, ARNT	2.34E – 02
IL-4 signaling	NFAT5, JAK1, NFATC3, SOS2, SOS1, PIK3R5, PIK3CB	2.51E – 02

Abbreviations: CNTF, ciliary neurotrophic factor; EGF, epidermal growth factor; HGF, hepatocyte growth factor; IL, interleukin; IRF, interferon regulatory factors; JAK/Stat, Janus kinase/signal transducer and activator of transcription; NFAT, nuclear factor of activated T cells; PDGF, platelet-derived growth factor; PKCθ, protein kinase C θ; PTEN, phosphatase and tensin homolog; TGF, transforming growth factor.

When canonical pathways were analyzed with Ingenuity Pathway Analysis, various pathways associated with cell growth, differentiation, cytokine and glucocorticoid receptor signaling and antibacterial responses were upregulated in monocytes (Table 1 and Supplementary Table 1). 'JAK/STAT signaling' (JAK1, JUN, SOS2, SOS1, PIK3R5, GNAQ, PIK3CB and STAT3, $P = 9.55E-03$) was among the upregulated pathways in CD14+ monocytes (Figure 1a). Similarly, 'IFN signaling' (IFIT3, IFIT1, IFITM3, OAS1, JAK1, MX1, BAX and IFITM1, $P = 5.62E-05$) was also prominent among upregulated genes (Figure 1b). Downstream genes in the IFN signaling pathway such as IFIT1/IFIT3 were upregulated over twofold. When 'IL-6 signaling' was analyzed, gp130 (2.0-fold) in the cell membrane, c-jun (1.71-fold) in the nucleus and STAT3 (1.64-fold) were also upregulated in monocytes from BD patients compared with healthy controls (HCs) (Figure 1c).

In $CD4^+$ T cells, genes related to developmental processes, differentiation and proliferation were activated. Canonical pathways associated with T-cell receptors such as ICOS, CD28 and glucocorticoid receptor signaling, inositol phosphate metabolism and Ca-induced apoptosis are shown to have upregulated molecules in $CD4^+$ T cells of BD patients (Supplementary Tables 2 and 3). JAK/STAT pathway genes in $CD4^+$ T cells were also among the prominent upregulated transcripts (JAK1, MAP2K2, PIK3C2A, SOCS2 and STAT2, $P = 1.78E-03$).

A recent study using RNA extracted from total PB mononucleated cells (PBMCs) demonstrated significant gene expression differences between BD patients and controls.[18] Our ability to directly compare these previously reported results with ours is limited as our study examined specific cell subsets in BD patients and controls. However, our data sets includes 76 and 13

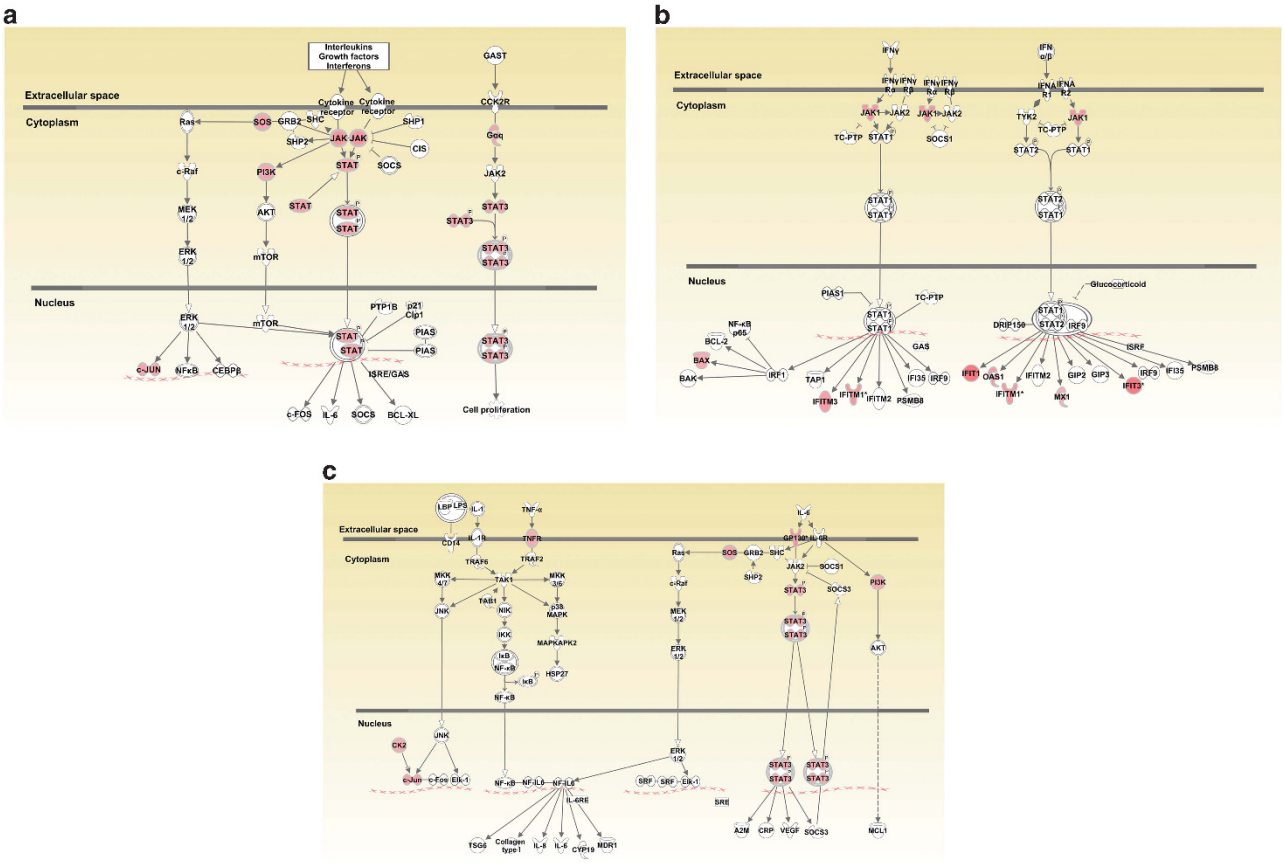


Figure 1. Canonical pathways activated in CD14+ monocytes in BD. (**a**) JAK/STAT signaling pathway. (**b**) IFN signaling pathway. (**c**) IL-6 signaling pathway. Red color indicates genes that are upregulated in patients compared to controls.

differentially expressed probes in monocytes and CD4+ T cells, respectively, that were also differentially expressed in the previously reported data in total PBMCs. Of particular interest is that two key genes in the neuregulin signaling pathway (*EREG* and *AREG*), which were among the top downregulated genes in PBMCs in BD patients,[18] were also significantly downregulated in BD monocytes in our study (Supplementary Table 4). Other relevant common downregulated genes in BD monocytes and total PBMCs include protein tyrosine phosphatase receptor type E (*PTPRE*) and phosphodiesterase 4D cAMP specific (*PDE4D*), among others.

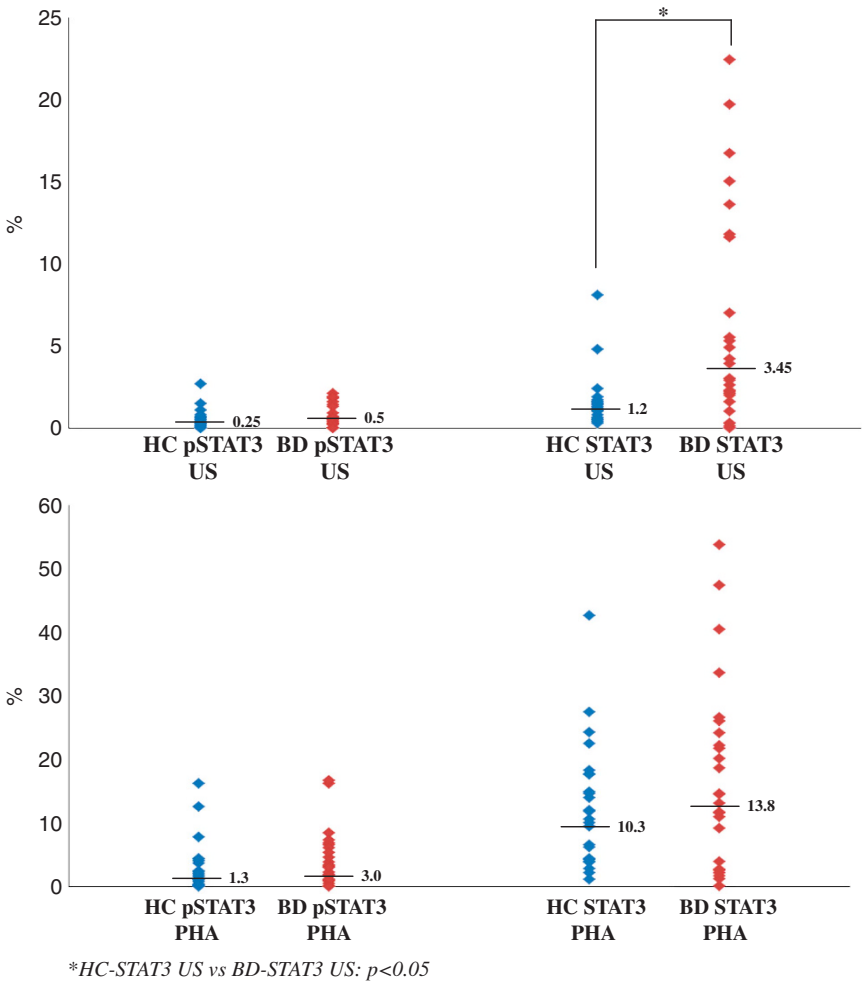
When patients with BD were compared in basal, unstimulated (US) and stimulated conditions (with phytohaemagglutinin (PHA)) for pSTAT3 and total STAT3 expressions, basal US total STAT3 expression was significantly higher in BD (1.2 (0.3–8.1) vs 3.45 (0–22.4), $P < 0.05$)(Figure 2). No correlations were observed between total STAT3 levels in BD patients and any disease manifestation, disease duration, age, gender and treatments.

After stimulations, both pSTAT3 and STAT3 expressions significantly increased compared with baseline, however no differences were observed between BD (pSTAT3: US: 0.5 (0–2.1) vs PHA: 3.0 (0–16.6); STAT3: US: 3.45 (0–22.4) vs PHA: 13.8 (0.1–53.7)) and HCs (pSTAT3: US: 0.25 (0–2.7) vs PHA: 1.3 (0–16.2); STAT3: US: 1.2 (0.3–8.1) vs PHA: 10.3 (1.1–42.6)) (Figure 2).

JAK/STAT signaling pathways are crucial for the activation of innate and adaptive immune systems. IFN-γR, IL-2R and IL-6R signals through JAK1, pairing with JAK2 or JAK3, whereas IL-12 and IL-23 activate through JAK2/Tyk2 pathway.[19] Downstream STAT1 is required for IL-2, IFN-γ and IL-6, whereas STAT3 is associated with IL-2, IL-6, IL-12 and IL-23. The anti-inflammatory cytokine IL-10 also activates the JAK1/STAT3 pathway, regulating SOCS3.[15] STAT3 was critical in modulating the balance of Th17 and regulatory T cells, as well as in promoting $CD4^+$ T-cell proliferation. STAT3 bound to multiple genes, especially IL-6, is involved in Th17-cell differentiation, activation, proliferation and survival, regulating both expression and epigenetic modifications. STAT3 also has an important role in the IFN-γ signaling pathway, which is highly involved in most autoimmune processes. Thus, STAT3 orchestrates multiple critical aspects of T-cell function in inflammation and homeostasis.[20]

JAK/STAT pathway-associated cytokines and T-helper subsets are shown to be activated in BD.[1] Both IL-12-activated, IFN-γ-secreting Th1 and IL-23-activated Th17 cell subsets are observed to be elevated in PB and tissues in BD.[3,4,21,22] Levels of IL-17, IL-23, IL-12/23p40 and IFN-γ in serum and the supernatants are significantly elevated.[10,23] The IL-6 signaling pathway, which is upregulated in our study, is implicated especially in the pathogenesis of neuro-BD, and IL-6 has been suggested as a biomarker in colony-stimulating factor analysis.[24]

US and PHA-stimulated pSTAT3 expressions, although higher in BD, were not significantly different between the study groups in our study. However, pSTAT3 expression is found to be upregulated in BD, in a different setting with anti-CD3/28 antibody stimulation, and suggested to be related to Notch pathway activation.[25] Most of total STAT3, observed to be elevated in our samples, seems to be unphosphorylated (U-STAT3). Recently, interest has increased in the functional roles of U-STATs. Ligand-dependent increases in the concentrations of U-STATs are shown to drive the expression of genes that are distinct from those activated by pSTATs. U-STAT3 binds to unphosphorylated NFκB (U-NFκB), in competition with IκB, and the resulting U-STAT3/U-NFκB complex is demonstrated to accumulate in the nucleus.[26] Following long-term IL-6 exposure, concentrations of endogenous



*HC-STAT3 US vs BD-STAT3 US: $p<0.05$

Figure 2. STAT3 and pSTAT3 expressions in PBMCs of BD patients and controls.

U-STAT3 is increased and it competes effectively with IκB for U-NFκB, to form a novel transcription factor that induces regulated on activation, normal T cell expressed and secreted expression.[27] This function of U-STAT3 seems clearly different from the absolute requirement for tyrosine phosphorylation that enables STAT3 dimers to bind to gamma-activated site motifs (IFN-activating sequences). STAT3 can also enter the nucleus independently of its phosphorylation, shuffling between cytoplasm and nucleus.[26] STAT3 has been shown to bind to some transcription factors such as cyclic AMP response-binding protein on the JunB promoter and has effects on cyclic AMP response-like sites in the C/EBPβ promoter and on the glucocorticoid response element. Recently, it was also demonstrated that U-STAT3 binds to AT-rich DNA sequence sites and recognizes specific DNA structures, such as four-way junctions and DNA nodes, within negatively supercoiled plasmid DNA.[28] These structures are important for chromatin organization and suggest a role for U-STAT3 as a chromatin/genome organizer. Our data might, therefore, suggest that upregulated U-STAT3 in BD patients can have intracellular effects other than phosphorylation in BD pathogenesis.

The JAK1/STAT3 pathway is also studied in some immune, inflammatory disorders (now termed autoinflammatory) with similar clinical and genetical features to BD, such as inflammatory bowel disease, spondyloarthropathies and psoriasis.[16,17] A common genetic background associated with JAK/STAT-activating cytokines such as IL-23R and IL-12R is present in BD, inflammatory bowel disease and psoriasis.[7] Recently, three genetic variants in JAK1 gene is reported to be associated with BD in Chinese patients with ocular disease.[29] However, a functional role of these single nucleotide polymorphisms could not be shown. Similarly, another study in the same population also showed that a STAT3 single nucleotide polymorphism seems to be associated with susceptibility to BD,[30] but not confirmed in a Spanish population.[31] STAT3 is also shown to be prominent in some animal models of chronic experimental uveitis and vasculitis, which have similarities to severe BD manifestations.[32,33]

In conclusion, we have demonstrated that JAK1/STAT3 signaling pathway is activated in BD, possibly through elevated serum and tissue expressions of Th1/Th17-type cytokines. In addition to current treatments such as IFN-α and anti-TNF-α agents that suppress Th17 cells, more direct therapies aiming JAK/STAT-associated cytokines such as ustekinumab (anti-IL-12/23) and recently approved tofacitinib that specifically inhibit JAK1/3 may be new therapeutic options for BD.[13,14,16,34]

PATIENTS AND METHODS

Patients

Nine patients with BD followed in the multi-disciplinary Behcet's Clinic of Marmara University Hospital and nine HCs were studied in the first phase of the study for the microarray analysis of CD14+ monocyte and CD4+ T lymphocytes. In the second phase, when the flow cytometric analysis of STAT3 and pSTAT3 levels were investigated, a larger group of patients with BD ($n=26$, 15 F/11 M, mean age: 35.3 ± 9.1 years) and HCs ($n=26$, 19 F/7 M, mean age: 33.9 ± 10.5 years) were recruited. All patients fulfilled the

International Study Group criteria for the classification of BD and had a mean disease duration of 6.9 ± 4.6 years.[35] Oral ulcers were present in all patients, 96% ($n = 25$) had cutaneous lesions, 81% ($n = 21$) had genital ulcers, 31% ($n = 8$) had musculoskeletal involvement and 50% ($n = 13$) had pathergy positivity. Among major manifestations, 19% ($n = 5$) of the patients had vascular, 12% ($n = 3$) had ocular and 4% ($n = 1$) had central nervous system disease. Ten patients (38%) were under immunosuppressive treatment (prednisolone > 10 mg per day and/or azathioprine). Patients studied for microarray analysis had similar characteristics to the whole group (9/9 patients with mucocutaneous, 3/9 with musculoskeletal, 2/9 with vascular and 1/9 with ocular manifestations). All patients had at least one active BD-associated disease manifestation at the time of blood sampling. The study was approved by the local Ethical Committee of Marmara University and informed consent was taken.

Sample collection and microarray analysis

PBMCs were isolated from fresh blood samples using density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). Monocytes and T-helper cells were purified using magnetic bead separation from PBMCs (Miltenyi Biotec, Cologne, Germany). The purity of isolated cell populations was confirmed by flow cytometric analysis using fluorochrome-conjugated antibodies against CD4 for T-helper lymphocytes and CD14 for monocytes and was over 90% for both cell populations. Total RNA of purified cell populations was extracted using RNeasy Mini kit (Qiagen, Hilden, Germany) according to the protocol recommended by the manufacturer. Total RNA (> 50 ng μl^{-1} (80–156)) from monocytes was available from eight patients and nine controls, and from CD4+ T cells from all nine patients and three controls. Total RNA was then hybridized to GeneChip Human Genome U133 Plus 2.0 microarrays (Affymetrix, Santa Clara, CA, USA) following manufacturer's protocols.

Gene expression data normalization and statistical analysis

Raw microarray data were processed using Affymetrix Expression Console version 1.1 (http://www.affymetrix.com/estore/browse/level_seven_software_products_only.jsp?productId=131414&categoryId%2520=35623#1_1) to extract gene level intensities using Robust multi-array average method. The subsequent analysis was performed using the R statistical environment (http://www.R-project.org) and BioConductor packages.[36] Quality control of the data included a comparison of boxplots of gene expression level among the conditions and hierarchical clustering of condition-specific gene expression profiles to identify potential outliers. The data were quantile normalized and deposited in GEO along with raw.CEL files (GEO accession number GSE61399). All data conform with the Minimal Information About a Microarray Experiment (MIAME) guidelines.

Differentially expressed probes were identified by significance analysis of microarrays.[37] A fold change cutoff of 1.5 was imposed to filter out less differentially expressed probes, and the delta was adjusted to keep false discovery rate $< 10\%$. Owing to the fact that some probes may detect expression of different exons, probe lists were converted to gene lists keeping all gene names. Gene lists were analyzed by Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, USA, http://www.ingenuity.com), a web-based bioinformatics tool. Each gene list was tested against the full Affymetrix Human Gene 1.0 ST Array data set to identify significantly overrepresented functions and canonical pathways as compared with the same number of randomly selected genes. The most significant functions and canonical pathways were selected and genes overrepresented in the functions and pathways were identified.

Flow cytometric analysis of STAT3 and pSTAT3 levels

For intracellular STAT3 and phosphorylated STAT3 (pSTAT3) level analysis, freshly isolated PBMCs were suspended in complete Roswell Park Memorial Institute medium 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and 100 U ml^{-1} penicillin and streptomycin (all from Sigma-Aldrich Inc., St Louis, MO, USA). PBMCs (1×10^6) in 1 ml were either left US or were stimulated with 10 µg ml – 1 PHA, Sigma-Aldrich Inc.) for 72 h. PMBCs were then washed and fixed with BD Cytofix buffer (BD Biosciences, Franklin Lakes, NJ, USA) for 20 min. Following the washing step, fixed cells were permeabilized by using BD Phosflow Perm Buffer III (BD Biosciences) for 10 min. US and PHA-stimulated cells were then stained with allophycocyanin-conjugated anti-STAT3 (M59-50) monoclonal antibody that recognizes STAT3 regardless of phosphorylation status. For the detection of phosphorylated STAT3, phycoerythrin-conjugated anti-STAT3 (pS727) monoclonal antibody that recognizes the S727-phosphorylated form of STAT3 isoform 1 was used. All samples were also stained with isotypic controls. STAT3 and pSTAT3 levels of PBMCs were analyzed with FACSCanto flow cytometry using Diva software (BD Biosciences). Results were expressed as median (range) of pSTAT3/STAT3-expressing mononuclear cells in PB and non-parametric Mann–Whitney *U*-test was used for comparisons.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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