
Differential expression of novel genes and signalling pathways of senescent CD8⁺ T cell subsets in Behçet's disease

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ABSTRACT

Objective. Behçet's disease (BD) is a rare disease characterised by recurrent mucocutaneous ulceration and chronic multi-systemic inflammation; however, its pathogenic mechanisms and biomarkers have not been fully discovered. Previously, we found that peripheral blood CD8⁺CD27⁺CD28⁺ T cell frequency was higher in patients with BD than in healthy controls (HCs). In this study, we used global gene expression analysis to identify candidate genes that might be related to pathogenesis or developed as biomarkers in two CD8⁺ T cell subsets from BD patients and HCs. **Methods.** We performed RNA sequencing analysis in CD8⁺CD27⁺CD28⁺ and CD8⁺CD27⁺CD28⁺ T cell subsets isolated from 18 patients with BD and healthy controls. Real time qPCR was used to validate the differential expression of genes in five patients with BD and healthy controls.

Results. We found that 1,103 genes and 652 genes were differentially expressed in the CD8⁺CD27⁺CD28⁺ and CD8⁺CD27⁺CD28⁺ T cell subsets of patients with BD, respectively. We validated the differential expression of COL5A1 in CD8⁺CD27⁺CD28⁺ T cells and TRPV3 and ARHGEF10 in CD8⁺CD27⁺CD28⁺ T cells. Furthermore, Ingenuity Pathway Analysis indicated that eleven pathways were more active in BD CD8⁺CD27⁺CD28⁺ T cells and more suppressed in BD CD8⁺CD27⁺CD28⁺ T cells than in the HCs.

Conclusion. Our study is the first transcriptome analysis of CD8⁺ T cell subsets in patients with BD and our results provide novel genes that might be related to BD pathogenesis.

Introduction

Behçet's disease (BD) is a chronic multisystemic inflammatory disease characterised by recurrent oro-genital, ocular, and skin lesions and the in-

volvement of multiple joints along with the gastrointestinal, cardiovascular, and central nervous systems (1-4). Although the exact pathogenesis of BD remains unclear, it is thought to be related to immune system dysfunction in genetically predisposed individuals and exhibits characteristics of both autoimmune and autoinflammatory diseases (5). The characteristics of BD related to autoinflammation include an association with the M694V MEFV mutation (6), an increase in C reactive protein (CRP) and erythrocyte sedimentation rate (ESR) during the active disease phase, and the dysregulation of IL-1 β (7, 8). Conversely, BD features associated with autoimmunity include an excessive T helper type 1 (Th1) response (9), a predicted role of human heat-shock protein (HSP) 60 as a candidate autoantigen (10), and association with IL-17 (11, 12).

Immunosenescence generally refers to changes in the structure and function of the immune system that occur during the aging process; however, increased senescent immune cell frequencies have been observed in several autoimmune diseases independently of aging (13-16). Senescent T cells are characterised by the loss of their proliferative capacity and the reduced expression of co-stimulatory molecules such as CD28, CD27, and CD40L (17-19). The frequency of these cells has been reported to increase in rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis (20, 21). Recently, we demonstrated that the frequency of senescent T cells increased in active BD (22); however, the phenotypic and functional characteristics of senescent T cells in patients with BD have not yet been fully elucidated.

Previous studies on gene expression in patients with BD have been limited to identifying the levels of several mRNAs or conducting gene microarrays; thus

far, no total RNA-sequencing has been conducted in relation to immunosenescent cells from patients with BD (23-26). Although there has been less focus on the role of CD8⁺ T cells in BD compared to CD4⁺ T cells, similar differential phenotypes have been observed in both CD4⁺ T cells and CD8⁺ T cells in patients with BD (22). Therefore, we took advantage of the higher frequency of senescent CD8⁺ T cells compared to senescent CD4⁺ T cells to perform whole transcriptome sequencing on senescent (CD8⁺CD27⁻CD28⁻) and non-senescent (CD8⁺CD27⁺CD28⁺) T cells isolated from healthy controls (HCs) and from patients with BD. Thus, we identified new sets of differentially expressed genes (DEGs) between the senescent and non-senescent CD8⁺ T cell populations of patients with BD and HCs.

Materials and methods

Subjects

We enrolled healthy controls (HCs) and patients with BD presenting for the first time or being monitored at the outpatient clinic of the Department of Dermatology, Ajou University Hospital between November 2017 and February 2018. Behçet's disease was diagnosed using the criteria of the International Study Group or the Behçet's Syndrome Research Committee of Japan (27, 28). A total of 18 patients with BD and 18 HCs were enrolled for transcriptome analysis, with a further five patients with BD and five HCs recruited for validation (Table I). BD patients with infectious or inflammatory diseases and other systemic diseases, including liver or renal failure, diabetes mellitus, and malignancies, and those with insufficient medical records were excluded. Informed consent was obtained from all subjects prior to enrolment. This study was approved by the Ajou University Hospital Institutional Review Board (IRB no.: AJIRB-BMR-GEN-14-462).

Isolation of CD8⁺ T cell subpopulations

Blood samples were collected from the subjects in standard 10 mL heparin-treated vacutainer tubes (Vacutainer® Tubes; Becton Dickinson, Stockholm,

Table I. Baseline demographics of study subjects and clinical characteristics of enrolled BD patients.

a. Transcriptome analysis			
	total number	male:female	mean age, yr. (SD, range)
BD	18	6:12	49.7 (9.0, 33-64)
HC	18	7:11	44.3 (4.1, 38-53)
b. Real-time qPCR			
	total number	male:female	mean age, yr. (SD, range)
BD	5	3:2	51.6 (6.2, 42-61)
HC	5	2:3	42.8 (3.0, 38-46)
c. Clinical characteristics of Behçet's disease patients			
	Number (%)		
	Transcriptome analysis	qPCR	
Symptoms at enrolment			
Oral ulcer	14 (77.8)	5 (100)	
Genital ulcer	5 (27.8)	3 (60)	
Skin lesion	11 (61.1)	3 (60)	
Ocular lesion	3 (16.7)	1 (20)	
Arthritis	2 (11.1)	0 (0)	
Gastrointestinal	0 (0)	0 (0)	
Cardiovascular	1 (5.6)	0 (0)	
Central nervous system	0 (0)	0 (0)	
Symptoms at sample collection			
Oral ulcer	13 (72.2)	2 (40)	
Genital ulcer	2 (11.1)	0 (0)	
Skin lesion	10 (55.6)	2 (40)	
Ocular lesion	2 (11.1)	0 (0)	
Arthritis	1 (5.6)	0 (0)	
Gastrointestinal	0 (0)	0 (0)	
Cardiovascular	1 (5.6)	0 (0)	
Central nervous system	0 (0)	0 (0)	
HLA-B51	9 (50)	5 (100)	
Systemic steroid treatment at enrolment	4 (22.2)	1 (20)	
	Mean ± SD		
ESR, mm/h	16.78 ± 13.48	9.80 ± 4.76	
CRP, mg/dL	0.73 ± 2.03	0.11 ± 0.10	

BD: Behçet's disease; HC: healthy control; SD: standard deviation; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein.

Sweden). Peripheral blood mononuclear cells (PBMCs) were isolated from each sample using Ficoll-Paque density gradient centrifugation (Ficoll Paque™ plus; Stem Cell Technologies, Vancouver, BC, Canada). CD8⁺ T cells were positively selected using CD8 microbeads with an MS column (Miltenyi Biotec Inc, CA, USA) and labelled with the following conjugated monoclonal antibodies: FITC-anti-CD8, allophycocyanin (APC)-H7-anti-CD27, and APC-anti-CD28 (BD Pharmingen; BD Biosciences, San Jose, CA, USA). Senescent CD8⁺ T cells (CD8⁺CD27⁻CD28⁻ T cells) and non-senescent CD8⁺ T cells (CD8⁺CD27⁺CD28⁺ T

cells) were sorted using fluorescence-activated cell sorting (FACS; BD FACS Aria III; BD Biosciences, Mountain View, CA, USA). The purity of each CD8⁺ population was over 90% (Supplementary Fig. S1).

Cell culture and stimulation

After sorting, cells were stimulated with anti-CD3 (500 ng/mL, clone OKT3; eBiosciences, San Diego, CA, USA) for 72 h in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco-BRL, Grand Island, NY, USA), and 10% fetal bovine serum at 37°C and 5% CO₂.

RNA extraction

Total RNA was extracted from the stimulated cells using an RNA isolation kit (NucleoSpin RNA XS kit; Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol.

RNA transcriptome analysis

Four pooled RNA samples (BD senescent CD8⁺ T cell sample, BD non-senescent CD8⁺ T cell sample, HC senescent CD8⁺ T cell sample, and HC non-senescent CD8⁺ T cell sample) were prepared by pooling equal amounts of RNA from each subject; pooled RNA was sent to Macrogen Inc. (Seoul, Korea) for RNA-sequencing analysis. Briefly, cDNA was produced using random hexamer-primed reverse transcription, and then 100 nucleotide paired-end sequenced using an Illumina HiSeq2500. Libraries were assessed by quantitative polymerase chain reaction (qPCR) and validated using an Agilent Technologies 2100 bioanalyzer. The RNA-seq reads were mapped to the human genome using TopHat (29), which traces split-read alignments across splice junctions, and determined using Cufflinks (30, 31). The Genome Reference Consortium GRCh37 genome assembly was used as a reference. Transcript counts were computed at the isoform and gene levels, and relative transcript abundance was expressed as fragments per kilobase of exon per million fragments mapped (FPKM) using Cufflinks.

Differentially expressed gene (DEG) identification

Transcripts with at least one FPKM value of zero in all samples were excluded from DEG analysis, with 1 added to each remaining FPKM value to simplify the log₂ transformation. Modified data were transformed and normalised using the quantile method. DEGs were adjusted to log₂ fold changel >2 for each comparison pair.

Ingenuity Pathway Analysis (IPA®)

Genes with a log₂ fold changel >2 were considered significant and submitted to Ingenuity Pathway Analysis for functional study and the identification of significant biological pathways (32).

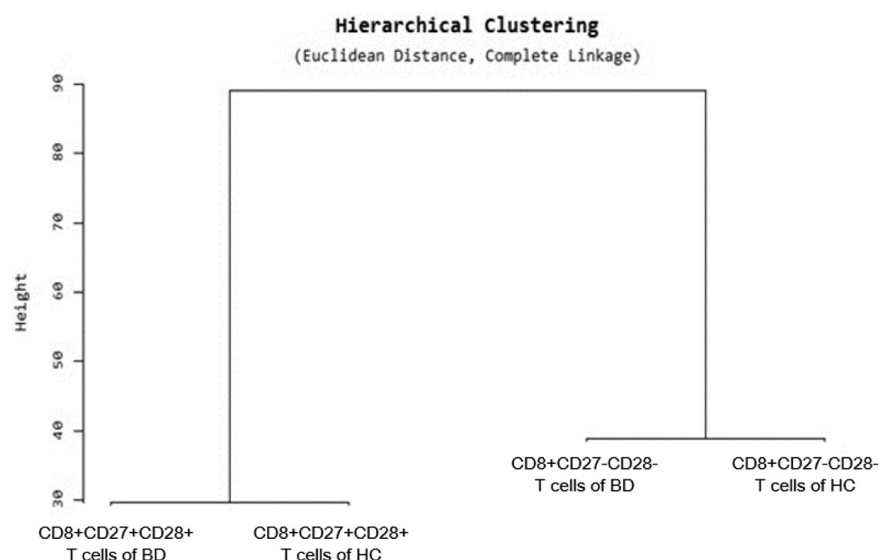


Fig. 1. Hierarchical clustering of four subsets of samples.

RNA transcriptome analysis grouped the subject groups and their CD8⁺ T cell subsets using the normalised value of gene expression level for each sample, to see which sample has similar levels of expression. (Distance metric: Euclidean distance; Linkage method: complete linkage).

BD: Behçet's disease; HC: healthy control.

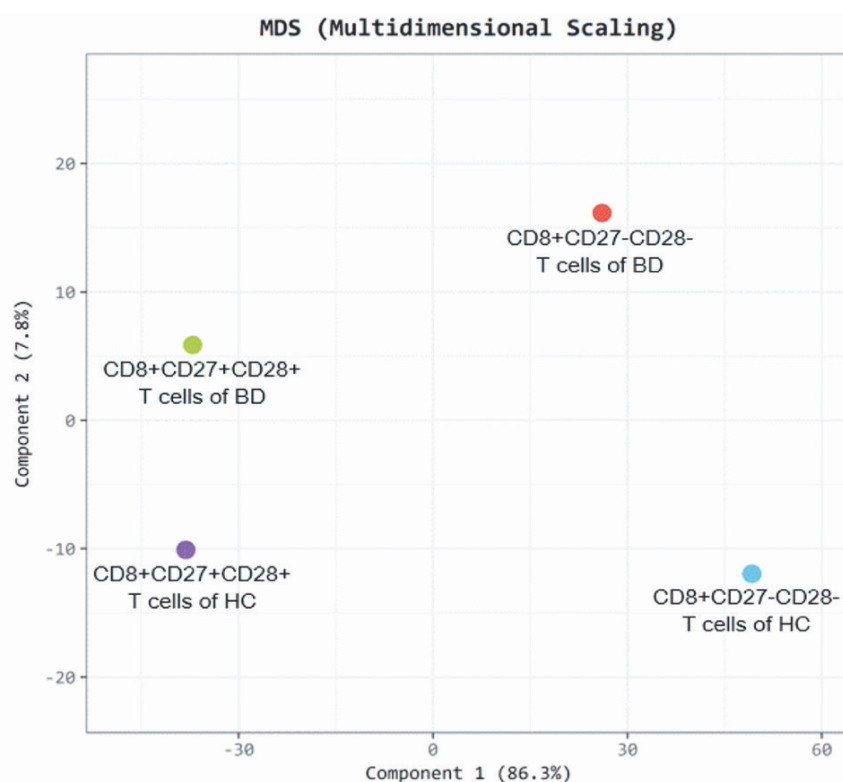


Fig. 2. Multidimensional scaling of four subsets of samples.

Two-dimensional space representation of the degree of similarity between samples using the normalised values, using two components that best describe the variability of the overall data. This enables the identification of outlier samples and similar expression patterns between biological replicates. BD: Behçet's disease; HC: healthy control.

Real-time quantitative PCR (qPCR)

cDNA was produced using oligo (dT) primers (Invitrogen, Carlsbad, CA, USA) with a Superscript™ III kit

(Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Real-time qPCR was performed using an ABI Prism 7000 Sequence

Detection System (Applied Biosystems, Foster, CA, USA) according to the manufacturer's instructions, with primers and internal probes for RGS11 (Hs01594651_m1), SHANK1 (Hs00211718_m1), AHRGEF10 (Hs00405154_m1), LOC102724428 (Hs00545020_m1), and UBE2F-SCLY (Hs00996436_m1) (Thermo Scientific) and SYBR Green Supermix for the TRPV3, COL5A1, CD302, and NBL1 genes, whose primer sequences were as follows: TRPV3, 5'-GTTAGCT-ACCCGCATTAAGCCTGA-3' and 5'-AGCAATTCTGGAATTCC-CAGCTC-3'; COL5A1, 5'-CCTG-CATTGTCTGTGGTGTGA-3' and 5'-AGCGCATGGCATGACTGAA-3'; CD302, 5'-GCCCAGATGATATC-CTACTAGGC-3' and 5'-GCAGAAAGCACAGGTGTCAACT-3'; NBL1, 5'-TCCACAGAGTCCCTGG-TTCACT-3' and 5'-GCTACAGTGC-AGGATCTTCTCC-3'. The $2^{-\Delta\Delta Ct}$ method was used for quantification.

Statistical analysis

Statistical analysis was performed using SPSS software (v. 22.0, IBM, Armonk, NY, USA). For dichotomous and nominal variables, we used Pearson Chi-square test and Fisher's exact test. To compare continuous variables, we conducted Mann-Whitney test. *p*-values of <0.05 were considered statistically significant.

Results

Transcriptome analysis was performed on 18 patients with BD and 18 HCs, with real-time qPCR performed on an additional five patients with BD and five HCs. The clinical characteristics of the patients with BD are summarised in Table Ic. More than 70% of patients exhibited mucocutaneous symptoms such as oro-genital ulcers or skin lesions, and of those who underwent transcriptome analysis, four (22%) were being treated with systemic steroids at the time of enrolment. The average ESR level and CRP of the patients with BD were 16.78 mm/h and 0.73 mg/dL, respectively, and 50% were positive for HLA-B51.

Following the transcriptome analysis, both hierarchical clustering (Fig. 1)

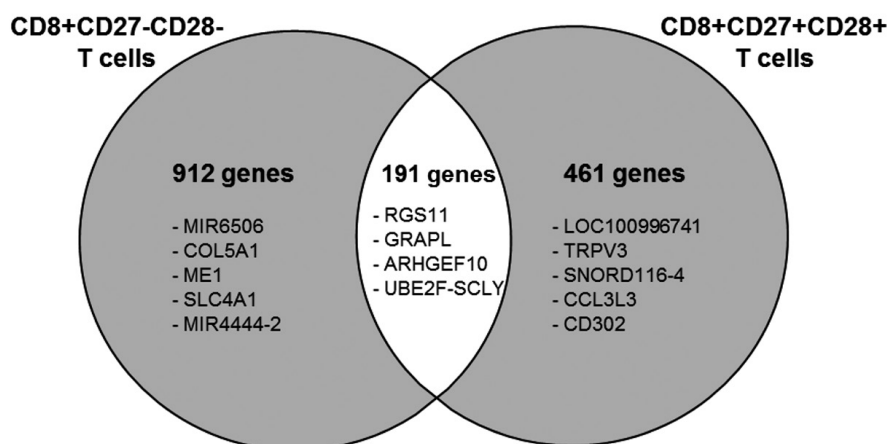


Fig. 3. Venn diagram of differentially expressed genes in senescent and non-senescent CD8⁺ T cells from patients with Behçet's disease compared to healthy controls. The mRNA levels of 1103 genes differed in the CD8⁺CD27⁻CD28⁻ T cells in patients with Behçet's disease and healthy controls, whereas 652 genes differed between their CD8⁺CD27⁺CD28⁺ T cells. Genes showing the largest fold change within each subset are marked within the Venn diagram.

Table II. Top ten genes based on the order of expression fold change in CD8⁺ T cells from Behçet's disease patients compared to healthy controls.

a. CD8⁺CD27⁻CD28⁻ T cell subset

Gene ID	Gene Symbol	Description	log ₂ fold change of RNA sequencing
4681	NBL1	neuroblastoma 1, DAN family BMP antagonist	131.4
8786	RGS11	regulator of G protein signalling 11	107.8
50944	SHANK1	SH3 and multiple ankyrin repeat domains 1	73.0
102465252	MIR6506	microRNA 6506	37.2
1289	COL5A1	collagen type V alpha 1 chain	36.9
4199	ME1	malic enzyme 1	32.9
57282	SLC4A10	solute carrier family 4 member 10	30.7
6571	SLC18A2	solute carrier family 18 member A2	29.4
5783	PTPN13	protein tyrosine phosphatase, non-receptor type 13	27.9
100846999	MIR4444-2	microRNA 4444-2	-88.7

b. CD8⁺CD27⁺CD28⁺ T cell subset

Gene ID	Gene Symbol	Description	log ₂ fold change of RNA sequencing
100996741	LOC100996741	uncharacterised LOC100996741	35.2
162514	TRPV3	transient receptor potential cation channel subfamily V member 3	23.6
8786	RGS11	regulator of G protein signalling 11	20.3
100033416	SNORD116-4	small nucleolar RNA, C/D box 116-4	16.5
9639	ARHGEF10	Rho guanine nucleotide exchange factor 10	-14.5
100526767	RNF103-CHMP3	RNF103-CHMP3 read-through	-15.5
100533179	UBE2F-SCLY	UBE2F-SCLY read-through (NMD candidate)	-26.3
414062	CCL3L3	C-C motif chemokine ligand 3 like 3	-31.7
9936	CD302	CD302 molecule	-47.1
102724428	LOC102724428	serine/threonine-protein kinase SIK1	-1634.7

Summary of top ten up- and down-regulated genes found in CD8⁺ T cells in BD patients compared to healthy controls. Positive and negative fold change values indicate up-regulation and down-regulation, respectively, of gene expression in senescent cells of BD patients relative to healthy controls.

and principal component analysis (Fig. 2) showed that degree of similarity in gene expression is higher between the senescent and non-senescent subsets

of HC and patients with BD than between the different subsets in the same subject group. Notably, differences in gene expression between patients

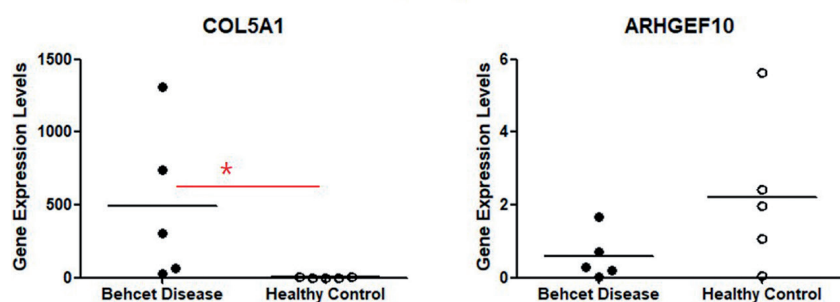
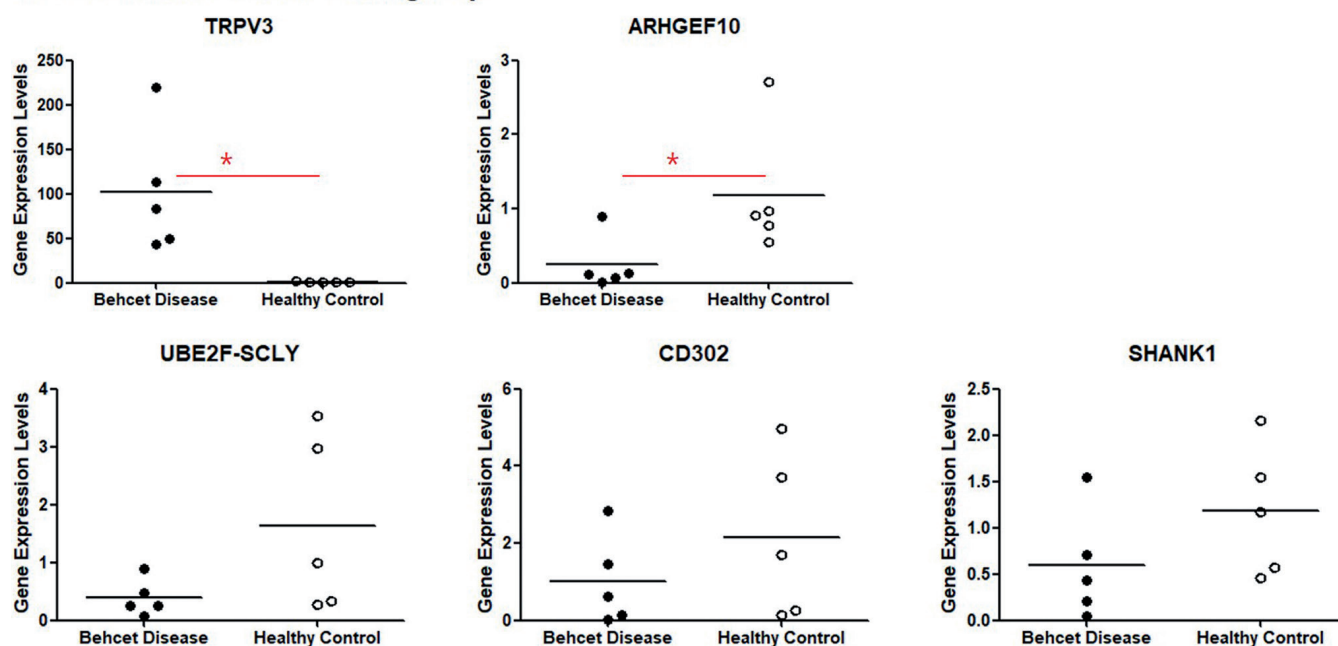
A. CD8⁺CD27⁻CD28⁻ T cell group**B. CD8⁺CD27⁺CD28⁺ T cell group**

Fig. 4. Validation of differentially expressed genes identified by RNA-sequencing.

A: Gene expression levels of the indicated genes in CD8⁺CD27⁻CD28⁻ T cells.

B: Gene expression levels of the indicated genes in CD8⁺CD27⁺CD28⁺ T cells.

Each dot represents a subject.

Bars represent the mean ($*p < 0.05$).

with BD and HC were greater in the CD8⁺CD27⁻CD28⁻ subset than in the CD8⁺CD27⁺CD28⁺ subset.

RNA sequencing revealed that 1,564 genes had a differential expression of greater than 2-fold between the patients with BD and the HCs. Figure 3 depicts a Venn diagram of the number of DEGs in the senescent and non-senescent CD8⁺ T cells of patients with BD compared to HCs. The mRNA levels of 1,103 genes differed between the CD8⁺CD27⁻CD28⁻ T cells of patients with BD and the HCs, whereas only 652 genes differed between their CD8⁺CD27⁺CD28⁺ T cells. Only 191 genes were common between the two subpopulations.

Table II shows the top ten up- and down-regulated genes with the highest absolute fold change values between patients with BD and the HCs within their senescent and non-se-

nescent CD8⁺ T cell subsets. Positive and negative log₂ fold change values indicate up- and down-regulated gene expression in patients with BD compared to the HCs. RGS11 was the only gene in the top ten for both subsets, showing positive fold change values in both.

To validate the RNA sequencing result, we performed real-time qPCR on eight genes (RGS11, SHANK1, ARHGEF10, LOC102724428, CD302, COL5A1, TRPV3, and UBE2F-SCLY) selected from the top DEGs, excluding miRNAs and uncharacterised genes. Figure 4 shows the genes that displayed consistency with the transcriptome analysis results. COL5A1 and ARHGEF10 had the same expression pattern as shown in the transcriptome analysis of the CD27⁻CD28⁻ subset in patients with BD compared to the HCs; COL5A1

displaying statistical significance (Fig. 4A). In the CD27⁺CD28⁺ subset in patients with BD compared to the HCs, TRPV3, ARHGEF10, UBE2F-SCLY, CD302, and SHANK1 also had the same expression pattern as seen in the transcriptome analysis with TRPV3 and ARHGEF10 displaying statistical significance (Fig. 4B).

To identify significant pathways, we submitted DEGs with log₂ fold change values >2 to IPA. The IPA results are presented in the supporting data 2 and show the top biological functions of the DEGs between BD and HCs, including “Diseases and Disorders”, “Molecular and Cellular Functions”, and “Physiological System Development and Function” alongside their *p*-values. The *p*-values presented in this table were determined by comparing the number of genes in each category with the total

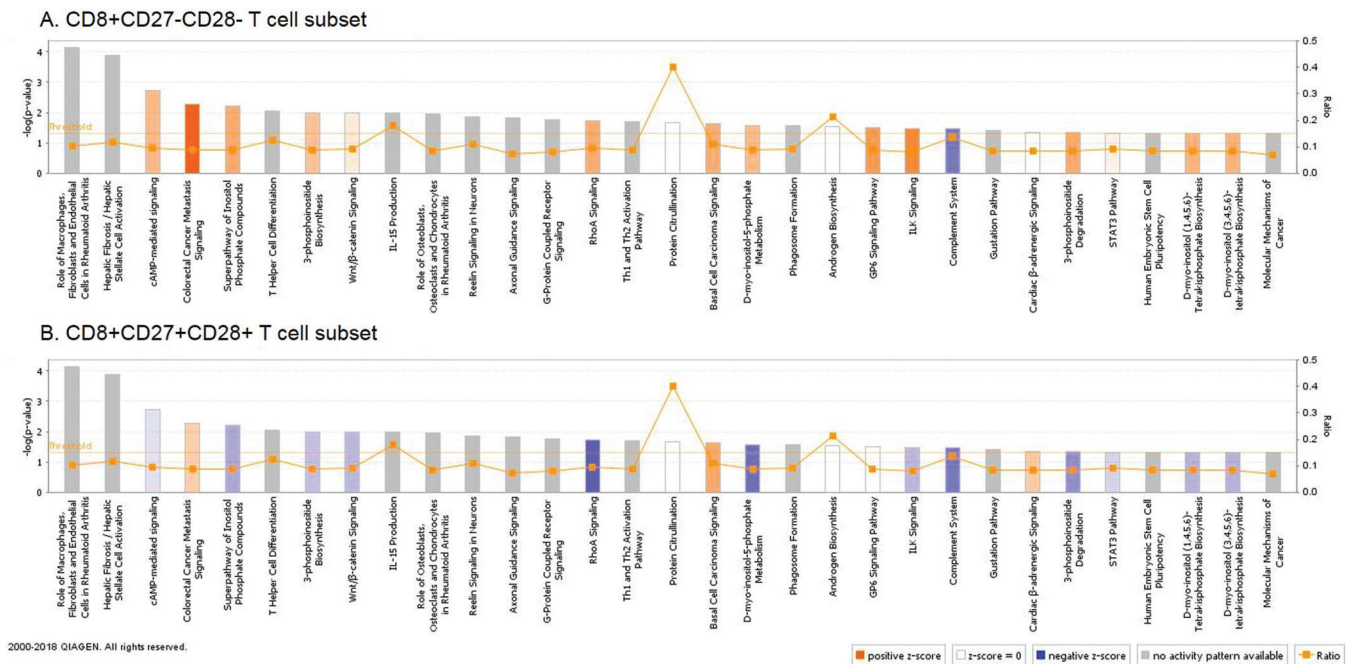


Fig. 5. Significant canonical pathways involving differentially expressed genes between patients with BD and healthy controls according to Ingenuity Pathway Analysis.

Canonical pathways were identified and pictorialised using Ingenuity Pathway Analysis (IPA®) software. A CD8⁺CD27⁻CD28⁻ T cells. B CD8⁺CD27⁺CD28⁺ T cells. The boxes in the picture are coloured orange for positive z-scores, blue for negative z-scores, white for zero, and grey where no activity pattern was found. Orange dots and straight lines represent the ratio between the DEGs found in our study and the corresponding genes in each pathway registered in the IPA software. For example, cAMP-mediated signalling shows approximately the same value on the Y-axis for both cell types but is coloured orange for CD27⁻CD28⁻ cells, and blue for CD27⁺CD28⁺ cells. This means that this pathway is more active in CD27⁻CD28⁻ cells from BD patients than in CD27⁻CD28⁻ cells from HC. Conversely, this pathway is less active in CD27⁺CD28⁺ cells from BD patients than in CD27⁺CD28⁺ cells from HC. In addition, the white colour means there is no difference between BD patients and HC in same cell type. A grey column indicates the IPA program could not find the activity pattern of the pathway with our DEG data.

number of known genes within the IPA software database; “Query Ingenuity Knowledge Base” (32).

The major canonical pathways identified by IPA are shown in Figure 5. Patients with BD demonstrated greater activity in eleven pathways in their CD8⁺CD27⁻CD28⁻ T cell subset, and also greater suppression in their CD8⁺CD27⁺CD28⁺ T cell subset, than that seen in with HCs. Within this set of affected pathways, cAMP-mediated signalling, RhoA signalling, and ILK signalling had positive Z-scores in the senescent cells of patients with BD and negative Z-scores in their non-senescent cells.

Figure 6 shows a detailed diagram of the cAMP-mediated signalling pathway, which was identified as the most significant pathway of the major canonical pathways. In our study, 24 DEGs corresponded to cAMP-mediated signalling pathways, including HTR6, P2RY14, TBXA2R, PDE7B, and AKAP12. Figure 6 shows cAMP-mediated signalling as being related to significant DEGs in patients with BD from the sample pop-

ulation. Genes highlighted in red and green were up- and down-regulated in patients with BD, respectively. Molecules coloured in orange and blue were excluded from our DEG list but were predicted to be activated and inhibited, respectively, by IPA analysis. DEGs detected in cAMP-mediated signalling pathways by IPA include G-coupled receptors, Rap1GAP, Gα/o, AO, PDE, EPAC, AKAP, and MKP1/2/2/4PKI.

Discussion

In this study, we profiled the whole transcriptome of CD8⁺ T cell subpopulations in patients with BD and HCs. Before sequencing, we divided the CD8⁺ T cell population into CD27⁻CD28⁻ and CD27⁺CD28⁺ subsets and compared the results of each subject group. Previous genetic studies on BD have used microarray methods or RNA sequencing on a limited number of pre-selected mRNAs (33-41). In contrast, we implemented a new “whole RNA sequencing” technique for the first time in BD. A study on psoriasis, which is

thought to share a similar pathogenesis to BD, suggested six novel DEGs (CXCL1, CXCL8 [IL-8], S100A8, S100A9, S100A12, and LCN2) related to psoriatic inflammation, and a study on ankylosing spondylitis demonstrated five DEGs (IL-6R, NOTCH1, IL-10, CXCR2, and TNFRSF1A) associated with therapeutic response by transcriptome analysis using PBMC samples (42, 43). In our study, we assessed the senescent and non-senescent CD8⁺ T cell subpopulations and found three novel DEGs in patients with BD compared to HCs.

We determined that DEGs with a log₂ fold change greater than two in each subset contained 191 genes that were common to both subsets, while the remaining 1,373 genes did not overlap. The top-ranked up- and down-regulated genes differed between the senescent (CD8⁺CD27⁻CD28⁻) and non-senescent (CD8⁺CD27⁺CD28⁺) T cell subsets, and we validated three of these genes using real-time qPCR. The genes that were significantly different in their

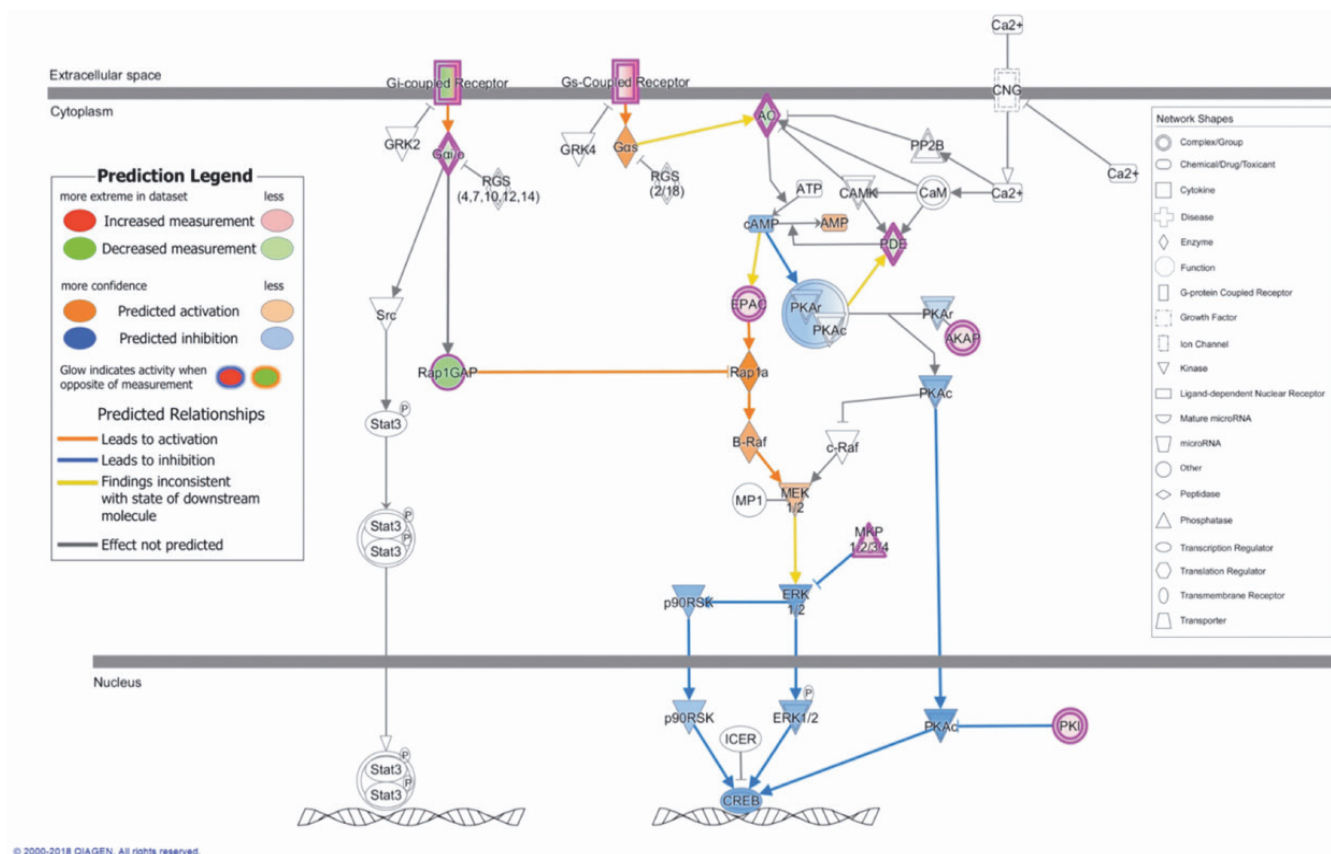


Fig. 6. cAMP-mediated signalling in the CD8⁺CD27⁻CD28⁻ T cells of patients with BD compared to healthy controls with activation predictions. Genes coloured in green were down-regulated and those in blue were predicted to be inhibited in patients with BD. Genes coloured in red were up-regulated and those in orange were predicted to be activated in patients with BD.

expression between patients with BD and the HCs by qPCR were COL5A1 in the CD8⁺CD27⁻CD28⁻ T cell subset and TRPV3 and ARHGEF10 in the CD8⁺CD27⁺CD28⁺ T cell subset. Although not significantly different, ARHGEF10 in the CD8⁺CD27⁻CD28⁻ T cell subset and UBE2F-SCLY, CD302, and SHANK1 in the CD8⁺CD27⁺CD28⁺ T cell subset also showed the same pattern in qPCR results as the transcriptome analysis. No studies have linked these genes to autoimmune or autoinflammatory diseases, such as BD; however, it seems worthwhile to evaluate their value as potential biomarkers for BD. There are several possible explanations for the differences between transcriptome analysis and qPCR results. First, we conducted qPCR and transcriptome analysis using samples from different patient populations. Second, individuals with very high variation in their expression levels may have been present in the pool and thus introduced error, which is a limitation of using pooling

methods. Third, patients with BD were not subdivided based on their disease activity, and this may have affected the results.

In this study, we identified 1,564 DEGs with varying fold change values, all of which were subjected to IPA. In total, eleven major DEG pathways analysed by IPA showed opposite activity patterns in CD8⁺CD27⁻CD28⁻ and CD8⁺CD27⁺CD28⁺ T cells, including the cAMP-mediated, RhoA, and ILK signalling pathways, which have recently been associated with immunoregulatory activities (44–49). Therefore, these eleven pathways may be key mechanisms by which senescent CD8⁺ T cells affect BD pathogenesis.

The most significant canonical pathway identified by IPA was the cAMP-mediated signalling pathway, which has been an area of increasing interest recently, with a particular focus on its effect on immune cell function. No studies have yet reported a link between cAMP-mediated signalling and BD;

however, our results suggest that it may connect BD with autoimmunity and autoinflammation. cAMP has an overall inhibitory effect on both innate and adaptive immune cell types, including neutrophils, macrophages, and T and B lymphocytes, and is also necessary for T cell activation and optimal immune function. Although transient increases in cAMP levels are triggered by T cell receptor engagement, continuous cAMP elevation suppresses T cell activation, proliferation, and chemotaxis (47). Therapeutic strategies to interrupt or enhance cAMP generation may have immunoregulatory potential in autoimmune and inflammatory disorders (50); for example, apremilast, a PDE-4 inhibitor that suppresses inflammation via cAMP-dependent mechanisms, demonstrated efficacy for treating oral ulcers in BD patients (51, 52). It is therefore possible that cAMP-mediated signalling pathway activation (sustained by senescent T cells) causes cellular dysfunction. As shown in Figure 6, our

data identified DEGs related to cAMP-mediated signalling pathways, including G-coupled receptors, Rap1GAP, Gai/o, AO, PDE, EPAC, AKAP, and MKP1/2/4/4PKI. Validating these molecules would confirm their association with BD; however, we were unable to verify them at the protein level due to the large number of cells required. Further experiments should validate these proteins and conduct qPCR for cAMP-signalling pathway-related genes with a larger sample size.

Figures 1 and 2 demonstrate that gene expression differed more between the T cell subsets than between HC and BD patients. For supplemental information, the DEGs that displayed differential expression between CD8⁺CD27⁺CD28⁺ and CD8⁺CD27⁺CD28⁻ T cell subsets are also listed in the supporting data 3. Notably, differences in gene expression between BD and HC was greater in CD8⁺CD27⁺CD28⁻ T cells than in the CD8⁺CD27⁺CD28⁺ T cells. Therefore, we infer that the CD27⁺CD28⁻ T cell subset may have a greater impact on BD pathogenesis.

The main limitation of this study was the small sample size. BD is a very rare disease; therefore, it was difficult to enroll patients for the study. RNA sequencing required relatively large blood samples, and patient RNA samples were pooled to ensure there was sufficient RNA for sequencing. RNA sequencing could not be performed repeatedly in the same manner; therefore, we validated the transcriptome analysis results by performing real-time qPCR on several genes instead of repeating the RNA sequencing, as described by Wang *et al.*, who pooled samples to minimise variability between individual samples in their RA study (53). However, as the samples were pooled for RNA sequencing, it was not possible to identify differences in gene expression depending on the type of clinical symptoms or disease activities of individual patients. Moreover, it was difficult to analyse gene expression according to HLA-B*51 positivity or IL-10 genotype. In addition, the effect of medication on patient's gene expression could not be excluded.

This is the first study to investigate DEGs by performing RNA sequencing

on the CD8⁺ T cells of BD patients and HCs, and is the first to separate senescent and non-senescent CD8⁺ T cells for RNA sequencing. Only 191 of the 1,564 DEGs were common to both T cell subsets, with just one (RGS11) common among the top-ranked up- and down-regulated genes. The difference between the senescent and non-senescent CD8⁺ T cells is therefore likely to be significant and there could be a masking effect when analysing all CD8⁺ T cells without subsetting. Furthermore, we validated several DEGs with large fold change values and predicted the signalling pathways that they may be involved with. In conclusion, our genetic profiling data may be useful for developing BD biomarkers to predict treatment responses and improve diagnosis, and for studying BD pathogenesis.

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