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Cell-Based Investigation of the Association of the rs4402765 Variant with *IL1A* and *IL1B* Genes in Behçet's Disease

Buneyan Tuzun [1,2], Irem Coskuntan [1,2], Burcu Salman Yaylaz [2], Mehmet Guven Gunver [3], Neslihan Abaci [1] ✉, Ahmet Gul [4] ✉

[1] Department of Genetics, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkiye
[2] Institute of Graduate Studies in Health Sciences, Istanbul University, Istanbul, Turkiye
[3] Department of Biostatistics, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Turkiye
[4] Division of Rheumatology, Department of Internal Medicine, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Turkiye

Abstract

Objective: In this study, we aimed to investigate the *IL1A* and *IL1B* mRNA expression profiles in association with the rs4402765 variant in monocytes and neutrophils to explore its role in the pathogenesis of Behçet's disease (BD).

Materials and Methods: Monocytes and neutrophils were isolated from peripheral blood samples obtained from 15 healthy Turkish participants and genotyped for rs4402765. THP-1 and HL-60 cell lines were used as controls to determine the doses and incubation times of the lipopolysaccharide (LPS) and aluminum hydroxide (alum) triggers. Isolated cells were stimulated with LPS and alum. *IL1A* and *IL1B* mRNA expression levels were analysed using by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Results: In monocytes stimulated with LPS for 8 h, the samples with the heterozygous rs4402765 genotype showed higher *IL1B* expression compared with the homozygous risk allele (CC) (p=0.016), but there was no statistically significant difference after 24 h of incubation (p=0.852). Increased *IL1A* expression was observed in neutrophils with the risk allele (CC) compared with the protective allele (GG) following LPS stimulation (p=0.002).

Conclusion: This study provides additional information about the functional effects of the rs4402765 variant in the IL-1 gene cluster, which may result in different expression profiles in monocytes and neutrophils, and the variability of cell-dependent expression warrants further studies.

Keywords Behçet's disease · *IL1A* · *IL1B* · rs4402765 · Monocyte · Neutrophil

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✉ Corresponding author: Neslihan Abaci neslihan.abaci@istanbul.edu.tr Ahmet Gul agul@istanbul.edu.tr

INTRODUCTION

Behçet's disease (BD) is a multifactorial relapsing inflammatory disorder of unknown etiology that manifests with oro-genital aphthous ulcers, uveitis, and inflammation in multiple organs such as skin, joints, blood vessels, intestinal tract, and brain (1). BD is relatively common in countries along the ancient "Silk Road," particularly Turkiye, and has a high sibling recurrence risk ratio (λs=11.4-52.5) (1–3). Although genome-wide association studies (GWAS) in different ethnic populations and other association studies have identified several BD-related genes, the major genetic susceptibility factor is known to be human leucocyte antigen (HLA)-B*51, a class I major histocompatibility complex (MHC) allele (3-4). Besides, the absence of pathognomonic clinical and laboratory findings in BD, which is quite complex due to its overlapping features with both autoimmune and autoinflammatory diseases (5), reveals the importance of elucidating the molecular mechanisms of the disease.

Dysregulation of innate and adaptive immune responses has been observed through external and internal triggering factors in the development of BD. Abnormally activated inflammatory response in BD has often been attributed to innate immunity (6-7). In this context, the contributions of monocytes and neutrophils to the pathogenesis of BD have become important research areas. Nowadays, neutrophil hyperactivation is a known molecular finding in BD and contributes to the disease manifestations (8-9). Despite technical challenges in studying monocytes due to the short time required for monocyte differentiation into macrophages (10-11), limited studies provide evidence for the role of changes in monocyte activation in the pathogenesis of BD. Recently, Zheng et al. found in their transcriptomic studies that there is a selective increase in monocyte subsets in BD, leading to abnormal production of proinflammatory cytokines, and that neutrophil hyperactivation is monocyte-dependent (12).

Considering the inflammatory characteristics, the changes in cytokine signaling and production are another notable findings in BD (7–13). Several studies have shown increased expression of proinflammatory cytokines, including the inflammasome-associated interleukin-1 beta (IL-1β) production in BD (14–18). Takeuchi et al. identified the rs4402765 variant located in the *IL1A*/*IL1B* locus (disease risk allele: C) associated with BD in the Immunochip analysis of the Turkish patients and controls, and it was replicated in the Iranian and Japanese cohorts (19). On the other hand, it was previously shown that the risk allele of the rs4402765 is associated with reduced production of IL-1α, and increased production of IL-1β (19). Evidence regarding this variant raises the possibility that the abnormal inflammatory response following exposure to pathogenic microorganisms may contribute to the risk of BD, and further studies are needed on the functional consequences of this variant on the expression of *IL1A* and *IL1B* genes.

In this study, we aimed to define the cell-specific mRNA expression profiles of different genotypes of the rs4402765 variant to investigate their effects on the inflammatory response.

MATERIALS AND METHODS

Study Design and Participants

This study was designed as an *in vitro* investigation and was conducted between August 2021 and August 2023. The study group consisted of 15 healthy Turkish controls (6 females and 9 males, mean age, 38.75 ± 14.00, who had no diagnosis of the inflammatory disorders including BD according to International Study Group (ISG) criteria (20) in their own or families' medical history.

Cell Lines

THP-1 (21-22) and HL-60 (23) cell lines were used to determine the dose and incubation times of lipopolysaccharide (LPS) and aluminum hydroxide ($Al(OH)_3$; alum) stimuli before starting the studies on the isolated monocytes and neutrophils.

Genotyping

The genomic region including the *IL1A* and *IL1B* intergenic polymorphism (rs4402765) was polymerase chain reaction (PCR)-amplified (Forward primer: 5′-AATGTATCCCCTTCTCCCTCA-3′ and Reverse primer: 5′-CCCCATTTACCTCCACACCAT-3′). Genotyping was performed by digesting the 522-bp PCR product with the NlaIII restriction enzyme. According to previously identified rs4402765 genotypes (19), participants were grouped as carrying homozygous susceptibility (CC), homozygous protective (GG), and heterozygous genotypes (GC).

Isolation of Monocytes and Neutrophils

Three different methods were used to prepare the peripheral blood cells:

1. Whole blood samples of the participants were collected in 10 mL EDTA tubes. Samples were diluted with phosphate-buffered saline (PBS) (Multicell, Canada). Peripheral blood mononuclear cells (PBMCs) and granulocyte layers were separated by Ficoll-Paque (Capricorn, Germany) density gradient difference (Ficoll-Paque density = 1.070 g/mL).

The isolated cells were washed twice in PBS before centrifugation (21000 rpm (~300 g) at RT for 25 min). Cells obtained from the PBMCs and granulocyte layer were measured using the ViCell Cell Counter XR (Beckman Coulter, USA). Following the determination of the cell counts, the cell suspension was centrifuged (10 min at 300 xg), and the supernatant serum was completely aspirated.

2. Monocyte isolation was performed based on the negative selection with the "Pan Monocyte Isolation kit" (Miltenyi Biotec, Germany) from the PBMC layer. A total of 10^7 cells were labeled with 10µl FcR blocking reagent, 10 µL biotin-antibody cocktail, and 20 µL anti-biotin MicroBeads. Unlabeled cells representing enriched monocytes were collected by washing Miltenyi Biotech MS (Miltenyi Biotec, Germany) columns 3 times with 500 µL MACS separation buffer.
3. The neutrophil isolation experiment based on the positive selection from the granulocyte layer was performed with the "CD66abce MicroBead Neutrophil isolation kit" (Miltenyi Biotec, Germany). According to the protocol defined by the manufacturer (Miltenyi Biotec, Germany), it was aimed to collect the granulocyte layer primarily with Ficoll-Paque phase difference; however, in this protocol, while the erythrocytes were lysed with Red Blood Lysis Buffer and only the granulocytes were expected to remain alive, it was observed that the neutrophils were suddenly activated and died. Afterwards, the protocol was modified and gelatin sedimentation buffer was used. First, 5 mL of blood was added as gelatin sedimentation buffer [4% gelatin (Sigma-Aldrich, USA), 0.1% dextran (MW: 7000 kDa, Sigma-Aldrich, USA), 0.9% NaCl (Sigma-Aldrich, USA)] and incubated at RT for 30 min. The leukocyte-rich orange layer was obtained by creating a phase difference (10 °C, 2300 rpm, 15 min DEC: 9 ACC: 9) on 3 mL Ficoll-Paque (Capricorn, Germany) and granulocyte- and erythrocyte-rich phases were obtained. Removal of erythrocytes from the medium was carried out with lysis buffer [4 mL ampoule water, 2 mL PBS (Multicell, Canada), 3% NaCl (Sigma-Aldrich, USA)]. Neutrophil isolation was performed using the obtained granulocyte layer. 10^7 cells were labeled with 10 µL CD66abce-Biotin and 20 µL Anti-Biotin MicroBead, respectively. Magnetically labeled neutrophil cells were collected by removing unlabeled cells with 1 mL of MACS separation buffer.

Cell Culture

Monocytes and neutrophils were cultured in RPMI 1640 (10% FBS, 100 U/mL penicillin, 50 µg/mL gentamicin, 100 µg/mL streptomycin, and 1% pyruvate) medium at 0.5×10^6 cells/well and 0.2×10^6 cells/well, respectively. Monocytes and neutrophils were stimulated by adding 10 µg/mL alum (aluminum hydroxide 2%; Alhydrogel®, InvivoGen, USA) and 100 ng/mL LPS to the culture medium. Cells were also cultured under control conditions [LPS(-) and alum (-)] without any stimulus.

In the LPS and alum dosage and incubation time experiments, THP-1 and HL-60 cells were seeded at $0.5\text{x}10^6$/well. THP-1 and HL-60 cell lines were treated with LPS (100 ng/mL, 300 ng/mL, and 500 ng/mL) and alum (10 µg/mL, 15 µg/mL, and 20 µg/mL). THP-1 cell line was stimulated at 8 and 24 h; HL-60 cell line was stimulated at 1 and 3 hours. IL-1β cytokine production amounts (pg/mL) were determined.

RNA Isolation and Reverse Transcription-Quantitative PCR (RT-qPCR)

RNA extraction from neutrophils and monocytes were performed using the "PureLink RNA Mini Isolation Kit" (Invitrogen™, USA) according to the manufacturer's guidelines. Subsequently, cDNA was synthesized from the isolated RNA using the "High-Capacity cDNA Reverse Transcription Kit" (Applied Biosystems™, USA), following the manufacturer's protocol. Primers were designed for the determination of *IL1A* (Forward: 5′-GGTAGTAGCAACCAACGGGAA-3′, Reverse: 5′-CAGCAGCCGTGAGGTACTGAT-3′) and *IL1B* (Forward: 5′-TCGACACATGGGATAACGAGG-3′, Reverse: 5′-CAACACGCAGGACAGGTACA-3′) gene expressions. RNA levels were normalized using 18s rRNA (Forward: 5′-CAACAGGTTACCCGCGTC-3′, Reverse: 5′-TGAAGGAGACTCTGGCATGC-3′) as a housekeeping gene. The mean value of the samples was determined based on the manufacturer's instructions for the Sensifast™ SYBR No-Rox Kit (Meridian Bioscience, USA), and the analysis was carried out using the LightCycler® 480 Instrument II for the RT-qPCR. Fold change in gene expressions were determined using the $2^{-(\Delta\Delta ct)}$ method (24).

Statistical Analysis

Distribution normalization in the data sets was achieved by log transformation. Repeated measures ANOVA analysis was applied to the transformed data. The statistical significance level (alpha) was selected as 5%. SPSS Statistics 28.0 (SPSS Inc., Chicago, IL, USA) was used. Statistical analyses were performed using the Kruskal-Wallis test.

RESULTS

Genotyping, Dose, and Incubation Time

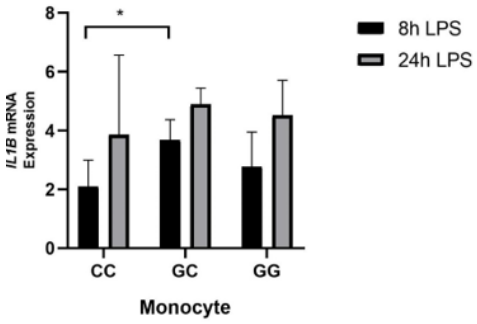
Genotyping for the rs4402765 variant revealed that 5 participants were homozygous for the risk allele (C/C; 4M, 1F), 5 were heterozygous (G/C; 2M, 3F) and 5 were wild type (G/G; 3M, 2F). THP-1 and HL-60 cell lines showed heterozygosity for the rs4402765 variant. As a result of the dose optimization experiments conducted with THP-1 and HL-60 cell lines, we stimulated monocytes at 8 and 24 h and neutrophils at 1 h and 3 h at different doses. Based on IL-1β ELISA results, we decided that the combination of 100 ng/mL LPS and 10 µg/mL alum was the optimum dose for the stimulation assays. Purity of monocytes and neutrophils were checked with Giemsa and May Grunwald's staining.

IL1B Expression Levels in Association with rs4402765 Genotypes

We compared cell-specific *IL1B* mRNA expression according to the rs4402765 genotypes. In monocytes stimulated with LPS for 8 h, we observed significantly higher *IL1B* mRNA expression in the GC group compared to the CC group (p=0.016); however, we did not encounter any statistical significance in terms of allele specificity at 24 h of incubation (p=0.852) (Figure 1a). In alum-stimulated monocytes, we could not find any difference between the genotypes (8 h alum, p=0.852; 24 h alum, p=0.18) (Figure 1b). *IL1B* mRNA expression levels did not show a significant difference between the incubation times with alum and LPS (1 h LPS, p=0.763; 2 h LPS p=0.112; 1 h alum p=0.755; 2 h alum p=0.403) (Figure 1c, Figure 1d).

IL1A Expression Levels in Different rs4402765 Genotypes

IL1A mRNA expression gave the most effective response in LPS-stimulated monocytes at the end of the 24th hour and increased almost 4-fold (p=0.228) (Figure 2a). There was no significant difference in *IL1A* mRNA expression levels between the groups after alum stimulation (8 h alum, p=0.667; 24 h alum, p=0.185) (Figure 2b). We found that *IL1A* mRNA expression in neutrophils was significantly higher in the CC group compared to the GG group at the 1st hour after LPS stimulation (p=0.002) (Figure 2c). We did not observe any difference at the 2nd hour (p=0.114) (Figure 2d).



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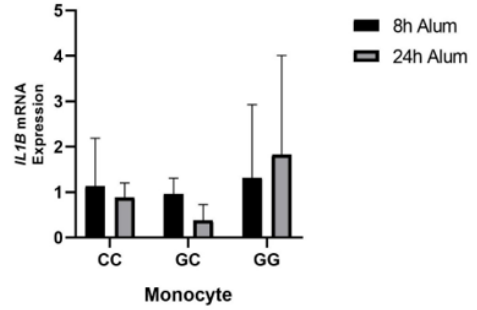

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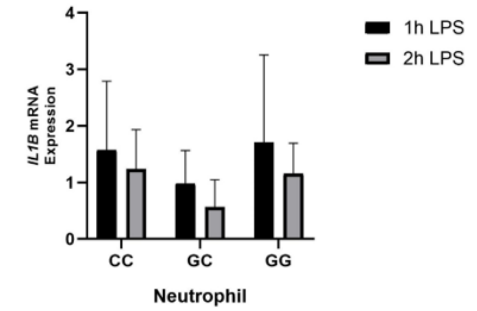

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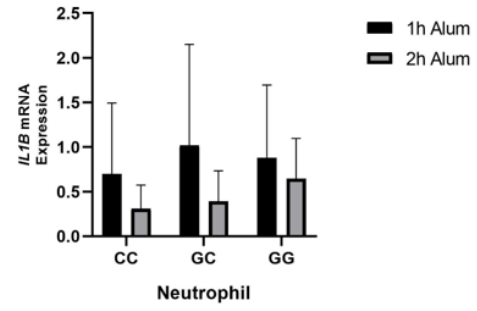
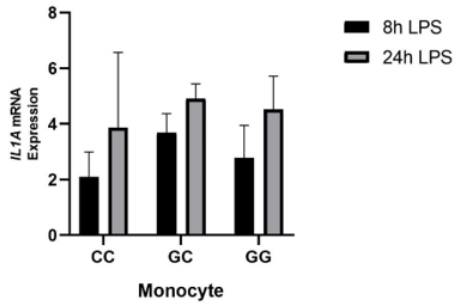


d

Figure 1. Comparison of *IL1B* expression levels in enriched monocytes and neutrophils of different genotypes. (a) *IL1B* mRNA in monocytes with LPS stimulation at the 8th and 24th hour. (b) *IL1B* mRNA expression in monocytes with alum stimulation at the 8th and 24th hour. (c) *IL1B* mRNA expression in neutrophils with different rs4402765 genotypes following LPS stimulation at the 1st and 2nd hour. (d) *IL1B* mRNA expression in neutrophils with alum stimulation at the 1st and 2nd hour. Means (horizontal bars) and standard deviation (error bars) are indicated. *p<0.05 (Kruskal-Wallis test).



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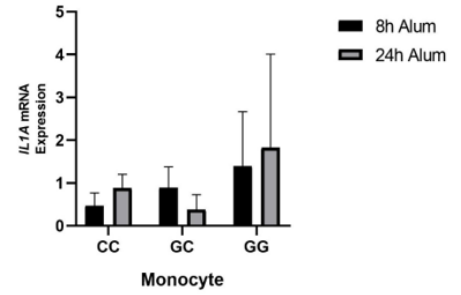

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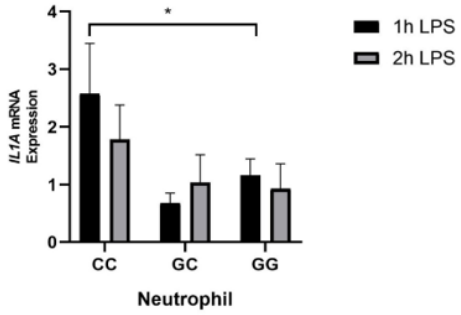

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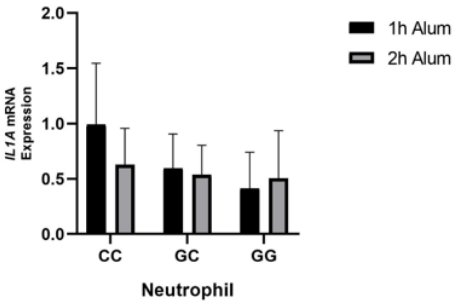

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Figure 2. Comparison of *IL1A* expression levels in enriched monocytes and neutrophils with different rs4402765 genotypes. (a) *IL1A* mRNA expression in monocytes with LPS stimulation at the 8th and 24th hour. (b) *IL1A* mRNA expression in monocytes with alum stimulation at the 8th and 24th hour. (c) *IL1A* mRNA expression in neutrophils with LPS stimulation at the 1st and 2nd hour. (d) *IL1A* mRNA expression in neutrophils with alum stimulation at the 1st and 2nd hour. Means (horizontal bars) and standard deviation (error bars) are indicated. *p< 0.05 (Kruskal-Wallis test.)

DISCUSSION

BD exhibits a highly complex pathology showing features of autoimmune and autoinflammatory diseases, and HLAB*51 carriage is considered an indicator of genetic susceptibility (1). The IL-1 signaling pathway is critically involved in the immunodysregulation of autoinflammatory diseases such as BD. Furthermore, clinical and molecular studies indicate innate immune response abnormalities in patients with BD and that single nucleotide polymorphisms (SNPs) contribute to BD susceptibility (14–18).

The immunogenetic profile of patients with BD varies in the presence of different triggering factors. One of the important findings is abnormal neutrophil activation in BD (25). In particular, the hyperactivation of neutrophils may occur with bacterial exposure and involvement of monocytes (26). Takeuchi et al. discovered as a result of GWAS in Turkish, Iranian and Japanese BD cases that the rs4402765 variant in the *IL1A*/*IL1B* locus contributed to the risk of BD (19). Therefore, it is considered one of the most important studies to examine the relationship between IL-1 and BD in detail. This study suggested that the *IL-1* gene family might play an important role in the disease's pathogenesis by causing an excessive inflammatory response to microbial triggers, and homozygous of the rs4402765 risk allele (CC) is associated with both increased IL-1β production and decreased IL-1α production in BD (19). Thus, we reasoned that SNPs may cause differences in BD development, genetic susceptibility, and prognosis. We hypothesized that functional polymorphisms may lead to cell-specific disruption of the *IL-1* pathway and focused on rs4402765.

rs4402765 (GRCh38:g.112811270G>C), located in the *IL1A*/*IL1B* locus that we targeted in our study, was identified as an intergenic and regulatory region variant in The Ensembl Variant Effect Predictor (27). Composing IL-1α and IL-1β, the IL-1 cytokines have been implicated in autoinflammatory conditions due to possible disruptions in IL-1 signaling. IL-1α is a unique cytokine with dual-function in regulating the inflammatory response at the transcriptional level and by binding to cell membrane receptors. *IL1B* expression is strictly controlled by regulatory mechanisms at both the transcriptional and post-transcriptional levels. Both cytokines are synthesized in blood monocytes and neutrophils (28-29). Given this information, unlike the study in which the rs4402765 SNP was discovered (19), we chose to isolate monocytes and neutrophils separately instead of PBMCs. Thus, we were able to investigate the variability of cell-specific responses across different SNP genotypes associated with BD.

In our dataset, we observed that *IL1B* mRNA expression in monocytes increased in the heterozygous of the rs4402765 risk allele compared to the homozygotes of the rs4402765 risk allele (p=0.016). Although the expression quantitative trait locus (eQTL) analysis in the Immunochip study (19) did not find a statistically significant result, we still do not fully describe the clinical significance of our finding that *IL1B* mRNA expression is higher in heterozygotes compared to homozygotes carrying the risk allele. Our findings indicate an interaction between the heterozygous rs4402765 risk allele and *IL1B* mRNA expression, with increased expression in monocytes and a protective effect in neutrophils upon stimulation with specific agents. Recent studies have identified enhancer-derived RNAs (eRNAs) as important regulatory elements involved in the transcriptional control of *IL1A* and *IL1B* genes. Notably, the expression of eRNAs is rapidly induced upon stimulation with LPS, highlighting their role in modulating the acute-phase inflammatory response mediated by IL-1 cytokines (30). We consider that one of the most important reasons for the different results of our study is that cell-specific responses may alter the enhancer-promoter interaction in IL-1 transcription in monocytes. Another possible reason could be that we isolated monocytes and neutrophils separately instead of PBMCs and examined cell-specific transcription because cell-based gene expression changes may not be detected in the entire lymphoblastoid cell population.

Although Immunochip study associated the rs4402765 risk allele with reduced *IL1A* expression in lymphoid cells, our analyses showed increased *IL1A* expression in LPS-stimulated neutrophils after 1 h in individuals homozygous for the risk allele compared those with the protective allele (GG) (p=0.002). In neutrophils, *IL1A* expression was found to be higher in individuals homozygous for the risk allele compared those carrying the protective allele, which is one of the most important findings of our study. Takeuchi et al. (19) reported that *IL1A* expression and protein levels were decreased. However, in our study, the higher *IL1A* expression in homozygous risk allele carriers showed a cell-specific difference in neutrophils compared fibroblasts and PBMCs. We suggest that these findings should be interpreted with caution until they are replicated in larger studies and across diverse ethnic groups.

We provide remarkable findings on the function of neutrophil hyperactivation and monocyte cells involved in the etiopathogenesis of BD. Moreover, stimulation of neutrophils with alum substance is a novel method that we used in our study. The major limitations of this study are (I) the small sample size, (II) the large number of variants related to IL-1α function, and (III) the variant heterogeneity in the transcription initiation site of the *IL1B* gene.

Our study reported distinct results from the literature regarding the effect of the rs4402765 variant, which was determined to be associated with BD, on the expression of *IL1A* and *IL1B* genes. Given the population-specific polymorphisms and their variable frequencies, ethnicity may generate different immune responses to BD risk. Given the established role of ethnicity in shaping immune responses and the population-specific differences in polymorphism frequency and function, particularly within the *IL1A*/*IL1B* locus, it is plausible that ethnic background contributes to distinct immunological pathways in BD pathogenesis (14, 15). Variations in allele frequencies, linkage disequilibrium patterns, and regulatory element activity may influence IL-1 cytokine expression and regulation, thereby altering the magnitude and character of inflammatory responses. However, limitations such as sample size and variant heterogeneity in the *IL1B* gene transcription site were noted. To fully understand the biological significance of the rs4402765 variant and its association with BD risk, a large-scale cross-sectional study with ethnicity-specific cohorts would be valuable. Future studies should explore not only gene-gene and gene-cytokine interactions at the *IL1A*/*IL1B* locus, where the rs4402765 variant is located, but also chromatin organization and its role in modulating immune responses. Additionally, investigating the molecular mechanisms induced by different stimuli—particularly enhancers, promoters, and epigenetic regulators—will provide deeper insights.

Ethics Committee Approval: The study was conducted in accordance with the latest revision of the Declaration of Helsinki and the approved Istanbul University Non-Interventional Clinical Research Ethics Committee (E-29624016-050.99-148703).

Authors Contributions: Conception/Design of Study- B.T., I.C., N.A., A.G.; Data Acquisition B.T., B.S.Y.; Data Analysis/Interpretation: B.T., I.C., M.G.G., N.A., A.G.; Drafting Manuscript- B.T., I.C., N.A., A.G.; Critical Revision of Manuscript- B.T., I.C., N.A., A.G.; Final Approval and Accountability- B.T., I.C., N.A., A.G.;

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Conflicts of Interest: The authors declare that there are no conflicts of interest.

Author Details

Buneyan Tuzun
[1] Department of Genetics, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkiye
[2] Institute of Graduate Studies in Health Sciences, Istanbul University, Istanbul, Turkiye
0000-0003-1032-1337

Irem Coskuntan
[1] Department of Genetics, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkiye
[2] Institute of Graduate Studies in Health Sciences, Istanbul University, Istanbul, Turkiye
0000-0003-0581-5295

Burcu Salman Yaylaz
[2] Institute of Graduate Studies in Health Sciences, Istanbul University, Istanbul, Turkiye
0000-0002-9144-3899

Mehmet Guven Gunver
[3] Department of Biostatistics, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Turkiye
0000-0002-4628-8391

Neslihan Abaci
[1] Department of Genetics, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkiye
0000-0002-9962-4010 neslihan.abaci@istanbul.edu.tr

Ahmet Gul
[4] Division of Rheumatology, Department of Internal Medicine, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Turkiye
0000-0001-8219-3720 agul@istanbul.edu.tr

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