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Abstract
Behçet’s disease (BD) is a type of vasculitis with many distinctive clinical manifestations and multifactorial immunopathogenesis. The cause of BD remains unknown, but it has been postulated that in a genetically predisposed or susceptible population, exogenous agents trigger the dysregulation of both autoinflammatory and autoimmune responses resulting in multisystem vasculitis. There are robust ongoing efforts across the globe to elucidate and identify signature markers to improve and assist in rapid diagnosis of the disease and to tailor the best therapy accordingly. While association of human leukocyte antigen (HLA)-B*51 (B*51:01 subtype) allele is well recognized as the strongest genetic susceptibility gene so far among genetically predisposed BD patients, further investigations using the latest technology have led to the identification of several novel single nucleotide polymorphisms (SNPs) and other associated genes involved in the pathogenesis. There are several “established” cytokines known to be involved in the pathogenesis of BD, which have been further implicated in the genome-wide association studies (GWAS)-based cytokine/receptor gene loci studies, as well as numerous “novel” cytokines, which are currently being studied and identified. This chapter offers insights into current knowledge and thoughts regarding the future of biomarkers in BD.

Keywords: Behçet’s disease, Behçet’s syndrome, biomarker, immunopathogenesis, immunogenetics, pathogenetics, etiopathogenesis, cytokines, HLA-B*51

1. Introduction
Behçet’s disease (BD) is a type of vasculitis characterized by recurrent inflammatory attacks causing many distinctive clinical manifestations, most commonly affecting the orogenital mucosa, skin, and the eyes [1–3]. The etiology has yet to be fully established, but it has been
postulated that a genetically predisposed or susceptible population, exposed to exogenous agents, may result in the dysregulation of both autoinflammatory and autoimmune responses. It is a complex disease that has been the subject of intense research and clinical interest.

In this era of precision medicine, there is a need to integrate biomarkers into clinical practice, which may serve as valuable predictive and prognostic tools to assist practicing physicians in making clinical decisions while managing complex diseases. This may also improve diagnostic capability, risk stratification, prediction of disease progression, assist in targeted therapy, and monitoring of response to treatment to improve patients’ overall clinical outcome.

This review chapter will look into the most relevant articles that have defined and clarified BD over the years, as well as review the most recent publications offering new insights in order to help fill the gaps in further understanding the disease. The chapter will focus on the etiology, immunopathogenesis, recent advances in search of potential biomarkers and targets for further research.

We will examine recent advances made that have shed new light on an old disease and explore the potential genetic (including genome-wide association studies (GWAS) and next-generation DNA sequencing, looking into the human leukocyte antigen (HLA) and non-HLA genetic associations) and molecular markers (including innate immune lymphoid cells and adaptive immune cells, cytokines, chemokines, other circulating biomarkers, and signaling molecules of inflammation) and their potential correlations with disease activity and therapy.

2. The pursuit for genetic susceptibility markers in Behçet’s disease

BD is a genetically complex and heterogeneous disease. The pursuit of gene discovery for causative genetic factors in BD spans over more than four decades since Professor Shigeaki Ohno first described the association of HL-A5 antigen observed in his Japanese BD cohort that was later renamed human leukocyte antigen (HLA)-B5 [4]. Other early evidence stems from the observation of familial clustering in BD families where more than one family member developed the disease [5–8], which provided further clues to a strong genetic predisposition to the disease.

2.1. The MHC I and HLA-B’51 and its association with BD

The major histocompatibility complex (MHC) has an expansive immune component including the HLA and plays a pivotal role in the genetic influences on susceptibility to autoimmunity. The ~3.5-Mb region has the highest density of genes in the human genome, the majority of which have fundamental roles in immunity [9].

A remarkably consistent body of evidence demonstrates association of HLA-B’51 (B’51:01 subtype) allele as the strongest genetic susceptibility gene so far among genetically predisposed BD patients. However, certain indigenous Amerindians have a high prevalence of HLA-B’51 but virtually no reported cases of BD. High level of recombination within the MHC is known
to have occurred in these Eastern populations before their migration into Beringia and it was suggested that disruption of the genetic loci in linkage disequilibria with HLA-B*51 might be one reason for the absence of disease in these high HLA-B*51-bearing populations [10]. These findings emphasize the fundamental roles and interplay of both genetic and environmental components in the development of the disease.

There have been conflicting views, however, on whether the disease association with HLA-B*51 is attributed to a role of MHC class I variant itself or if the association is found due to its linkage disequilibrium (LD) with another variant in the region [11].

2.2. The unifying concept of MHC-I-opathy

The understanding of the pathophysiology of BD is challenging, as it is at the crossroads between autoimmune and autoinflammatory syndromes [12]. A new perspective of a unifying concept of MHC-I-opathy was proposed recently comprising of BD and several clinically distinct spondyloarthropathies (such as ankylosing spondylitis and psoriasis)—all associated with MHC Class I alleles, such as HLA-B*51, HLA-B*27, and HLA-C*0602, and epistatic endoplasmic reticulum aminopeptidase 1 (ERAP-1) interactions.

McGonagle et al. have proposed that the MHC-I-opathies share an immunopathogenetic basis including barrier dysfunction in environmentally exposed organs such as the skin and aberrant innate immune reactions at sites of mechanical stress. This they argue can often trigger secondary adaptive immune CD8+ T cell responses with prominent neutrophilic inflammation that culminate in the initiation and chronicity of these diseases [13]. Further research and understanding into this unifying concept of MHC I driven inflammatory response may provide further targets for disease management.

2.3. Other MHC/HLA associations

The complexity and strong LD with the HLA-B*51 allele make it difficult to explore additional independent susceptibility loci within this region. Despite having the strongest genetic association, it remains unclear to this day whether disease susceptibility in BD is due to the HLA-B*51 itself or due to the genes located around the HLA which is in LD with it.

The MHC Class I chain-related gene A (MICA) is located in proximity and in between the HLA-B and tumor necrosis factor (TNF) genes on the short arm of chromosome 6. It has long been considered a major genetic susceptibility gene for BD and has been studied in many different populations since the first observation of a possible association by Mizuki et al. [14]. Lee et al. conducted a meta-analysis on the associations of MICA and BD and found statistically significant association in various ethnic populations [15]. However, due to its strong LD with HLA-B*51, it has been difficult to prove MICA as a primary susceptibility gene for BD. Furthermore, different GWAS did not find an independent association between MICA and BD [16–18].

Ombrello et al. performed stepwise conditional analysis and found independent genetic associations for HLA-B*15 and HLA-B*27 and risk, while HLA-B*49 and HLA-A*03 were protective.
Montes-Cano et al. demonstrated HLA-B*57 as a marker for risk in the Spanish population [20], while Meguro et al. showed that HLA-A*26 is a risk marker in the Japanese population [16]. Using a custom platform (Immunochip) that includes 8572 single nucleotide polymorphisms (SNPs) in the HLA extended region, Hughes et al. demonstrated that the robust HLA-B*51 association in BD is due to a strong association signal of an SNP rs116799036 in two independent BD cohorts (Turkish and Italian) from two ancestry groups, while also identifying two additional independent genetic associations with genome-wide significance ($P < 5 \times 10^{-8}$) in the HLA region: rs12525170 and rs114854070 [21].

2.4. Genomic strategies for a complex genetic disease beyond the typical Mendelian inheritance

It was difficult to venture beyond the MHC in the past: the tools to do so were unavailable as linkage studies were only suitable for Mendelian disorders. Intriguing new techniques have surfaced in recent times accelerating the pace for gene discovery, especially for complex diseases such as BD.

2.4.1. Genome-wide association studies (GWAS)

Being a complex disease, BD does not follow the typical Mendelian law of inheritance, rather of a dichotomous nature conforming the “polygenic threshold model” where the phenotypic expression is resultant upon genetic variation at multiple rather than a single loci, with the majority of the cases occurring sporadically. This is the basis foundation for the development of GWAS, which was initially thought to be the “comprehensive” option designed to identify genetic variants associated with such complex disease [22].

The advent of GWAS earlier in this century has dramatically improved our ability to identify and map successfully susceptibility loci associated with complex diseases such as BD, usually as single nucleotide polymorphisms (SNPs). It seems that these common genetic variants contribute to polygenic disease manifestations where phenotypic variance depends on contributions from several genetic variance.

Fei et al. [23] performed the first GWAS study in BD in a relatively small Turkish population and identified several novel candidate genetic loci (KIAA1529, CPVL, LOC100129342, UBASH3B, and UBAC2) that are associated with increased susceptibility to BD. In the same year, Meguro et al. [16] found that the main susceptibility locus in BD Japanese population remains in the MHC itself, wherein reside two independent loci: HLA-B*51 and HLA-A*26. Two large GWAS conducted in Turkey and Japan followed in 2010: Remmers et al. confirmed the association of HLA-B*51, identified a second, independent association within the MHC Class I region and also found association at IL10 [17], while Mizuki et al. identified IL23R-IL12RB2 and IL10 as Behçet’s disease susceptibility loci [18].

In 2012, GWAS performed by Kirino et al. identified novel susceptibility loci at chemokine receptors CCR1-CCR3, signal transducer and activator of transcription 4 (STAT4), killer cell lectin-like receptor K1 subfamily K, member 1 (KLRK-1), killer cell lectin-like receptor
subfamily C, member 4 (KLRC4), and ERAP1 in a Turkish population [24], thus apparently supporting the emerging concept delineating common pathogenic mechanisms for BD, ankylosing spondylitis, and psoriasis. In the same year, Hou et al. also identified STAT4 as a novel susceptibility locus for BD in the Chinese population in their GWAS and functional studies [25].

One of the difficulties of using GWAS in cohorts of mixed ethnicity is due to the rarity and unequal distribution of disease prevalence among different ethnic background. This was overcome by novel statistical approaches, demonstrated by Kappen et al. [26] who confirmed the central role of the HLA region in the disease and validated the association of IL2A gene by meta-analysis with previous work.

2.4.2. Next-generation sequencing (NGS) and candidate gene analysis

Despite discoveries of many unimpeachable associations with GWAS, it became apparent that the approach alone could not explain the full range of heritability or genetic susceptibilities to complex diseases [27] and at best could only identify moderate proportions of genetic variants contributing to the disease heritability. Among the growing menu of techniques, targeted next-generation sequencing is the latest promising technology in search of rare genetic variants with fewer alleles (minor-allele frequency), which likely carry a greater larger impact on disease manifestations and with larger deleterious biological effects [28, 29]. Next-generation exome sequencing has the ability to generate millions of short reads of sequence, ranging from 50 to 500 bp, in parallel. It can be targeted in key regions of the genome-to make quick discoveries [29].

2.5. Non-MHC I susceptibility genes

Early data revealing the contributory influence of non-HLA susceptibility genes came from studies in the 1990s; however, some of the associations were weak or inconclusive. In 2009, Karasneh et al. published the first systematic whole genome linkage analysis from 28 Turkish BD families, which provided evidence for non-HLA susceptibility loci with the strongest evidence seen for 12p12–13 and 6p22–24 [30].

The revelation of GWAS unfolded associations with genome-wide significance ($P < 5 \times 10^{-8}$) in the interleukin 23 receptor (IL23R)-IL12RB2, IL12A, IL10, ubiquitin-associated domain containing 2 (UBAC2), STAT4, CCR1-CCR3, KLRC4, ERAP1, TNF alpha-induced protein 3 (TNFAIP3), and fucosyltransferase 2 (FUT2) loci [17, 18, 22–26]. Some of these results have been replicated since in case-control candidate gene studies from Iranian, Chinese and Spanish European populations [30–36].

Targeted next-generation sequencing revealed the additional involvement of rare non-synonymous variants in toll-like receptor 4 (TLR4), nucleotide-binding oligomerization domain-containing protein 2 (NOD2), and the Mediterranean Fever Gene (MEFV) [37]. Recently, Ognenovski et al. used whole exome sequencing in BD of European descent for the first time and identified and replicated two novel putative protein-damaging genetic variants within LIMK2 and NEIL1, which may influence cytoskeletal regulation and DNA repair [38].
The associations with ERAP1, IL23R, IL10, and MEFV variations suggest that BD may share susceptibility genes and inflammatory pathways with spondyloarthritis [39], while the TLR and FUT polymorphisms that affect response to invasive pathogens have led to an increase interest in responses to microbiomes [40].

3. Molecular markers of BD

3.1. The cytokines network of BD

The term “cytokine” was first introduced in 1974 by Cohen et al. [41] to describe a polypeptide mediator superfamily central in the immune system generation and regulation. An entwined network comprising of interleukins (ILs), interferons (IFNs), tumor necrosis factor (TNF), chemokines, and other mediators primed to regulate the immune system, however, due to several factors such as imbalance of its receptor expression and dysregulation of its functions, generates the pathologic systemic inflammatory and/or immune responses seen in various autoimmune and autoinflammatory disorders.

Pro-inflammatory and anti-inflammatory cytokines have been shown to be involved in patients with BD (as discussed in more detailed below). Several studies demonstrated elevated levels of cytokines in local lesions indicating its involvement in the disease local immune responses [42–46]. Evidence from the GWASs further implicated several cytokines underlying the pathogenesis of BD [17, 18, 22–26]. Moreover, the successful use of various anti-cytokine therapies in BD patients provides additional evidence that cytokines play a crucial role in its pathogenesis [47–64]. These overall observations highlight the fundamental role of cytokines as key players in the pathogenesis of BD.

There are several established cytokines that are known to be involved including IL1β, TNFα, IL6, IL10, and IL23. Various new promising candidate’s cytokines identified to be associated with BD include IL21, IL22, IL33, IL37, and several others, all of which be described as detailed below.

3.1.1. The main pro-inflammatory cytokines

Despite the pleiotropic nature of most cytokines, this group of cytokines primarily promotes inflammation. Several pro-inflammatory cytokines have been implicated in BD.

3.1.1.1. The interleukin-1 (IL1) family: IL1β, IL18, IL33

All cells of the innate immune system express and/or are affected by IL1 family members, which play a key role in the differentiation and function of polarized innate and adaptive lymphoid cells [65]. Among the 11 cytokines in the family, IL1β is the principal pro-inflammatory cytokine, leading to the expression of many chemokines and secondary mediators of inflammation and upregulating innate immunity in response to infectious agents [66]. The levels of IL1β have been shown to be elevated in several studies [45, 46, 49, 67, 68], including in synovial
fluids of BD patients [45, 46]. A proof-of-concept study by Güll et al. strongly implicated IL1β and BD with significant improvement seen especially in patients with uveitis treated with IL1β-regulating antibody [47]. Recently Tugal-Tutkun et al. demonstrated rapid control of uveitis in BD patients without the need for high-dose corticosteroid in a prospective, open-label, randomized phase II trial [48] supporting several other previous studies of the proven efficacy of IL1β to induce stable clinical remission among BD patients [49–52].

Other pro-inflammatory cytokines in the IL1 family implicated in BD especially in the last decade include IL18 [42, 69–71], an important component of polarized Th1 cell and natural killer (NK) cell responses and of the interplay between macrophages and NK cells [65]. Identified in 2005 by Schmitz et al, IL33 was noted to have the capability to activate NF-κB and MAP kinases, and induces the expression of IL-4, IL-5, and IL-13 in vivo, leading to severe pathological changes in mucosal organs [72]. In several of his studies, Hamzaoui et al. demonstrated higher levels of IL33 in sera of active BD patients compared to BD patients in remission [73–75], and this was supported by Kim et al. [76] who found elevated IL33 in BD patients with erythema nodosum (EN) and EN-like skin lesions. Surprisingly, Koca et al. found contrasting results of lower IL33 levels in active BD Turkish patients compared to the inactive patients and healthy controls (HC) but did find significantly higher levels of IL33 among BD patients with uveitis [77].

3.1.1.2. The tumor necrosis factor (TNF) superfamily

Among the 19 TNF superfamily cytokines that has so far been identified, the first member of the family, TNFα, which was the first to be discovered, is the most highly investigated. Levels have been shown to be elevated in studies from different populations [49, 69, 78–83]. Meta-analysis by Touma et al. [84] found TNF (-238A/G, -1031C/T, and -857T/C) polymorphisms are associated with susceptibility to BD, while an updated meta-analysis by Zhang et al. confirmed a significant association between the TNF−308A/G polymorphism and BD susceptibility [85]. Treatment has also been shown to be highly effective [53, 54].

There is not much information about the other members of the TNF superfamily and its association with BD, but despite the limitation, isolated studies have shown several other TNF family member to be associated with BD in different ethnic populations: Cantarini et al. found significantly higher serum soluble TNFR and soluble CD40L [86], Shaker et al. demonstrated higher levels of B cell activating factor (BAFF), A proliferation producing ligand (APRIL), and B cell maturation antigen (BCMA) in BD patients [87] and Düzgün et al. found elevated soluble CD30 levels in active BD patients compared to controls [88].

Besides TNFα, other members of the TNF superfamily may offer options as potential targets and therapeutic candidates in BD; however, more research is needed in this field to prove its efficacy and safety profile.

3.1.1.3. Interleukin-6 (IL6)

Several studies have shown higher levels of serum IL6 in active BD compared to inactive BD and HC [71, 89, 90], and interestingly in neuro-BD patients, IL6 was noted to be markedly
elevated in the cerebrospinal fluid (CSF), but not in the sera [91, 92]. Blockage of IL6 signaling with tocilizumab in BD patients despite looking promising in the treatment of neuro-BD [55–58] has revealed mixed results for non-neurological manifestations [58, 59, 93, 94] and is currently undergoing further evaluation in controlled clinical trials.

3.1.2. The main anti-inflammatory cytokine: type I interferon (IFNα)

Being the oldest cytokine discovered exactly 60 years ago, interferon-α (IFNα) has been the scope of investigation in many inflammatory diseases including BD. Despite initially thought to have mainly pro-inflammatory effects, it is becoming clearer that IFNα display a more complex function and its anti-inflammatory properties have led to its use as one of the treatment modalities in BD since the mid-1980s. Different studies by Hamzaoui, Kötter, and Pay et al. in their respective Tunisian, German and Turkish populations demonstrated higher levels of IFNα among BD patients [71, 95, 96]. In 2010, Liu et al. published their in vitro experiments demonstrating the ability of IFNα to inhibit IL17 expression and increase IL10 production by PBMCs and CD4+ T cells [97]. Successful uses of IFNα-2a and -2b as treatment modalities have been reported [60–62], and more recently, Lightman et al. reported the successful use of pegylated IFNα-2b in BD resulting significant reduction in corticosteroid use and improvement of quality of life [63]. The exact mechanism of IFNα, however, is still largely unknown.

3.1.3. Helper and regulatory T lymphocytes involvement in BD

There have been remarkable advances leading to our current understanding on the lineage commitment and plasticity of helper CD4+ T cells. The “naïve” CD4+ T cells in the presence of its associated cytokines differentiates into distinct T helper (Th) cells populations-Th1, Th2, Th17 or Th22: tailoring their responses to address specific threats accordingly. On the other hand, CD4+ CD25+ regulatory T cells (Tregs), derived from the thymus or differentiation from naïve T cells, downregulate Th responses and are critical for the preservation of immune tolerance and maintaining balance in the immune system.

3.1.3.1. Th1, Th17, and Th22 cell-associated cytokines

In the early 1980s, Ohno et al. demonstrated for the first time significantly higher levels of IFNγ in Japanese BD population [98]. Ahn et al. in their case series showed that the levels of IFNγ were elevated in aqueous humor and serum in BD patients with uveitis, which was then suppressed with combined low-dose cyclosporine/prednisone treatment [99]. Many other studies similarly found elevated serum IFNγ in active BD patients [44, 71, 83, 100–106], especially in BD patients with uveitis [44, 83, 102–105].

Other Th1 cell-associated cytokines associated with BD include IL2 and IL12. Despite conflicting results for IL2 levels in the ocular fluid of active BD patients with uveitis [106, 107], it was found to be significantly elevated in the serum of BD patients [108] and in active disease [109]. The alpha-chain of the IL2R that is shed from the surface of T cells by proteolytic enzymes to form the soluble sIL2R, which retains affinity to IL2, is also found to be significantly higher in active BD [46, 79, 110–113] and specifically in BD patients with uveitis [112, 113]. Serum IL12
levels were also found to be elevated in BD patients with active uveitis [114–116] and other active manifestations [42, 117, 118].

IL23 influences Th17 cell responses but shares a common p40 subunit with IL12 [119], and like IL12 has also been shown to be elevated in BD patients with active disease [101, 105, 120, 121]. Ustekinumab, a therapeutic agent, targeting both IL12 and IL23 cytokines has been shown to be therapeutic in BD [122], and subsequently a phase 2 open-label study to evaluate the proof-of-concept of ustekinumab in BD (STELABEC) has been recently registered in France. Both IL12 and IL23 stimulates nonreceptor Janus kinase 2 (JAK2) and tyrosine kinase 2 (TYK2) activity, leading to phosphorylation of STAT family members, with IL12 particularly activating STAT4 homodimers and IL23 predominantly activating STAT3 [119, 123–125]. Tulunay et al. demonstrated that the JAK1/STAT3 signaling pathway is activated in BD, and several other studies have shown similar findings [126]. Tulunay further suggested that more direct therapies aimed at JAK/STAT-associated cytokines such as ustekinumab (anti-IL12/23) and recently the approved tofacitinib that specifically inhibits JAK1/3, may be new therapeutic options for BD [126].

Th17 and Th22 are the “newer” helper-T cell subsets that secrete pro-inflammatory cytokines IL17 and IL22, respectively. Both are also implicated in the pathogenesis of BD, and their levels were markedly increased in BD patients [97, 105, 127–133] including active uveitis [107, 127, 128]. Chi et al. in their study demonstrated that production of IL17 was successfully inhibited by treatment with cyclosporine [127]. Another interesting finding in one of the studies above is increased levels of CCL20, an essential potent chemoattractant for the recruitment of Th17 lymphocytes [129]. Sugita et al. established Th22-type T cell clones from ocular samples taken from BD patients with active uveitis, which produced large amounts of IL22 and TNFα [106]. Sugita also demonstrated that IL22 in the presence of retinal antigens were able to produce high levels of IL22 in mice with experimental autoimmune uveitis [106]. From the therapeutic point of view, Liu et al. demonstrated significantly higher levels of IL17 in active BD patients, and stimulation with IFNα significantly decreases this IL17 production [97].

IL21 is one of the more recently identified type I cytokines that has been shown to tilt the balance between Th17 cells and regulatory T cells (Tregs) [134]. Geri et al. found markedly increased IL21 in active BD patients’ sera and in the CSF of active neuro-BD patients. He further demonstrated increased Th17 and Th1 differentiation and decreased frequency of Tregs cells after stimulation of CD4+ T cells with IL21. Conversely, IL21 blockade with an IL21R-Fc restored the Th17 and Tregs homeostasis in BD patients, which might represent a potential target for novel therapy [135].

3.1.3.2. Th2 cell-associated cytokines and Tregs

The studies on Th2 cell-associated cytokines and their contribution in the pathogenesis of BD have been rather conflicting. Several studies found lower or no significant differences of the related cytokines in BD patients compared to HC [45, 106, 109, 119, 136, 137]. However, studies by Hamzaoui et al. found increased serum levels of Th2 (IL4 and IL13) cytokines, and Takeuchi et al. found elevated IL4 and IL10 in BD patients [71, 83], while studies from Raziuddin and Aridogan et al. demonstrated high levels of IL4, IL10 and IL13 in active BD
patients [138, 139]. Liu et al. and Guenane et al. in separate studies found significantly higher levels of IL10 in uveitis patients with BD compared to HC and idiopathic uveitis, respectively [97, 114]. Dalghous and his colleagues observed the presence of IL4 cytokines in oral lesions only from BD patients compared to RAS patients [140], while Ben Ahmed et al. found elevated levels of IL10 comparable to the increased IFNγ levels in active lesions of BD patients [141].

The possible role of Tregs in the pathogenesis of BD has gained considerable interest in recent times. Hamzaoui and Gündüz both demonstrated decreased Tregs level in clinically active BD patients [43, 142], Nanke et al. suggested that a decreased percentage of Tregs in peripheral blood of BD patients might be a predictive marker of ocular attack [143], while Sugita et al. demonstrated that Tregs level increased significantly with infliximab therapy but not with colchicine or cyclosporine in BD patients with uveitis [144]. Another subset of Tregs expresses high levels of CD52 glycoprotein [145], and together with other CD52-bearing cells (T cells, B cells, monocytes, macrophages, NK cells, dendritic cells, and granulocytes) are molecular targets of CAMPATH-1. A humanized antibody of IgG1 CAMPATH-1H/alemtuzumab has been successfully used in BD [146, 147] including in an open trial involving 18 BD patients with complete or partial remission achieved in 84% of patients [146].

3.1.3.3. Gammadelta (γδ) T cells

Gammadelta (γδ) T cells are innate-like lymphocytes that express a unique T cell receptor (TCR) γ and δ chain. Despite constituting only a small proportion (1–5%) of lymphocytes, they are more widespread within epithelial-rich tissues, such as the skin, intestine and reproductive tract, where they can comprise up to 50% of T cells [148]. They are a unique population of T cells that have features of both innate and adaptive immunity and express characteristics of conventional T cells, natural killer cells, and myeloid antigen presenting cells [149].

The relationship between γδ T cells and BD has been noted in several studies since the early 1990s. Increased γδ T cells levels were seen in BD patients compared to HC [99, 150–155] and in active BD compared to inactive BD [99, 151, 155, 156]; however conversely, several studies did not show any significant difference with HC [157–159]. Hasan et al. postulated that these discrepancies might be due to the activation status of the disease, as a reflection of local tissue inflammation compared to peripheral blood γδ T cells and such variation might be dependent on several other factors including disease severity, usage of medications such as immunomodulatory agents, and perhaps other variables, namely, age, gender, ethnicity, and/or environmental factors [149]. Their roles in the pathogenesis and potentially as therapeutic targets remain to be elucidated.

3.1.4. Other cytokines

IL37 is part of the IL1 family (discovered in 2009 and formerly identified as IL1F7) but has emerged as an inhibitor of innate immunity [160]. It has been shown to be significantly lower in BD patients compared to HC, with pronounced inhibition in active patients, and was associated with increased production of IL1β, IL6, and TNFα in LPS-stimulated PBMCs [161, 162]. Furthermore, in vitro experiments revealed that supplementing IL37 in BD patients significantly
suppresses these three pro-inflammatory cytokines [163]. There have also been suggestions of associations between BD and other cytokines such as IL15 and IL27; however, the data are still inadequate and sparse [44, 164–166].

3.1.5. Chemokines

The attraction of leukocytes to tissues is essential for inflammation and is controlled by chemokines, which are chemotactic cytokines [167]. Saruhan-Direskeneli et al. observed significantly higher α-chemokine CXCL10/IP10 CSF levels in neuro-BD patients compared to patients with non-inflammatory neurological disease (NIN) and multiple sclerosis, whereas CXCL8/IL8 was increased in neuro-BD compared to NIN [168]. El-Asrar et al. found higher levels of CXCL9/MIG, CXCL10/IP10 and CXCL11 in BD patients’ serum with uveitis [169], while its receptors CXCR3 expression were observed by Dalghous et al. to be higher in oral lesions biopsied form BD patients [140]. Recently, Ambrose et al. demonstrated significantly higher production of CXCL10/IP10 in blood monocytes of BD patients stimulated with IFNγ compared to HC, rheumatoid arthritis, and systemic lupus erythematosus controls [170]. There is even more robust evidence for CXCL8/IL8, a potent neutrophil chemoattractant, being implicated in BD pathogenesis, with some of the authors proposing that it could be a marker for vascular involvement and a more reliable marker for disease activity than the C-reactive protein or erythrocyte sedimentation rate [171–174].

In regard to β-chemokines, Ozer et al. found significantly elevated levels of MCP1/CCL2, MIP1α/CCL3, and RANTES/CCL5 in active BD serum than in HC [175]. Similarly, Kökçam and Kim and their respective colleagues in two separate studies demonstrated high levels of MIP1α/CCL3 [176, 177] while Kaburaki et al. and Do et al. found higher levels of MCP1/CCL2 in BD patients compared to HC [178, 179]. In CSF of neuro-BD patients, Saruhan-Direskeneli et al. and Miyagishi et al. both demonstrated significantly higher levels of MIP1α/CCL3 compared to NIN [168, 180].

The emerging evidence of a complex cytokine and chemokine network interplay involved in the pathogenesis of BD, the identification of candidate gene including cytokine polymorphisms and the proven potency of anti-cytokines treatment shed more light on the fundamental role of cytokines and chemokines in BD. Perhaps cytokine and/or chemokine gene therapy, which has been used in cancer therapy, though not extremely impressive but nonetheless promising, may offer a novel yet powerful approach in the treatment of BD in the future.

3.2. The innate immune network

3.2.1. Neutrophil hyperfunction and endothelial cell activation

Becatti et al. demonstrated significant enhancement in leukocyte reactive oxygen species (ROS) production particularly by neutrophils in BD patients and only neutrophil-derived ROS (but not lymphocyte- or monocyte-derived ROS) showed a significant correlation with fibrinogen carbonyl content, highlighting neutrophil activation as the promoter of fibrinogen oxidation and thrombus formation in BD [181] supporting similar finding in several previous
studies [182, 183]. Increasing evidence supports a role for neutrophil/lymphocyte ratio (NLR) as a cheap and simple disease activity marker in BD. NLR has been proposed as a surrogate marker for endothelial dysfunction and inflammation, and several authors have proposed the use of NLR as part of evaluation for disease activity in BD [184–187].

3.2.2. Innate lymphoid cells (ILCs)

Groundbreaking studies over recent years have formally identified innate lymphoid cells (ILCs) as a distinct arm of the innate immune system, comprising of the classic cytotoxic natural killer (NK) cells, lymphoid tissue inducer cells, and non-cytotoxic ILC populations [188]. Besides controlling tissue homeostasis, it has the ability to promote inflammation at mucosal and surface barriers [188]. Yamaguchi et al. reported that NK cells are actively involved in the induction and maintenance of disease remission in BD patients, through NK2 polarization [189] while Takeno et al. from their study concluded that abnormal killer inhibitory receptor (KIR) expression of NK cells may be associated with the development of BD [190]. Furthermore, ILCs have been recently shown as an important source of cytokines production [188, 191, 192]. The recent discovery of this latest group of diverse immune cells with its many emerging diverse roles in autoimmunity and inflammation may redefine or rather perhaps reinforce our understanding of the pathogenesis of BD in the future.

3.3. Inflammasomes

The inflammasome has been shown to be a key regulator of IL1β and IL18 via direct activation of caspase-1 [173–194]. Liang et al. demonstrated production of IL-1β was significantly decreased in ocular BD patients after the Nod-like receptor protein 3 (NLRP3) inflammasome was downregulated [195] while Kim et al. showed that the basal and LPS-induced expressions of NLRP3 inflammasome components were significantly increased at both mRNA and protein levels in BD patients [196]. Conversely, Türe-Özdemir and his team were not able to find any difference in DC and neutrophils of BD patients compared to HC after stimulating caspase-1 activation [197].

3.4. Autoantibodies

There have been several studies implicating certain autoantibodies in BD, but all are neither non-specific nor sensitive and are of limited clinical significance. Among them the most described were anti-\textit{Saccharomyces cerevisiae} antibodies (ASCA) [198–201] and anti-endothelial cell antibodies (AECA) [202–205]. ASCAs were linked more to gastrointestinal manifestations in BD [198–202] and their healthy relatives [201]. AECA has been implicated with vascular [202, 204] and intestinal [205] involvement in BD and several studies demonstrated that alpha-enolase is the target antigen of AECA in BD patients [203–205]. These positive results, however, were not replicated in certain other ethnic populations [206–208].

3.5. The host-microbe interaction in BD

The role of microbial triggers in BD has long been postulated since the disease was first described. Microbial heat shock proteins (HSPs) show significant homology with human
mitochondrial HSP and molecular mimicry is suggested as the mechanism of pathology exacerbating BD when patients were exposed to these foreign antigens. While both streptococci and herpes simplex virus have garnered the most particular interest among researchers [209–214], evidence of exposure to other microbes such as staphylococci and mycobacteria has also been reported [215, 216]. Nonetheless, conflicting reports have been published and a specific pathogen has yet to be identified.

3.5.1. Microbiome: the rapidly re-emerging hypothesis in inflammatory disorders

Microbiome is a term first described by Lederberg in 2001 describing the microbial ecosystem [217], but it was Metchnikoff more than a centenary ago who hypothesized that the microbiota might influence the balance between pro-inflammatory and regulatory host responses and that alterations in the composition of the microbiota (a process that is known as dysbiosis) could jeopardize host immune responses [218]. Recent evidence indicates the possible contribution of the intestinal microbiota to immunological diseases outside the gut [218, 219].

The advent of the 16S ribosomal RNA (16S rRNA) sequencing technology over the past quarter century has identified a comprehensive human microbiota far more comprehensive than we ever imagined. Despite the emergence of newer application such as metagenomics [220], due to the nature of the rRNA genes that are highly conserved and evolutionarily stable but differ in their hypervariable regions enables identification of species, and owing to a confluence of methodological advancements, 16S rRNA has re-emerged as a stand-alone molecular tool [221].

BD patients seem to exhibit specific microbiome signature. Consolandi et al. compared fecal microbiota of BD patients and HC and found significantly depleted Roseburia and Subdoligranulum genera and butyrate production in BD patients [222] while Shimizu et al. also demonstrated gut dysbiosis in BD patients with significantly increased genera Bifidobacterium and Eggerthella and decreased genera Megamonas and Prevotella compared to HC [223].

Several authors also identified salivary dysbiosis among BD patients. Seoudi et al. found in BD patients an increased colonization of *Rothia dentocariosa* at non-ulcer oral sites, while the ulcer sites were highly colonized with *Strep salivarius* compared to recurrent aphthous stomatitis (RAS) patients, and with *Strep sanguinis* compared to HC, who were more highly colonized with *Neisseria* and *Veillonella* [224]. Coit et al. found significantly less diverse microbial structure in stimulated saliva samples in BD patients both with and without immunosuppressant [225].

3.6. Other possible markers

There are other possible molecular markers that have been or are still under investigations. Fecal calprotectin (FC) were demonstrated to be significantly elevated in intestinal BD in several studies [226–228], and interestingly Özşeker et al. demonstrated high fecal FC levels in asymptomatic but endoscopically proven BD patients with intestinal involvement [226]. Vascular endothelial growth factor (VEGF) levels have been observed in BD patients, particularly in active BD [229–232]. Several studies provided evidence for the increased levels of markers for endothelial activation or dysfunction such as vascular and intercellular adhesion molecules VCAM1, ICAM1, Selectins, and YKL40 in BD [233–238]. Various authors explored the association between certain genetic mutations and thrombosis in BD; however, results
have been inconclusive [239–244], and current data indicate that the pathogenesis of thrombosis in BD is not due to a coagulation abnormality [245].

The immunomodulatory role of vitamin D is of increasing interest, and several in vitro studies have demonstrated downregulation of inflammation by vitamin D [246–249]. Moreover, hypovitaminosis D had been implicated in various inflammatory disorders including BD [250–255]. Further investigations from different ethnic populations may provide further insights to this potentially clinically relevant knowledge of vitamin D as a potential suppressor of inflammatory response in BD.

4. Gaps and future directions

Efforts to develop biomarkers in BD have been confounded by substantial impediments and challenges and the greatest is probably due to the rare nature of the disease, while others include the complex role of the susceptibility genes and related cytokines, chemokines and other signaling molecules, variability of duration and severity of the disease, as well as variations between different geographical areas and the limited number of patient samples.

Standardization and quality assurance are significant hurdles and collaboration between laboratories at different centers to standardize protocols and assays is essential. There are clear similarities and differences across different ethnic groups phenotypically and at a genetic or molecular level. So far, clinical data trials support the critical role of innate cytokines TNF, IL1, and IL6 in the development of inflammatory episodes of BD, and targeting T cells or B cells may provide favorable results [256]. In this era of personalized and precision medicine, collaborative efforts nationally or internationally are needed to assemble adequately powered cohorts to perform further population- or regional-based molecular and genetic studies.

One possible way to move forward is broadening the classification criteria to combine objective clinical indicators and biomarkers, but despite the emergence of these candidate markers, there is still a lack of sufficient widespread evidence to support their implementation and incorporation into the contemporary classification criteria. In the meantime, it must be noted that BD remains fundamentally a clinical diagnosis.

Many questions remain a conundrum including (1) which patients will develop a more severe form of disease, (2) who will be resistant to certain therapy, (3) which patients with recurrent aphthous ulcers will progress to develop BD, and (4) who will benefit the most from a particular therapy. The search remains a highly scientific priority, but until we find the biomarkers, likelihood is, many of these questions will remain uncertain.

5. Conclusions

It has been a long, challenging journey in search of biomarkers in Behçet’s disease. There are clear genetic and molecular similarities and variability between different ethnic populations.
Collaborative efforts nationally or internationally are needed to assemble sufficiently powered sample size to perform further population- or regional-based molecular and/or genetic studies in the search for the elusive “magic bullet” as the signature marker that will revolutionize the field of BD.

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