PD-1 on CD8$^+$T cells are low in Behçet's disease and is not normalised with type-I interferon

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Abstract

Objective

Programmed cell death-1 (PD-1) protein on CD8$^+$T cells may affect disease development as a regulatory molecule through various mechanisms. On the other hand, type-I interferons (IFN-I) are used in autoimmune and autoinflammatory diseases to induce regulatory mechanisms. This study aims to investigate PD-1 molecules on CD8$^+$T cells in Behçet's disease (BD) and their response to IFN-I.

Methods

BD (n=37) patients were compared with age- and sex-matched healthy (n=24) and diseased controls (n=29). Peripheral mononuclear cells (PBMCs) were stained for PD-1, CD45RA and CCR7 molecules on CD3$^+$CD8$^+$T cells. Cells from patients and controls were further activated in vitro by anti-CD3 and interferon (IFN-α/2α1b) and the induction of PD-1, TIM-3 and TIGIT on CD8$^+$T cells was analysed.

Results

PD-1 on CD8$^+$T cells with potentially regulatory functions were significantly lower in BD patients, regardless of disease activity, than in controls. CD3 stimulation of PBMCs induced PD-1 expression in all groups, whereas the addition of interferon to stimulated CD8$^+$T cells produced differential responses: the increase of PD-1 on CD8$^+$T cells in controls was not observed in BD patients. The responsive increase of similar co-inhibitory molecules, TIM-3 and TIGIT, in controls was not seen in BD patients either.

Conclusion

The decreased PD-1 expression on CD8$^+$T cells, along with the decreased PD-1, TIM-3 and TIGIT responses of CD8$^+$T cells upon IFN-I stimulation, demonstrated an altered immune response in BD.

Key words

CD8$^+$T cells, programmed cell death protein-1 (PD-1), type-I interferon, Behçet's disease, altered immune response

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Introduction

Behçet's disease (BD) is a chronic, multisystemic inflammatory disorder characterised by recurrent mucocutaneous ulcerations. BD is frequently observed in Eastern Mediterranean and East Asian countries, Turkey being one of the countries with the highest prevalence of BD (1). The disease usually affects young adults and can cause significant morbidity. The relatively strong genetic association with HLA-B*51 detected in BD implicates a possible role of CD8+T cell-mediated mechanisms in the disease development (2, 3).

BD's pathogenesis is attributed to a complex genetic background that induces a pro-inflammatory state, increased activation of the innate immune system and the perpetuation of inflammation through adaptive immune response (4). In BD, both $CD4^+$ and $CD8^+$T cells producing T helper 1 (Th1) related pro-inflammatory cytokines, including interleukin-2 (IL-2), IL-12, and interferon-γ (IFN-γ), are increased in the peripheral blood and inflammatory tissues (5). Th17 and T cytotoxic IL-17 (Tc17) cells are also elevated in patients with BD (6,7). These alterations are accompanied by increased levels of other pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and IL-6 in BD (8). Genome-wide association studies have also identified IL-10 as a key susceptibility gene in BD, with variants linked to reduced IL-10 production potentially impairing anti-inflammatory responses and contributing to disease pathogenesis (9). Moreover, a gain-of-function polymorphism in *IFNGR1* gene has been associated with BD, leading to enhanced *IFNGR1* expression in monocytes and amplified IFN-γ signalling, which may further promote immune activation and inflammatory responses in genetically predisposed individuals (10).

Accessory molecules with co-stimulatory and co-inhibitory functions expressed on the surface of T cells are essential regulators of immune responses to both pathogens and autoantigens, acting independently of the specific antigen/MHC complex and T cell receptor (TCR) (11). Co- inhibitory pathways, such as the PD-1:PD-L1 axis, are acting in the balance between activating and regulatory pathways in the peripheral tolerance mechanisms (12, 13). A weak engagement between PD-1 expressed on T cells and its ligands PD-L1 or PD-L2 on antigen-presenting cells (APCs) appears to contribute to an exaggerated and heightened T cell response (14). Functional studies show that repression of PD-1 expression by binding of T-bet to the *PDCD1* promoter enhances Th1 and $CD8^+$ cytotoxic T lymphocyte (CTLs) responses through increased IFN-γ and IL-17 production, underscoring the key regulatory role of PD-1 in immune control (15,16). In HLA class I-associated diseases such as ankylosing spondylitis (AS) and psoriasis, PD-1 expression on active $CD8^+$T lymphocytes was reduced (17,18). In a previous study, when PD-1 as well as CTLA-4 were evaluated in BD, not PD-1, but CTLA-4 levels on T cell subsets were lower than controls after stimulation. Moreover, reduced PD-L1 mRNA levels in blood mononuclear cells as well as diminished PD-L1 presence in skin lesions were observed in active BD patients. These changes in co-inhibitory molecule patterns might indicate their possible role in the development of BD (19).

Type-I interferon (IFN-I) family is consisted of evolutionarily conserved innate cytokines with pleiotropic effects (20). Besides their well-known antiviral activity, they also control cell growth, apoptosis and innate immunity (21). Possible mechanisms have been proposed to explain these diverse and paradoxical effects of IFN-Is. IFN-I may be effective by modulating the balance between pro- and anti-inflammatory mediators secreted by APCs and T cells (22). Co-regulatory molecules, such as PD-1/TIM-3/LAG3 are induced on human T cells with IFN-I exposure (23). IFN-I's are used as biologic therapies for multiple sclerosis (MS). The proportion of $PD\text{-}1^+CD8^+$T cells was significantly increased in the peripheral blood of MS patients under IFN-β treatment, and the transcription of multiple co-inhibitory genes, including *PDCD1*, *CTLA4* and *TIGIT* were upregulated. The immune response suppressing properties of the PD-1 on $CD8^+$T cells

were also emphasized in this study (24). The use of IFN-α has also been shown to be effective in the treatment of BD, especially in uveitis refractory to conventional immunosuppressive (IS) treatments (25-27).

In this study, we evaluated CD8+T cells expressing PD-1 molecule for their possible involvement in BD, in diseased- and healthy control groups. Moreover, we performed *in vitro* stimulation experiments with IFN-I to examine its role on inhibitory receptors (PD-1, TIGIT and TIM-3) of CD8+T cells and cytokine (IL-10, TNF-α and IL-6) responses in BD patients and controls.

Table I. Demographic features of the study groups.

	BD n=37	HCs n=24	AChR-MG n=29
Age (mean ±SD)	44.7 ± 11.8	45.3 ± 13.5	48.3 ± 17.7
Women (%)	20 (54)	13 (54.1)	16 (55.2)
Active disease (%)	18 (49)	-	-
Oral ulcer (%)	18 (49)		
Pathergy (%)	18 (49)		
Mucocutaneus lesion (%)	10 (27)		
Vascular involvement (%)	3 (8)		
Ocular involvement (%)	4 (11)		
Articular involvement (%)	9 (24)		
Immunosuppressive therapy (%)	9 (24)		11 (38)
Methylprednisolone (%)	3 (8)		-
Prednisolone (%)	-		10 (34)
Azathioprine (%)	3 (8)		4 (14)
Sulfasalazine (%)	2 (5)		-
Adalimumab (%)	2 (5)		-

BD: Behçet's disease; HCs: healthy controls; AChR-MG: acetylcholine receptor antibody positive myasthenia gravis; n: numbers in study groups; SD: standard deviation.

Material and methods

Study groups

Blood samples from 18 active-BD (a-BD), 19 inactive-BD (ia-BD) patients and 24 healthy controls (HCs) were included in this study (Table I). All patients fulfilled the International Study Group criteria for the classification of BD (28). Patients with oral aphthosis and additional BD manifestations such as genital ulcers, skin lesions, uveitis or arthritis at the time of blood collection were accepted as clinically active and patients without these findings during this study as inactive patients. Totally, 9 (24%) patients were on treatment with IS (ISP-BD). The remaining 28 (76%) patients were not receiving IS treatment (ISN-BD) (Table I).

As the diseased-control group, 29 patients with the clinical and electrophysiological findings, and the presence of acetylcholine receptor antibodies were included as the myasthenia gravis (AChR-MG) group. Healthy donors without known diseases were included in the HCs group. Age and gender distributions of the patients and HCs were balanced to be not different from each other.

This study was approved by the Clinical Trials Ethical Committee of Istanbul Faculty of Medicine (24.01.2024/1517). Peripheral blood samples were obtained from the donors after acquiring written informed consent.

Phenotypic analyses of CD8+T cell population

Peripheral blood mononuclear cells (PBMCs) were separated with gradient centrifugation procedure on a lymphocyte separation medium (Capricorn). Freshly isolated cells (1×10^6) were re-suspended in 100 mL staining buffer with and were stained with the following fluorophore-conjugated antibodies: anti-CD8 (FITC, Biolegend), anti-CD3 (APC, Biolegend), anti-PD-1 (PE, Biolegend), anti-CD45RA (PE-Cy5, Biolegend), and anti-CCR7 (PE-Cy7, Biolegend) antibodies by incubating at +4°C for overnight (29). After staining, the cells were washed with 1 mL of PBS at 300 x g and then analysed by flow cytometer (Attune Acoustic Focusing Cytometer, Applied Biosystems, United States of America). Data were analysed using Flow-Jo software version 10. Cells were initially gated on singlets to exclude doublets, followed by lymphocyte gating based on forward and side scatter axes, and subsequent identification of target cell populations (Supplementary Fig. S1). Geometric mean fluorescence intensity (MFI) was also measured in the target populations for the respective markers.

In vitro IFN-I stimulation

Separated PBMCs from BD (n=15; a-BD n=2; ia-BD n=13; ISP-BD n=8; ISN-BD n=7), AChR-MG patients (n=17) and HCs (n=15) were cultured in RPMI medium containing 1% L-glutamine, 1% penicillin/streptomycin, and 10% foetal bovine serum in 96-well U-bottom plates as $3x10^5$ cells per well. The concentration and incubation time for anti-CD3 (clone: HIT3a, Biogems) and IFN-α/2α1b (11020-IF-010-R&D) were determined with optimisation experiments. The doses of IFN-α/2α1b and anti-CD3 antibody were tested by comparing concentrations between 3-300 ng/mL and 0-10 μg/mL CD3 in pre-study experiments, and 30 ng/mL and 0.03 μg/mL were chosen. Similarly, culture durations of 1, 2 and 4 days were evaluated, and 4 days was chosen for the analyses. The wells were coated with anti-CD3 for 4 hours before the addition of the cells. Then the cells were incubated *in vitro* at 37°C with 5% CO_2 with or without IFN-α/2α1b (30 ng/ml) stimulation in addition to CD3 for 4 days. At the end of the culture period, the cells were stained with anti-CD8 (FITC, Biolegend), anti-PD-1 (PE, Biolegend), anti-TIGIT (PE-Cy7, Biolegend), anti-TIM-3 (APC, Biolegend) antibodies for 30 minutes at room temperature. To evaluate the effect of IFN-I on the molecules in question, the differences between the stimulation with anti-CD3 alone and the stimulation with anti-CD3 combined with IFN-I were calculated, as the induced effect and expressed as Δ values.

Cytokine measurement in the culture supernatants

IL-10, TNF-α and IL-6 levels were measured in cell culture supernatants

(either without stimulation, or stimulated with anti-CD3, with or without IFN-I) by multi-analyte bead-based flow cytometry (LEGENDplex™, BioLegend). According to the protocol, samples and standards were incubated with fluorescently labelled beads. After the incubation, samples and standards were washed to remove unbound substances and then transferred to the flow cytometer for analysis. The detection limits in the cell culture medium for IL-10, TNF-α, and IL-6 were 0.8±0.7 pg/mL, 1.1±0.9 pg/mL, and 1.97±2.6 pg/mL, respectively. The obtained data were evaluated quantitatively using analysis software (LEGENDplex™, BioLegend).

Statistical analysis

Non-parametric tests are applied for the comparisons of disease groups and controls (Kruskal-Wallis). Based on the differences in selected parameters, the Mann-Whitney U-test was used to evaluate the statistical differences between groups. The obtained parametric data were expressed as mean ± standard deviation values, and non-parametric data were expressed as median. Statistical significance was defined as $p<0.05$. The statistical analysis of the data was performed using GraphPad Prism 10.4.1. Graphs of the data were also generated using same software and presented with median values and interquartile ranges.

Results

Decreased PD-1 on CD8+T cells in BD

The proportion of cells carrying PD-1 molecule on CD8+T cells was lower in BD (14.9%) compared to HCs (24.2%, $p=0.0027$) (Fig. 1). When BD patients were divided according to disease activity, PD-1 on CD8+T cells was significantly decreased both in ia-BD (17.9%, $p=0.02$) and a-BD (12.6%, $p=0.005$) patients compared to HCs (24.2%) with no significant difference between patient groups (Fig. 1). Likewise, stratification based on IS treatment also demonstrated significantly decreased frequency of PD-1 on CD8+T cell in both ISP-BD (14.9%, $p=0.008$) and ISN-BD (15.1%, $p=0.018$) patients compared with HCs (24.2%). ISP-BD and ISN-BD patients revealed similar distributions (not shown). MFI levels of PD-1 on CD8+T cells did also not reveal any difference between groups (Data not shown). When compared with the diseased-control group, frequency of PD-1 on CD8+T cells was also significantly decreased in BD compared to AChR-MG (19.3%, $p=0.02$, Fig. 1).

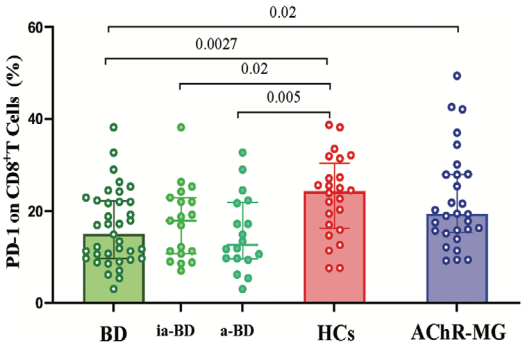


Fig. 1. Proportions (%) of PD-1+ cells in CD8+T cells among PBMCs in BD (n=37; a-BD: n=18, ia-BD: n=19), HCs (n=24) and AChR-MG (n=29) groups. Disease groups as well as disease subgroups (a-BD and ia-BD) are compared with HCs. Subgroups of BD have also been compared with each other (a-BD *vs*. ia-BD).
a-BD: active Behçet disease; ia-BD: inactive Behçet disease; HCs: healthy controls; AChR-MG: acetylcholine receptor antibody positive myasthenia gravis.

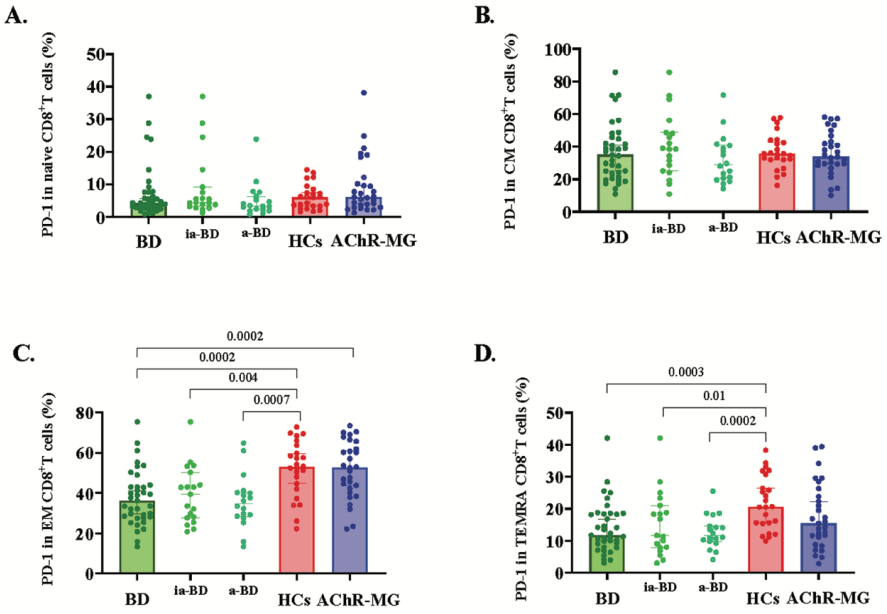


Fig. 2. Proportions (%) of PD-1+ cells among CD8+T cell subsets. **A.** naive (CCR7+ CD45RA+), **B.** central memory (CM) (CCR7+ CD45RA−), **C.** effector memory (EM) (CCR7− CD45RA−) and **D.** TEMRA (CCR7− CD45RA+) cells in CD8+T cell measured in BD (n=37; a-BD: n=18, ia-BD: n=19), HCs (n=24) and AChR-MG (n=29) groups. Disease groups as well as disease subgroups (a-BD and ia-BD) are compared with HCs. Subgroups of BD have also been compared with each other (a-BD *vs*. ia-BD).
a-BD: active Behçet disease; ia-BD: inactive Behçet disease; HCs: healthy controls; AChR-MG: acetylcholine receptor antibody positive myasthenia gravis.)

Cell subsets of PD-1+CD8+T cells

Both gene expression profiling and phenotypic analyses revealed that PD-1 is predominantly expressed on effector memory (EM) CD8+T cells, rather than on terminally differentiated effector memory RA (TEMRA) T cells (30). To determine the cell subgroups, PD-1+ cells among CD8+ T cells were classified as naive (CCR7+ CD45RA+), central memory (CM: CCR7+ CD45RA−), effector memory (EM: CCR7− CD45RA−), and TEMRA (CCR7− CD45RA+) populations. When the distribution of PD-1 on these CD8+T cell

subgroups is evaluated, proportions of PD-1+ cells both among EM (36.2 *vs.* 52.9%) and TEMRA CD8+T cells were lower in BD (11.7% *vs.* 20.6%) compared to HCs (p=0.0002, p=0.0003) (Fig. 2). This decrease was observed both in ia-BD (39.4%, p=0004) (Fig. 2C) and a-BD (34.8%, p=0.0007) (Fig. 2) patients compared to HCs (52.9%) in EM as well as ia-BD (11.7%, p=0,01) and a-BD (11.7%, p=0.0002) in TEMRA subgroups. No significant difference was detected between a-BD and ia-BD patients in these cell subgroups. Percentages of PD-1+ cells among naive and CM CD8+T cell were similar in all groups (Fig. 2).

EM cell group among CD8+T cells was more prominent in all BD, HCs and AChR-MG groups in this study (Suppl. Fig. S2). The distribution was also similar in both a-BD and ia-BD groups.

Differential IFN-I responses of BD patients in vitro

Several attempts have been published to clarify the possible immunomodulatory effects of IFN-I in BD (25,31). Understanding the role of IFN-I on T cell responses could help to utilise this cytokine better for its beneficial effects on immune regulation of BD. Since the co-inhibitory receptors such as PD-1, TIGIT, and TIM-3 may modulate CD8+T cell activity, assessing their expression in response to IFN-I stimulation can reveal disease-specific alterations of the immune mechanisms. To evaluate the effect of IFN-I on CD8+T cells, CD3 stimulation with or without IFN-I was applied to PBMCs and the changes of surface markers were measured. To demonstrate the IFN-I effect specifically, the differences of the induced molecules on the CD8+T cells were determined by directly comparing the group stimulated with both anti-CD3 and IFN-I to the group stimulated with anti-CD3 alone (Fig. 3A). The resulting differences in responses were compared between the groups. CD3 stimulation alone induced the expression of PD-1, as well as other co-inhibitory molecules such as TIGIT, TIM-3 on CD8+ T cells across all groups (Fig. 3). However, the addition of IFN-I to the CD3-stimulated PBMCs elicited

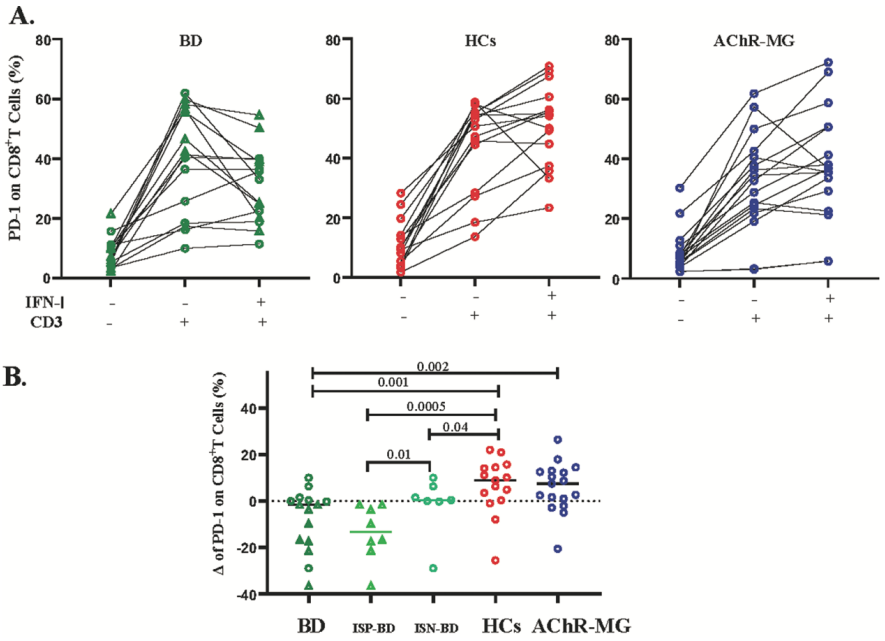


Fig. 3. PD-1 induction in PBMCs with IFN-I. **A.** Proportions (%) of PD-1+ CD8+T cells in three cell culture conditions (no stimulation, only CD3 and CD3+IFN-I stimulation) were measured in BD (n=15; ISP-BD: n=8, ISN-BD: n=7), HCs (n=15) and AChR-MG (n=17) groups. **B.** Changes (Δ) of % PD-1 on CD8+T cells with or without IFN-I in the same groups. Δ values were calculated as the difference between CD3+IFN-I stimulated minus CD3 stimulated alone. Disease groups as well as disease subgroups (ISN-BD and ISP-BD) are compared with HCs. IS subgroups of BD have also been compared with each other (ISN-BD *vs.* ISP-BD).
ISP-BD: BD treated with IS; ISN-BD: IS-negative BD; HCs: healthy controls; AChR-MG: acetylcholine receptor antibody positive myasthenia gravis.

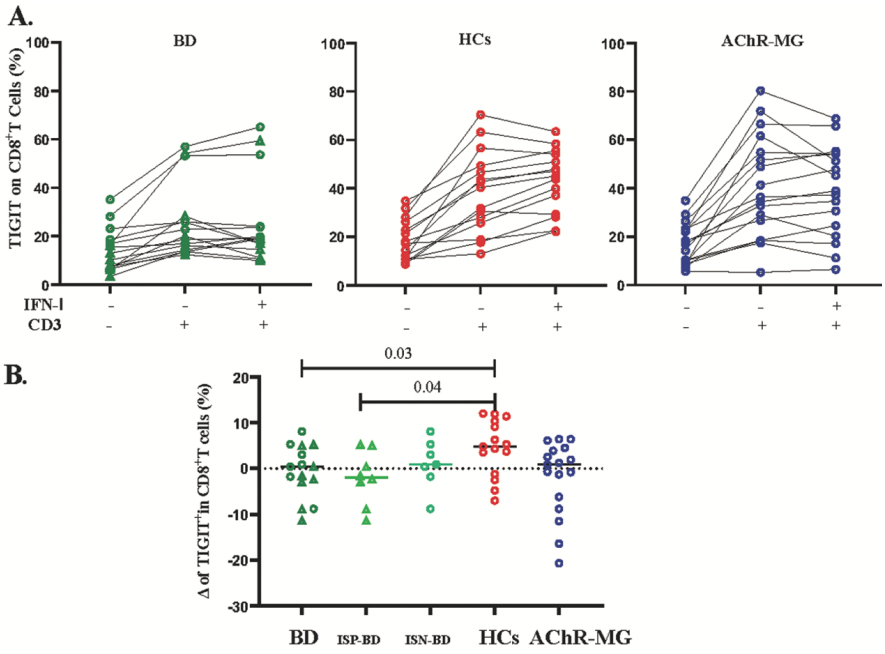


Fig. 4. TIGIT induction in PBMCs with IFN-I. **A.** Proportions (%) of TIGIT on CD8+T cells in three cell culture conditions (no stimulation, only CD3 and CD3+IFN-I stimulation) were measured in BD (=15; ISP-BD: n=8, ISN-BD: n=7), HCs (n=15) and AChR-MG (n=17) groups. **B.** Changes (Δ) of % TIGIT on CD8+T cells with or without IFN-I in the same groups. Δ values were calculated as the difference between CD3+IFN-I stimulated minus CD3 stimulated alone. Disease groups as well as disease subgroups (ISN-BD and ISP-BD) are compared with HCs. IS subgroups of BD have also been compared with each other (ISN-BD *vs.* ISP-BD).
ISP-BD: BD treated with IS; ISN-BD: IS-negative BD; HCs: healthy control; AChR-MG: acetylcholine receptor antibody positive myasthenia gravis.

differential responses; while an increase of PD-1 on CD8+T cells was notably absent in patients with BD, an upregulation was observed in both HCs and diseased-control (AChR-MG) groups. PD-1 expression on CD8+T cells decreased by 1.6% in the BD group, whereas it increased by 8.9% in HCs group and 7.4% in patients with AChR-MG. Comparisons between groups revealed that PD-1 expression on CD8+T cells of BD patients was reduced, whereas increases were observed in both the HCs (p=0.001) (Fig. 3B) and AChR-MG (p=0.002) (Fig. 3B) groups. Stratification of BD patients demonstrated 13.2% decrease of PD-1 expression on CD8+T cells in the ISP-BD and a slight increase of 0.4% in the ISN-BD (p=0.01) (Fig. 3B), emphasising the effect of immunosuppression on PD-1 induction on CD8+T cells. The changes observed in both ISP-BD and ISN-BD were significantly lower than those in HCs (p=0.0005 and p=0.04, respectively) (Fig. 3B).

On the other hand, TIGIT expression on CD8+T cells showed a minimal increase of 0.4% in BD, compared to 4.8% in HCs and 0.9% in AChR-MG groups (Fig. 4A). Statistical analysis indicated that the change in BD was significantly lower than in HCs (p=0.03) (Fig. 4B), while no significant difference was detected between BD and AChR-MG (Fig. 4B). Subgroup analysis of BD patients revealed 2% reduction in TIGIT expression on CD8+T cells in the ISP-BD, whereas a modest increase of 0.9% was observed in the ISN-BD group. Comparative statistical analysis demonstrated that the change in TIGIT expression in ISP-BD patients was significantly lower than that observed in HCs (p=0.04) (Fig. 4B). In contrast, no statistically significant difference was identified between the ISP-BD and ISN-BD subgroups (Fig. 4B).

Similarly, TIM-3 expression on CD8+T cells decreased by 0.5% in BD, whereas it increased by 5% in HCs and 0.9% in AChR-MG (Fig. 5A). The differences in changes of TIM-3 expression between BD and HCs was significant (p=0.04) (Fig. 5B), whereas no significant difference was found between BD and AChR-MG. Evaluation of BD subgroups showed 2.9% decrease in TIM-3 expression on CD8+T cells among ISP-BD patients, while a slight increase of 1.4% was noted in the ISN-BD group. The magnitude of change in TIM-3 expression was significantly lower in the ISP-BD subgroup compared with HCs (p=0.02) (Fig. 5B). No significant difference was observed between ISP-BD and ISN-BD patients (Fig. 5B).

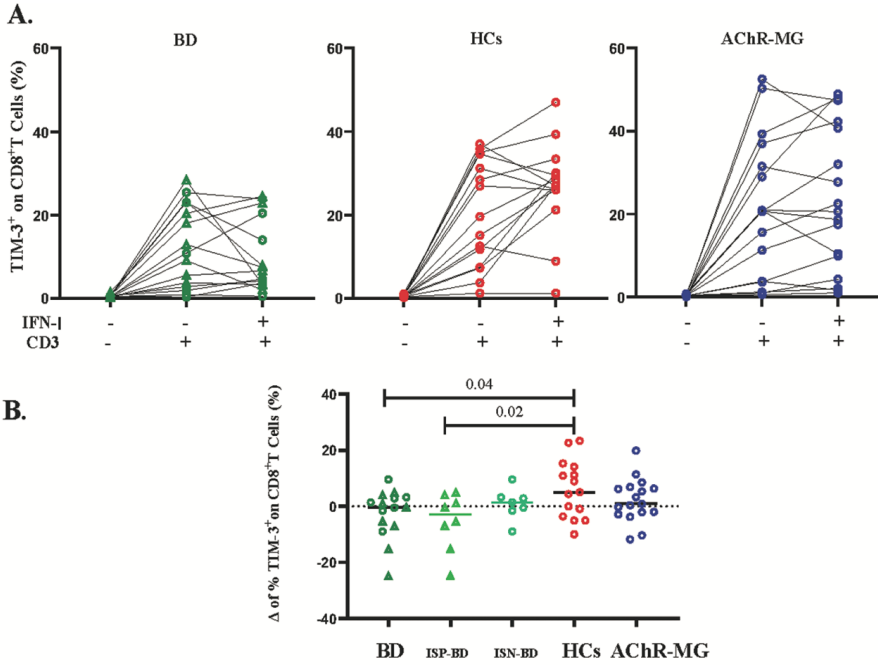


Fig. 5. TIM-3 induction in PBMCs with IFN-I.
A. Proportions (%) of TIM-3 on CD8+T cells in three cell culture conditions (no stimulation, only CD3 and CD3+IFN-I stimulation) were measured in BD (n=15; ISP-BD: n=8, ISN-BD: n=7), HCs (n=15) and AChR-MG (n=17) groups. **B.** Changes (Δ) of % TIM-3 on CD8+T cells with or without IFN-I in the same groups. Δ values were calculated as the difference between CD3+IFN-I stimulated minus CD3 stimulated alone. Disease groups as well as disease subgroups (ISN-BD and ISP-BD) are compared with HCs. IS subgroups of BD have also been compared with each other (ISN-BD *vs*. ISP-BD).
ISP-BD: BD treated with IS; ISN-BD: IS-negative BD; HCs: healthy control; AChR-MG: acetylcholine receptor antibody positive myasthenia gravis.

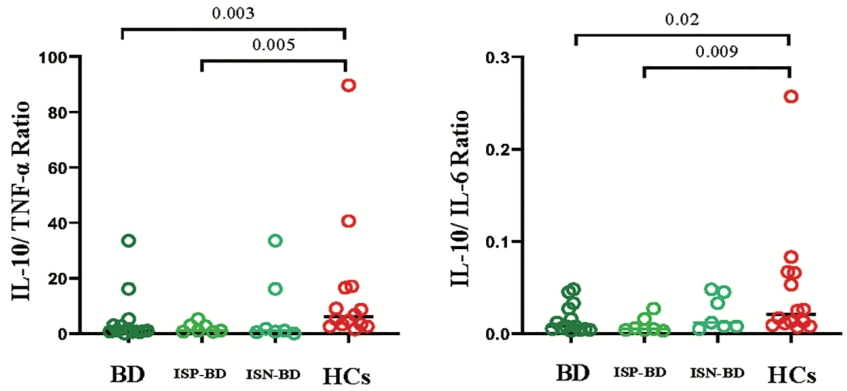


Fig. 6. IL10/TNF-α and IL10/IL-6 ratios in supernatants of none-stimulated PBMCs in BD (n=14; ISP-BD: n=7, ISN-BD: n=7) patients and HCs (n=14). BD as well as disease subgroups (ISN-BD and ISP-BD) are compared with HCs. IS subgroups of BD have also been compared with each other (ISN-BD *vs*. ISP-BD).
ISP-BD: BD patients treated with IS; ISN-BD: IS-negative BD patients; HCs: healthy controls.

The effects of IFN-I on cytokines in BD

IFN-I regulates immune responses by modulating cytokine production, including increasing IL-10 and inhibiting pro-inflammatory cytokines like

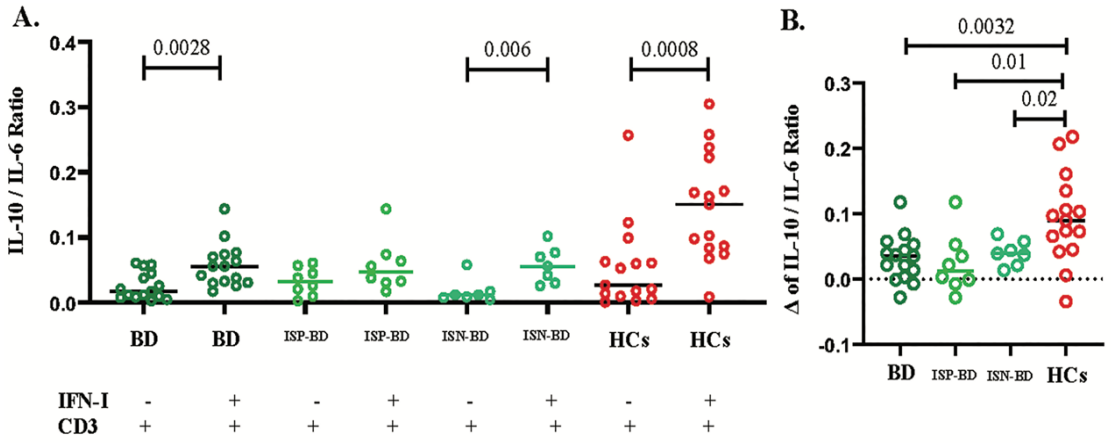


Fig. 7. Effects of IFN-I on IL10/IL-6 ratio
A. IL10/IL-6 ratios with or without IFN-I in BD (n=15; ISP-BD: n=8, ISN-BD: n=7) patients and HCs (n=15). **B.** Changes (Δ) of IL10/IL-6 ratios with or without IFN-I in the same groups calculated as the difference between CD3+IFN-I stimulated minus CD3 stimulated alone. BD as well as disease subgroups (ISN-BD and ISP-BD) are compared with HCs. IS subgroups of BD have also been compared with each other (ISN-BD *vs*. ISP-BD).
ISP-BD: BD patients treated with IS; ISN-BD: IS-negative BD patients; HCs: healthy controls.

TNF-α and IL-6 (22). To assess whether this regulatory function is altered in BD, we examined basal cytokine levels and anti-/pro-inflammatory cytokine ratios with the effect of IFN-I stimulation. Basal cytokine (IL-10, IL-6, TNF-α) levels in unstimulated cell culture supernatants revealed that TNF-α was significantly higher in patients with BD compared to HCs (30.7 *vs*. 4.8 pg/mL, p=0.04) (Suppl. Fig S3). IL-6 (2.5 *vs*. 1.8 ng/mL) and IL-10 (36.5 *vs*. 59.5 pg/mL) levels were not different in BD compared to HCs (Suppl. Fig. S3). Subgroup analysis based on IS treatment status demonstrated TNF-α levels of 15.2 pg/mL in the ISP-BD and 39.8 pg/mL in the ISN-BD. Notably, TNF-α levels were significantly elevated in the ISN-BD group compared with HCs (p=0.01) (Suppl. Fig. S3). IL-6 concentrations were 1.7 and 2.8 ng/mL in the ISP-BD and ISN-BD groups, respectively, showing no significant differences relative to HC. Similarly, IL-10 levels measured 28.1 pg/mL in ISP-BD and 72.8 pg/mL in ISN-BD, without statistically significant differences compared with HCs (Suppl. Fig. S3).

When basal cytokine production levels were also evaluated as a ratio of "anti-inflammatory/inflammatory" cytokines, both IL-10/TNF-α (BD = 1.1, *vs*. HCs=5.9; p=0.003) (Fig. 6) and IL-10/IL-6 ratios (BD=0.008 *vs*. HCs=0.021; p=0.02) (Fig. 6) were significantly lower in BD compared to HCs. Comparisons according the IS usage revealed that these differences were mainly observed in the ISP group for both ratios (IL-10/TNF-α ratio in ISP-BD=1.1; p=0.005 and IL-10/IL-6 ratio in ISP-BD=0.005; p=0.009) (Fig. 6).

When the effect of IFN-I stimulation on cytokine production is compared between the groups, the IL-10/IL-6 ratio increased in BD (0.02 *vs*. 0.06, p=0.0028) (Fig. 7) as well as ISN-BD (0.012 *vs*. 0.056, p=0.006) (Fig. 7) and HCs (0.03 *vs*. 0.15, p=0.0008) (Fig. 7). However, the increase induced by IFN-I was lower in BD, as well as ISP-BD and ISN-BD groups compared to HCs (0.04, 0.012, and 0.03 *vs*. 0.09, p=0.0032; p=0.01; p=0.02) (Fig. 7).

Discussion

BD is an inflammatory disease of unknown aetiopathogenesis. In this study, CD8+T cells of BD patients were compared for their co-inhibitory molecules with HCs and with a typical autoimmune disease. Frequency of PD-1+ CD8+T cells of BD patients was lower, regardless of disease activity and IS usage. This finding of BD differentiated it from HCs as well as AChR-MG patients. In addition, BD patients exhibited a reduced capacity to induce PD-1 expression on CD8+T cells in response to IFN-I stimulation, together with a relatively diminished anti-inflammatory cytokine profile. The reduced presence of PD-1 on CD8+T cells may point to an altered immune response that normally would constrain excessive immune activation observed in BD.

PD-1 is a member of the CD28 family of immune modulators. It is upregulated on CD8+ and CD4+T cells upon activation. PD-1 binds to its ligands PD-L1 or PD-L2. Ligation of PD-1 results in dephosphorylation of signalling molecules downstream of the TCR, thus dampening T cell sensitivity to antigenic stimulation (30). It not only inhibits T cell receptor signalling, but also suppresses the expression of the co-stimulatory molecule CD28 (31). In chronic infections and malignancies, exhausted CD8+T cells express PD-1 prominently (32). In recent years, antibodies against PD-1 and its ligands have become important therapeutic modalities in various malignancies. However, the role of PD-1 in chronically activated inflammatory responses and its therapeutic potential are less clear. Experimental studies indicate that the absence of PD-1 expression on T cells is associated with increased production of IFN-γ and IL-17, highlighting an essential regulatory role of PD-1 in controlling Th1 and Th17 immune responses (15). The finding of decreased PD-1 on CD8+T cells in this study may also contribute to augmented Th1 and Th17 responses in BD. In a study by Onofrio *et al*. the frequency of CD8+T cells expressing inhibitory receptors such as PD-1, CD160, TIM-

3 and BTLA was decreased in PBMCs of untreated rheumatoid arthritis (RA) patients. RA patients who responded to treatment showed an increased frequency of inhibitory receptor-expressing CD8+T cells, which correlated with decreased inflammatory cytokine production (33). These receptors on CD8+T cells may play a role in restraining excessive inflammation in chronic inflammatory conditions and prevent the immunopathological consequences of sustained inflammatory activity, which may be dysfunctional in BD. The lower capacity to regulate by CD8+T cells may well be considered as complementary to excessive inflammation in BD.

In a recent study, decreased frequency of PD-1 on CD8+T cells of MS patients were recovered with IFN-β treatment *in vivo*. Moreover, IFN-β induced PD-1 expression on CD8+T cells when PBMCs were stimulated *in vitro* with anti-CD3/CD28. In the same study, circulating CD8+T cells positive for PD-1 expressed higher IL-10 in individuals treated with IFN-β. Furthermore, transcriptomic profiling of isolated CD8+T cells expressing PD-1+ identified a gene module enriched in co-inhibitory receptors. Functionally, these cells demonstrated upregulation of PD-1 expression and enhanced IL-10 secretion (24). Similar with these observations, a reduction in the frequency of PD-1+CD8+T cells in patients with BD was found in this present study, regardless of disease activity. However, unlike the published report, the IFN-I response *in vitro* did not result in an upregulation of PD-1 expression on CD8+T cells in BD patients, reflecting the inefficiency of this stimulation of CD8+T cells in this disease. High proportion of BD patients under IS-treatment in the study group may also be confounding for this result by effecting IFN responsiveness in patients. However, ISN-patients have revealed lower responses to IFN-I.

IFN-I has been reported to be an effective treatment for severe ocular involvement in BD, especially with significant visual loss or retinal disease, often achieving sustained remission even post-discontinuation (27). The proportions of CD8+ γδ T and NK cells were reduced following IFN-I (α2a) treatment, supporting the possible immunomodulatory role of this therapy (34). Recently, reduced IL-10 production of Treg cells was restored in response to IFN-I in Behçet's uveitis, as Th17-mediated inflammatory responses were suppressed (25). In another study, the effects of IFN-α were examined in BD by the cytokine production and an increase in IL-10/IL-6 ratio in both healthy individuals and patients with BD was observed. Notably, IFN-α stimulation enhanced IL-10 production in memory CD4+T cells, indicating a direct immunomodulatory effect (22). Although a similar increase of IL-10/IL-6 ratio was observed in this current study with the application of IFN-I to CD3-activated PBMCs, the increase remained at a lower level in CD8+ T cells of BD patients compared with HCs.

In contrast to the antibody-mediated framework of AChR-MG, the pathogenesis of BD appears centred on CD8+T cell dysregulation. The fact that PD-1+ on CD8+T cells remain unchanged in MG, yet are altered in BD, highlights a fundamental difference, positioning BD as a distinct clinical entity outside the realm of typical autoimmune diseases.

This study has several limitations when interpreting the findings. Firstly, a subset of BD patients was receiving heterogeneous IS treatment. Although subgroup analyses were performed, the relatively small sample size and treatment variability may have influenced immune responses, particularly CD8+T cell responsiveness to IFN-I and thus represent a potential confounding factor. Secondly, *in vitro* stimulation experiments were conducted using total PBMCs rather than purified CD8+T cells. As PBMCs include multiple immune cell populations such as monocytes and dendritic cells that are highly responsive to type-I interferons, the observed effects may be partially mediated through indirect mechanisms, rather than reflecting purely intrinsic CD8+T cell responses. While the use of PBMCs provide endogenous co-stimulation, this cannot fully replace controlled CD28-mediated signalling. Thirdly, the study does not include functional assays to directly assess the regulatory capacity or cytokine producing properties of PD-1+CD8+T cells. Cytokine measurements were also performed in cultures of bulk PBMCs, precluding identification of the specific cellular sources. In addition, no mechanistic analyses of IFN-I signalling pathways (such as IFN receptor expression or downstream activation) were performed. Therefore, the observations of PD-1+ cells among CD8+T cells, receptor expression, and cytokine profiles remain associative rather than causal.

In conclusion, this study demonstrates a reduced frequency of PD-1 on CD8+T cells in BD compared with healthy and diseased controls, a difference that appears to persist irrespective of IS treatment and disease activity status. In addition, BD patients exhibit a diminished capacity to upregulate these co-inhibitory receptors on CD8+T cells following *in vitro* IFN-I stimulation, together with altered cytokine profiles in PBMCs supernatants, including relatively reduced anti-inflammatory cytokine balance. Altered CD8+T cell responses to IFN-I in BD may reflect a disease associated disturbance in immune regulation. This finding may indicate a disturbance in mechanisms that normally constrain excessive immune activation, potentially contributing to ongoing immune dysregulation in BD. Further studies are needed to clarify the biological significance of these observations and to better define the potential role of PD-1 on CD8+T cells as targets for immunomodulatory strategies.

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