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Expression of Bcl-2 in inflammatory sites from patients with active Behçet's disease

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Behçet's disease (BD) is a current systemic vasculitis of unknown aetiology. Eyes, skin, joints, the oral cavity, genital system, blood vessels, central nervous system and lung are usually involved. Defective regulation of programmed cell death (apoptosis) may play a role in the development of (BD), and the proto-oncogene Bcl-2 is involved in the control of apoptosis in immunocompetent cells. We therefore wished to investigate the expression of Bcl-2 in the peripheral lymphocytes and in two inflammatory sites of patients with active BD: bronchoalveolar lavage (BAL) and cerebrospinal fluid (CSF) lymphocytes. Levels of Bcl-2 expression in the lymphocytes of patients with BD and, for comparison, in the lymphocytes of healthy controls and non-inflammatory neurological diseases (NIND), were studied by two-colour cyto-fluorography and RNA analysis. In BD patients, a significant proportion of T cells expressed increased amounts of Bcl-2 protein, both in peripheral blood and in inflammatory sites. Mononuclear cells of patients with BD showed increased amount of Bcl-2 messenger RNA. The in vitro incubation of T lymphocytes with IL-10, significantly increased the Bcl-2 expression, specifically in T lymphocytes from inflammatory sites. In active BD, stimulation of HSV-1 T lymphocytes slightly increased Bcl-2 expression, not significantly different from unstimulated HSV-1 T cells. The occurrence of circulating T lymphocytes with abnormally high Bcl-2 expression in peripheral circulation and in inflammatory sites may be explained in part by the increased *in vivo* activation levels, and by aetiopathological agent(s): our findings seem to indicate an important role in the chronic inflammation in BD.

Key words: Beh et's disease, Inflammatory sites, Bcl-2, Vasculitis

Key Indexing Term: Bcl-2 in Beh et's disease inflammatory sites

Introduction

Beh et's disease (BD) is a polysymptomatic, recurrent systemic vasculitis with a chronic course and an unknown aetiology.[1] Several organs or systems can be involved and the resultant symptomatology and severity depend on the system affected.[1] The main clinical features are: oral aphthous, genital ulcers, ocular lesions, skin lesions, arthritis, central nervous system manifestations, vasculitis[1] and other less frequent findings, such as pulmonary manifestations.[2] Increasing evidence indicates that immunological processes and a variety of cytokines may contribute to the pathophysiological process in BD.[3] We have recently reported that patients with BD have a dysregulation in programmed cell death.[4,5]

Programmed cell death (apoptosis) is important in down-modulation of immune responses after activation and proliferation of inflammatory cells. It has been suggested that dysregulated apoptosis of lymphocytes may be linked to the development of autoimmune diseases: Sjögren's syndrome,[6] systemic lupus erythematosus (SLE)[7] and rheumatoid arthritis (RA).[8]

Bcl-2 is a proto-oncogene that regulates apoptosis of several cell types. Bcl-2 plays a role in the maintenance of the immune system, since inactivation of Bcl-2 in mice leads to the disappearance of the lymphoid system.[9] Lymphocyte activation in active BD was increased, in particular in the inflammatory sites,[1] so it is possible that the expression of apoptosis-regulating proteins, like Bcl-2, is

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dysregulated in these lymphocytes. On this basis we studied Bcl-2 expression in inflammatory sites: bronchoalveolar lavage and cerebrospinal fluid.

Patients and Methods

Patients

BD was determined according to the criteria of the International Group for Beh et's disease.[10] BD patients were receiving treatment including steroids and colchicine. Characteristics of BD patients are shown in Table 1. Different patient groups were used for flow cytometry and nucleic acid hybridization studies.

Bronchoalveolar lavage was isolated from 10 patients with active BD with lung involvement (chronic cough associated to interstitial shadows on chest X-ray or pulmonary aneurysms).[11] Cerebrospinal fluid lymphocytes were isolated from five patients with neurological manifestations.[4]

As controls we studied peripheral blood mononuclear cells (PBMC) and cerebrospinal fluid (CSF) lymphocytes from 10 patients with non-inflammatory neurological diseases (NIND) and the bronchoalveolar lavage cells (BAL) from 12 persons undergoing routine investigations for bronchial carcinoma and whose lung X-rays were normal.

Reagents

Anti-human bcl-2 (isotype IgG) monoclonal antibodies (mAb) were purchased from Dako. Anti-CD3 mAb OKT3 was from Becton-Dickinson (Mountain View, CA, USA). The negative control antibody 11G2 was mouse IgG_1 mAb to chicken Bu-1b alloantigen.[12] Fluorescein isothiocyanate (FITC)-conjugated anti-human CD3, and mouse IgG were purchased from Becton-Dickinson. Unspecific mouse IgG (I-5381) was purchased from Sigma (St Louis, MO, USA) and streptavidin–phycoerythrin (PE) (7100–09) from Southern Biotechnology Associates (Birmingham, AL, USA). Purified human recombinant IL-2, IL-10, and neutralizing anti-IL-10 mAb were from Genzyme Diagnotics.

Table 1. Clinical features of patients with Behçet's disease

Patient	Age/sex	Disease activity	Symptoms at the time of sampling
1	40/M	Active	Or ulcer – Gen ulcer – Uveitis – Pulm.
2	45/M	Remission	No symptom
3	36/F	Active	Or ulcer – Gen ulcer – Uveitis – EN
4	37/F	Active	Or ulcer – Gen ulcer – Uveitis
5	28/M	Active	Or ulcer – Gen ulcer
6	57/M	Active	Or ulcer – Gen ulcer – Pulm
7	26/M	Active	Or ulcer – Gen ulcer – Uveitis – Pulm
8	45/M	Active	Or ulcer – Gen ulcer – Arthritis
9	47/M	Active	Or ulcer – Gen ulcer – Uveitis – CNS – Arthritis
10	63/M	Partial remission	Or ulcer
11	29/F	Remission	No symptom
12	32/M	Active	Or ulcer – Gen ulcer – Uveitis – Arthritis
13	35/M	Active	Or ulcer – Gen ulcer – CNS
14	56/M	Active	Or ulcer – Gen ulcer – Uveitis
15	58/M	Active	Or ulcer – Gen ulcer – Arthritis
16	33/M	Active	Or ulcer – Gen ulcer – Uveitis
17	34/M	Active	Or ulcer – Gen ulcer – Uveitis – Pulm
18	21/M	Active	Or ulcer – Gen ulcer – EN
19	18/M	Active	Or ulcer – Gen ulcer – Arthritis
20	40/M	Active	Or ulcer – Gen ulcer – Uveitis
21	49/F	Active	Or ulcer – Gen ulcer
22	42/M	Active	Or ulcer – Gen ulcer – Uveitis – EN
23	35/M	Partial remission	Or ulcer – Gen ulcer – Arthritis
24	27/M	Active	Or ulcer – Gen ulcer – Uveitis – Arthritis
25	29/M	Active	Or ulcer – Gen ulcer – Uveitis – Pulm
26	28/M	Active	Or ulcer – Gen ulcer – Uveitis – CNS
27	17/M	Active	Gen ulcer – Uveitis – Arthritis
28	36/F	Active	Or ulcer – Gen ulcer – EN
29	34/M	Active	Or ulcer – Gen ulcer – Uveitis – Pulm
30	52/M	Active	Or ulcer – Gen ulcer – Uveitis – Pulm
31	46/M	Active	Or ulcer – Gen ulcer – Uveitis – CNS
32	43/M	Active	Or ulcer – Gen ulcer – Uveitis – CNS
33	27/M	Active	Or ulcer – Gen ulcer – Uveitis – Pulm
34	36/M	Active	Or ulcer – Gen ulcer – Uveitis – Arthritis – Pulm
35	38/M	Active	Or ulcer – Uveitis – Arthritis – Pulm

Or ulcer, oral aphthous ulceration; Gen ulcer, genital ulceration; EN, erythema nodosum; CNS, central nervous system involvement; Pulm, pulmonary manifestation, artery aneurysm.

Cell preparations and cultures

Peripheral blood, cerebrospinal and bronchoalveolar lymphocytes were isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density-gradient centrifugation and washed twice with PBS at +4°C. Lymphocytes were cultured at 10^6 cells/ml per well in Iscove's modified Dulbecco's medium (Gibco) supplemented with 10% fetal calf serum and 50 µg/ml gentamycin. Cells were cultured in 24-well plates (Costar, Cambridge, MA) at 37°C in a 5% CO_2 incubator (Jouan). IL-2 (50 U/ml), IL-10 (100 U/ml) or anti-IL-10 mAbs (2 µg/ml) were added at the onset of the cultures. After a culture period of 7 days, cells were harvested and washed twice with 1% bovine serum albumin (BSA). Cells were stained and analysed using flow cytometry as described below.

Generation of HSV-1 stimulated T cells

Four millilitres of cells at 2×10^6 cells/ml were cultured in tissue culture flasks, in the presence of UV-inactivated HSV-1, in an incubator with 5% CO_2 and 95% air for 6 days, as previously reported.[13] On the day of testing, the cells were washed and the number of viable cells was determined by trypan blue exclusion. The cells were resuspended in RPMI 1640 medium supplemented with 25 mM Hepes buffer and 10% heat-inactivated FCS.

Immunofluorescence staining and flow cytometric analysis

A total of 10^6 cells were incubated for 30 min with anti-Bcl-2 monoclonal antibody (mAbs), followed by incubations with biotinylated anti-mouse IgG and streptavidin–PE in 1% BSA, 0.3% saponin, followed by two washes in 1% BSA, 0.1% saponin. For two-colour analysis, cells were then incubated with unspecific mouse IgG for 15 min and stained with FITC-conjugated mAbs for 30 min without saponin. The negative controls included the replacement of anti-Bcl-2 mAb with a negative control antibody (11G2) of the same isotype and the staining of cells without saponin permeabilization. Cells with light scatter characteristics of lymphocytes were gated and analysed using LYSYS II software from a FACScan (Becton-Dickinson) flow cytometer.

RNA isolation and nucleic acid hybridization

Total RNA was extracted from PBMC and inflammatory sites cells (BAL and CSF mononuclear cells) as described by Chirgwin *et al.*[14] Briefly, cells were homogenized in 4 M guanidium isothiocyanate and RNAs were purified with ultracentrifugation through caesium chloride cushions. To verify the integrity of RNA preparations, a small amount of RNA from each sample was electrophoresed in 1% agarose/formaldehyde and stained with ethidium bromide. Five micrograms of total RNA were dot blotted onto a nylon membrane (Hybond-N+; Amersham International). The filter was hybridized and washed under stringent conditions according to the manufacturer's protocol. Human Bcl-2 cDNA was excised from pN2-H-BCL2 vector.[15] It recognizes the appropriate size 8.5-kb RNA. The glyceraldehyde 3-phosphate dehydrogenase cDNA clone pHGAPDH was used as a reference probe.[16] The probes were labelled with [^{32}P]dATP (PB.10204; Amersham), using a random priming DNA labelling kit (70200; USB, Cleveland, OH, USA), to obtain specific activities of $1 - 2 \times 10^9$ dpm/µg. After labelling, probes were run through Sephadex G50 spin columns. The autoradiograms from the sequential hybridization of a dot blot filter with Bcl-2 and GAPDH probes were analysed with a scanning densitometer, and the densitometric readings were normalized to the amount of RNA in each sample.

Results

Bcl-2 immunofluorescence

To quantitate the expression of Bcl-2 protein in the T lymphocytes from patients with active BD, we studied

Table 2. Bcl-2 expression as mean fluorescence intensity (MFI) in peripheral blood T lymphocytes (PBMC), bronchoalveolar lavage T lymphocytes (BAL) and cerebrospinal fluid lymphocytes (CSF) from controls and active BD with pulmonary manifestations and neurological involvement

Sample	No. of patients	Mean fluorescence intensity ± S.D.	Bcl-2-positive lymphocytes ± SD (%)		
			Medium	+IL-2	+IL-10
Control PBL	25	56 ± 17	72 ± 6	84 ± 22*	96 ± 12*
Active BD-PBL	35	92 ± 8†	87 ± 8	90 ± 15	87 ± 8
Control BAL	12	28 ± 14	52 ± 6	80 ± 3*	74 ± 10*
Active BD-BAL	10	46 ± 10†	78 ± 3	74 ± 10	90 ± 7*
NIND-CSF	10	32 ± 14	53 ± 7	72 ± 6*	70 ± 3*
Active BD-CSF	05	63 ± 6†	74 ± 5*	94 ± 4*	96 ± 15*

Percentage (%) of Bcl-2 expression was tested after IL-2 and IL-10 stimulation.
*P<0.001, percent of Bcl-2 expression in active BD after IL-2 and IL-10 incubation compared to the control incubated with medium alone.
†P<0.001, MFI expression in active BD compared to the respective control sites.

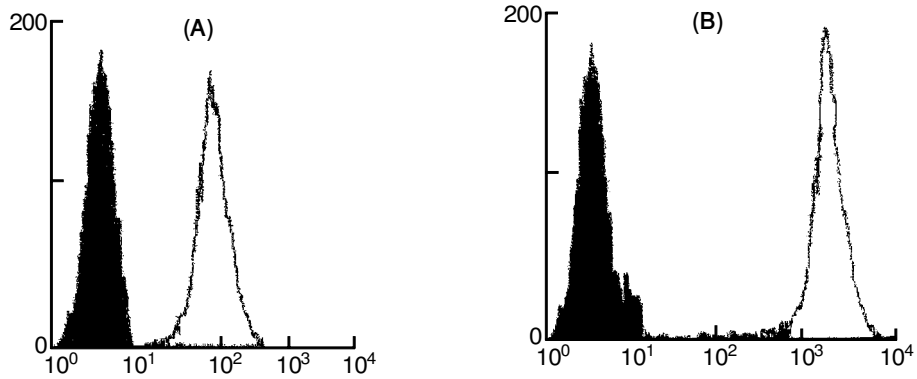

FIG. 1. Immunofluorescence histogram showing Bcl-2 expression lymphocytes of bronchoalveolar lavage from controls (A), and patients with active Behçet's disease (BD) with pulmonary manifestations (B). The black histograms indicate fluorescence intensity of control antibodies. Data are representative of 10 normal controls and eight active BD. Bcl-2 was expressed in CD3+ BAL-T lymphocytes at a higher intensity.

Bcl-2 expression in 35 peripheral blood mononuclear cells (PBMC), 10 BAL and five CSF from patients with active BD, using flow cytometry. As shown in Table 2, Bcl-2 expression in PBL from active BD was high compared to healthy controls. In every lymphocyte sample, from CSF and BAL of active BD >80% were Bcl-2 positive when compared to CSF from NIND and BAL from healthy controls. In addition, the MFI of Bcl-2-positive cells in the BAL and CSF from active BD were clearly increased when compared to BAL of healthy controls and CSF from patients with NIND (Figs 1 and 2).

Effect of IL-2 and IL-10 on Bcl-2 expression

The Bcl-2 expression analysed by flow cytometry was studied in cultured T cells incubated with IL-2 and IL-10. The addition of IL-2 did not change significantly the percentage of Bcl-2 expression on peripheral T cells and T lymphocytes from inflammatory sites: bronchoalveolar lavage and cerebrospinal fluid lymphocytes. However, IL-10 increased significantly the Bcl-2 expression (Table 2), specifically in T lymphocytes from inflammatory sites. The increased Bcl-2 expression was abrogated when anti-IL10 was added.

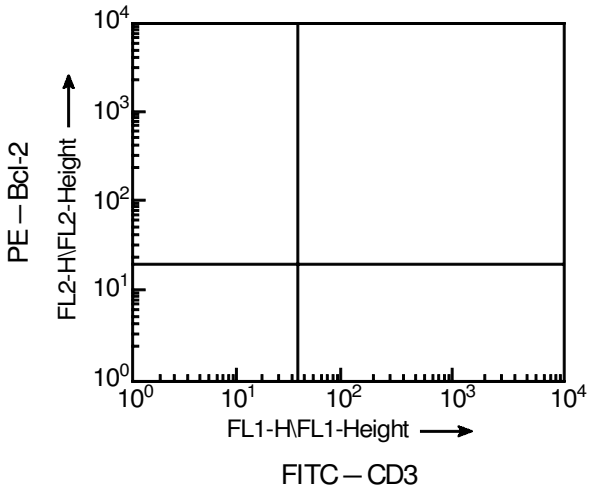

FIG. 2. Results of double staining experiments on the lymphocytes of representative bronchoalveolar lavage from patients with active Behçet's disease with pulmonary manifestations. Freshly isolated lymphocytes were stained with FITC-conjugated anti-human CD3 monoclonal antibody. Following this step cells were permeabilized and then stained with biotinylated polyclonal rabbit IgG anti-human Bcl-2, as described in Patients and Methods. As control for the expression of Bcl-2, cells were incubated with biotinylated rabbit IgG and then with PE-conjugated streptavidin.

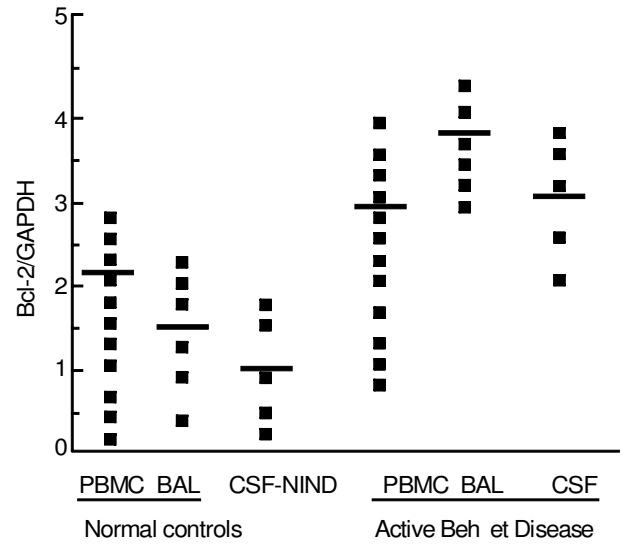

FIG. 3. The expression of Bcl-2 mRNA in peripheral blood, bronchoalveolar lavage (BAL) and cerebrospinal fluid (CSF), cells from patients with active Behçet's disease (BD). Seven micrograms of total RNA were blotted onto nylon membrane and hybridized sequentially with Bcl-2 and GAPDH probes. The dots represent the ratios of densitometric reading of Bcl-2 and GAPDH expression. Each dot represents one patient. Mean values are indicated by a line.

Table 3. Bcl-2 expression as mean fluorescence intensity (MFI) in peripheral blood T lymphocytes (PBL) after HSV-1 stimulation, in healthy controls (n = 10), patients with NIND (n = 7) and patients with active BD (n = 12). Lymphocytes were cultured in the presence of UV-inactivated HSV-1 in an incubator with 5% CO_2 for 6 days. On the day of testing cells were washed and resuspended in RPMI 1640 medium

Sample	Mean fluorescence intensity ± S.D.	
	Before HSV-1 stimulation	After HSV-1 stimulation
Healthy Controls	53 ± 12	84 ± 6*
NIND	42 ± 16	82 ± 10*
Active BD	82 ± 9.5	97 ± 22

*P<0.001 compared to non-HSV-1-stimulated cells.

Bcl-2 mRNA expression

The increased expression of Bcl-2 in active BD at the inflammatory sites could be demonstrated at the RNA level. Bcl-2 mRNA expression was studied in active BD in 12 PBMC, six BAL and five CSF samples. As a control, we used 10 PBMC and six BAL from healthy controls and five CSF from patients with NIND. The expression of Bcl-2 mRNA in PBMC from active BD was increased compared to healthy controls. BAL and CSF from active BD exhibited an increase in the expression of Bcl-2 mRNA compared to BAL from controls and CSF from patients with NIND (Fig. 3).

Bcl-2 expression after HSV-1 stimulation

The expression of Bcl-2 proto-oncogene was quantified in peripheral blood T cells after HSV-1 stimulation (Table 3) in active BD, compared to NIND and healthy controls. In healthy controls and patients with NIND, Bcl-2 expression exhibited a drastic increase after HSV-1 stimulation (P<0.001). In active BD, Bcl-2 expression increased slightly, but was not significantly different from unstimulated HSV-1 T cells.

Discussion

Our findings indicate that Bcl-2 is overexpressed in patients with active BD, both in PBMC and inflammatory sites: BAL and CSF. This conclusion is based on results obtained using flow cytometry and nucleic acid hybridization. An increased expression of Bcl-2 has been previously demonstrated in active BD,[4] and in SLE.[17] The mRNA level expression of apoptosis-related gene product did not differ between SLE cells and normal donor cells. Expression of Fas/APO-1 protein was increased in freshly isolated SLE T lymphocytes compared with normal donor T lymphocytes, whereas Bcl-2 protein was up-regulated after a 3-day culture period.[18] Dysregulation of the apoptosis system in RA is controversial.[19] Increased Bcl-2 expression, and in general programmed cell death dysregulation, seemed to lead to autoimmune disease and/or viral processes.

Some autoimmune mechanisms are involved in the etiopathogenesis of BD,[1] although there are disagreements as to whether BD should be classified as an autoimmune disease.[20] The main immunological finding in autoimmune disease is T cell hypofunction.[21] BD is essentially characterized by T cell hyperactivity.[22] Taking into account flow cytometry results and nucleic acid hybridization, we can conclude that there is a certain similarity between BD and SLE in the apoptosis defect, characterized by increased Bcl-2 expression.

A possible explanation for the increased Bcl-2 expression in the peripheral blood and inflammatory sites in BD, was the increased production of IL-2,[23] because IL-2 has been shown to increase Bcl-2 expression in active T cells.[24] On the other hand, IL-10 has demonstrated to modulate the expression of Bcl-2 in leukaemia cells.[25] IL-10 is highly produced by PBMC3 and plasma[26] from patients with BD. Therefore, we studied the roles of IL-2 and IL-10 in the regulation of Bcl-2 expression by culturing lymphocytes with exogenous IL-2, IL-10 or neutralizing anti-IL-10 mAb. Our *in vitro* data suggest that IL-2 cytokine is not responsible for the increased expression of Bcl-2 in BD inflammatory sites and peripheral blood lymphocytes. However, IL-10 is able to increase *in vitro* expression of Bcl-2, in peripheral blood lymphocytes and in inflammatory lymphocytes. This increase was abrogated when lymphocytes were incubated with anti-IL-10.

In patients with SLE, a prominent role for IL-10 in the induction of apoptosis was observed,[27] as neutralizing anti-IL-10 mAb markedly reduced cell death in active SLE patients by 50%.[27] Using Western blotting, it has been demonstrated that exposure of IL-10 to T cells, enhanced the expression of Bcl-2, inhibiting T cell apoptosis, and this is associated with a normal proliferative function.[28]

In patients suffering from viral infection, the induction of apoptosis may result in virus clearance. In contrast the inhibition of apoptosis may result in virus cell transformation and viral persistence. Epidemiological data favour an infectious cause, and T cell hypersensitivity for BD: *Streptococcus sanguis*,[29] parvovirus B19,[30] and bacterial superantigens,[31] have been associated with BD. Antibodies to herpes simplex virus 1 (HSV-1), were found to be increased in patients with active BD.[32],[33] In a limited number of patients with BD, a HSV-1–DNA fragment was found in peripheral blood lymphocytes by polymerase chain reaction (PCR). HSV DNA was found in biopsy samples taken from genital ulcers by applying PCR, and the 289-bp protein of HSV DNA was also detected in these lesions, but not in controls.[33],[34]

The *in vivo* antigenic stimulation in active BD is responsible for : (i) the activation of T lymphocytes which is characterized by a divergent cytokine production profile of mixed Th1/Th2 cell types;[35] (ii) the switch of naïve T cells to memory lymphocytes;[22] and (iii) the alteration of the inflammatory sites, with destruction of the host tissues. All these factors might account for the mechanisms of cell death observed in our patients, which were characterized by increased soluble CD95/APO-1,[4] and increased expression of Bcl-2 in the peripheral circulation and in the inflammatory sites. Our results with regard to the lack of equilibrium in apoptosis mechanisms in BD, was corroborated with the insufficient expression of Fas on activated $CD4^+$ cells in BD.[36] $CD4^+$ T cells from patients with active BD, were characterized to mediate specific cytotoxicity against HSV-1.[13]

Our data confirmed that HSV-1 increased Bcl-2 expression in active BD, undergoing apoptosis in response to a variety of different agents. The question is, whether Bcl-2 is able to interfere with Fas-induced apoptotic cell death? Data indicate that Bcl-2 is capable of inhibiting both the perforin/granzyme B- and Fas-induced apoptotic cell death.[37] Confirmation should be established in the BD context.

Based on these data, we conclude that the specific programmed cell death in BD might be explained, at least in part, by the increased *in vivo* activated levels of lymphocytes in peripheral blood and in inflammatory sites. The increased Bcl-2 protein expression in active BD are non-specific for the disease, and may be explained at least in part by the increased *in vivo* activation levels, or autoimmune vasculitides combined with *in vivo* induction by aetiopathological agent(s). These findings[4,5,36] seem to play an important role in the chronic inflammation in BD.

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