Increased Notch pathway activation in Behçet’s disease

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Abstract

Objective. Behçet’s disease (BD) is a refractory inflammatory disorder with unknown causes. Since the Notch pathway is critically involved in the immune response, the present study was undertaken to investigate the role of this pathway in BD.

Methods. Hes-1, Notch 1/C1504, Jagged-1, DLL-1 and DLL-4 expression, frequency of IFN-γ and IL-17 expressing Th cells, Notch intracellular domain (NICD), phosphorylation of signal transducer and activator of transcription 3 (STAT3) and the production of IFN-γ and IL-17 were examined by real-time PCR, flow cytometry and ELISA. Notch blockade was performed using the γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT). Transfection with miR-23b mimics and inhibitor was used to examine the effect of miR-23b on Notch pathway activation.

Results. Active BD patients showed an increased activation of the Notch pathway in association with a higher Th17 response. Notch blockade preferentially inhibited Th17 responses. The effect of Notch blockade on the Th17 response was associated with a lower level of STAT3 phosphorylation. miR-23b was significantly decreased in CD4+ T cells from active BD patients. CD4+ T cells transfected with miR-23b showed a reduced expression of NICD and a reduced frequency of IL-17- and IFN-γ-expressing T cells.

Conclusion. The present study suggests that an increased activation of the Notch pathway may contribute to the pathogenesis of BD. Decreased expression of miR-23b may be involved in activation of the Notch pathway in BD. Manipulation of the Notch pathway may offer a novel therapeutic approach for BD.

Key words: autoinflammatory conditions, Behçet’s, ophthalmic, cell receptor–ligand interaction, signalling and activation, microRNA, cytokines and inflammatory mediators, inflammation, T cells, lymphocytes, molecular biology.

Introduction

One of the main global causes of blindness is uveitis [1]. Behçet’s disease (BD) is one of the most prevalent uveitis entities seen in China [2]. It is a refractory inflammatory disorder characterized by oral aphthae, genital ulcers, recurrent uveitis and multiple skin lesions. BD is characterized by exacerbations and remissions, and autoinflammatory pathways are presumed to play a vital role in its pathogenesis [3].

Although it was initially thought that Th1 cells were the main cells mediating BD, the important role of Th17 cells in the pathogenesis of BD has emerged in recent years [4–6]. The pathways involved in the triggering of Th17 cells are not fully understood, but lately Notch signalling has been shown to be involved in the differentiation of Th17 cells [7]. Notch receptor family members (Notch1–4) are type I transmembrane proteins. Their ligands include the two related families, Dll (Dll1, Dll3, Dll4) and Jagged (Jagged1, Jagged2) [8]. Following ligand binding, the intracellular part of the Notch receptor is released as the Notch intracellular domain (NICD) by a metalloproteinase and γ-secretase, which travels to the nucleus and then
modulates the expression of a Notch target gene such as Hes-1 [9]. The Notch pathway is critically involved in lymphocyte development [10] and Notch-1 mutations have been found in >50% of patients with T cell acute lymphoblastic leukaemia, an observation that has highlighted a critical position for this pathway in regulating T cell growth [11]. Previous studies have suggested that the Notch pathway plays a critical role in Th1- and/or Th2-mediated immune responses [12, 13], but as mentioned above, it was recently shown that the Notch pathway was also involved in Th17 lymphocyte differentiation [7].

The Notch pathway is thought to play a critical role in the pathogenesis of several clinical autoimmune diseases, including SSc [14], RA [15, 16] and GCA [17]. Inhibition of the Notch pathway has been shown to attenuate the severity of experimental autoimmune encephalomyelitis [18] as well as experimental autoimmune uveoretinitis [19], a classic experimental model of uveitis.

The role of the Notch pathway in clinical uveitis is not fully understood and was therefore the subject of our current study. We chose BD as a clinical uveitis entity because it is a well-defined disease, it has a relatively high prevalence in China and further insight into its pathogenesis may offer new approaches to prevent visual disability in the individuals suffering from it [2].

Our results show for the first time an increased activation of the Notch pathway in patients with active BD. Further functional experiments showed that Notch pathway blockage could attenuate both Th17 and Th1 responses in lymphocytes obtained from these patients.

Materials and methods

Subjects

BD patients with active uveitis (n=26) and BD patients without active uveitis (n=8) (defined as active BD and inactive BD, respectively) visiting our uveitis clinic between May 2011 and June 2012 were included in the study. All BD patients strictly fulfilled the criteria of the International Study Group for BD [20]. The distribution of demographic characteristics and clinical features of the included BD patients are shown in Table 1. Age-matched healthy individuals (n=33) served as normal controls. Active intraocular inflammation was defined in this study largely depending upon the presence of anterior chamber cells and retinal vasculitis instead of visual impairment. Several complications of ocular BD can influence visual acuity, such as complicated cataracts. The 26 active BD patients enrolled in the present study were all on their first visit to our hospital. Eleven of them did not use any immunosuppressive agents before visiting us because of fears of the side effects of immunosuppressive agents or not being referred to a hospital. The other 15 patients only used a low dose of systemic corticosteroids (< 20 mg/d) earlier and stopped using these drugs for about 2 weeks when visiting our clinic and during sampling. We routinely treat BD patients using systemic corticosteroids in combination with cyclosporin, CYC or chlorambucil for >1.5 years. When the intraocular inflammation was under control, the drug dose was gradually reduced. All drugs were usually stopped 6 months later, after complete control of the intraocular inflammation. After the patients stopped using all medications for at least 2 months, we collected blood samples from these inactive BD patients. All BD patients and controls provided written informed consent. The local ethical committee of the First Affiliated Hospital of Chongqing Medical University approved the study and all the procedures met the tenets of the Declaration of Helsinki.

Cells isolation and culture

Peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood by Ficoll-Hypaque density gradient centrifugation. CD4+ T cells were purified using human CD4+ T cell microbeads following the manufacturer’s instructions (Miltenyi Biotec, Palo Alto, CA, USA). CD4+ T cells were resuspended in RPMI 1640 complete medium (10% fetal calf serum, RPMI 1640 and 1% penicillin/streptomycin) and seeded into 24-well plates at a concentration of 1 x 10^6 cells/ml. CD4+ T cells were incubated with N-[N-(3,5-difluorophenacetyl)-1-alanyl]-S-phenylglycine t-butyl ester (DAPT) or its vehicle dimethyl sulfoxide (DMSO; 10 μmol/l [17, 21]; Sigma-Aldrich, St Louis, MO, USA) in the presence of anti-CD3/CD28 antibodies (5:1; Miltenyi Biotec).

Real-time quantitative PCR measurement

Total RNA (including miRNA) was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. Real-time quantitative PCR was performed on the ABI7500 Fast System (Applied Biosystems, USA). The primers for Hes-1, Notch1-4, Jagged-1, DLL-1, DLL-4 and β-actin detection are shown in supplementary Table S1, available at Rheumatology Online. miRNA primers for miR-23b, miR-27b, miR-34a and U6 small nuclear RNA (Ribobio, Guangzhou, China) were used for miRNA detection. Due to patent protection, the miRNA primer sequence is not provided by the product manufacturer. The relative expression of miRNAs or genes was calculated using the 2^[-ΔΔCT] method as described previously [22].

miRNA target prediction analysis

PicTar (http://pictar.mdc-berlin.de/), miRanda( http://www.microrna.org) and TargetScan (http://www.targetscan.org) were used to identify the miRNAs for targeting Notch1 3’UTR. To optimize the prediction accuracy, the potential target miRNAs should be predicted by all three programs and the targeted sequence should be conserved among species.

Measurement of cytokines by ELISA

IFN-γ and IL-17 secretion were examined using Duoset ELISA development kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer’s instructions with a detection limit of 15.6 pg/ml.
Flow cytometry
Following treatment, CD4+ T cells were examined for various markers as follows. CD4+ T cells were fixed, subsequently permeabilized and treated with anti-Notch1-PE (clone:mN1A), anti-IL-17-FITC, anti-IFN-γ-APC or anti-pSTAT3-PE antibodies or appropriate isotypes. All the antibodies were purchased from eBioscience (San Diego, CA, USA) except for anti-pSTAT3 (BD Biosciences, San Jose, CA, USA).

The transfection rate of miR-23b mimics or inhibitor was indicated by siR-RiboTM Transfection Control (Ribobio, Guangzhou, China). Cy3-labelled CD4+ T cells were detected using flow cytometry 48 h after transfection. Data collection was conducted on a FACScan flow cytometer and the data were analysed with FlowJo 7.6 software (Tree Star, Ashland, OR, USA).

Cell transfection with miRNA mimics and inhibitor
miR-23b mimics and miR-23b inhibitor (Ribobio) were used for the overexpression and inhibition of miR-23b in purified CD4+ T cells, respectively. Negative control mimics or inhibitor (Ribobio) were used as matched controls. CD4+ T cells were seeded into 24-well plastic plates at a concentration of 1 × 10⁶ cells/ml. To silence or overexpress miR-23b, CD4+ T cells were transfected with miR-23b inhibitor or mimics at a concentration of 200 nM (inhibitor) or 100 nM (mimics) using an X-tremeGENE transfection kit (Roche, Mannheim, Germany) following the manufacturer’s instructions and cultured for 48 h.

Statistical analysis
The data were analysed by SPSS 13.0 (IBM, Armonk, NY, USA) using the nonparametric Mann–Whitney U-test, Student’s t-test and one-way analysis of variance (ANOVA). P-values <0.05 were considered statistically significant. Data are expressed as mean (s.d.).

Results
Increased activation of the Notch pathway associated with signal transducer and activator of transcription (STAT3) phosphorylation promotes Th17 response in BD patients
The expression of the Notch target gene Hes-1 in PBMCs and CD4+ T cells was significantly increased in active BD patients as compared with normal controls or inactive BD patients (Fig. 1A and B). Notch1 gene expression and the active form of Notch1 (NICD) in CD4+ T cells were also significantly increased in active BD patients as compared with controls (Figs. 1C and 2A). CD4+ T cells stimulated with anti-CD3/CD28 led to even higher Notch1 expression and again the highest expression was observed in cells obtained from active BD patients (Fig. 2B). The expression of the other three Notch receptors did not reveal a detectable difference between BD patients and normal controls (supplementary Fig. S1A and C, available at Rheumatology Online). We also found that the expression of Jagged-1 and DLL-1, but not DLL-4, was significantly increased in active BD patients as compared with controls (Figs. 1C and 2A). CD4+ T cells stimulated with anti-CD3/CD28 led to even higher Notch1 expression and again the highest expression was observed in cells obtained from active BD patients (Fig. 2B). The expression of the other three Notch receptors did not reveal a detectable difference between BD patients and normal controls (supplementary Fig. S1A and C, available at Rheumatology Online). We also found that the expression of Jagged-1 and DLL-1, but not DLL-4, was significantly increased in active BD patients (supplementary Fig. S2, available at Rheumatology Online). These results show for the first time an increased activation of the Notch pathway in active BD patients.

We performed another set of experiments to investigate the role of the Notch1 pathway in lymphocyte differentiation in BD patients. Confirming our earlier results [23], we showed an increased frequency of IFN-γ-expressing and IL-17-expressing CD4+ T cells (supplementary Fig. S3A and B, available at Rheumatology Online) and an elevated production of IFN-γ and IL-17 in lymphocyte culture...
supernatants in active BD patients compared with controls (supplementary Fig. S3C, available at Rheumatology Online). Our results also confirmed that the ratio of Th17/Th1 cells was increased in active BD patients compared with normal controls (supplementary Fig. S4, available at Rheumatology Online).

Further experiments with the Notch pathway inhibitor DAPT showed a decreased expression of NICD and

Fig. 1 Increased activation of the Notch pathway in BD patients

(A) Hes-1 mRNA expression in PBMCs from active BD patients (n = 8), inactive BD patients (n = 8) and normal controls (n = 8).

(B) Hes-1 and (C) Notch1 mRNA expression in CD4+ T cells from active BD patients (n = 10) and normal controls (n = 10).

Fig. 2 The Notch1 pathway shows increased expression in BD patients

(A) The active form of Notch1 [Notch intracellular domain (NICD)] expression [mean fluorescence intensity (MFI)] in CD4+ T cells from active BD patients (n = 10) and normal controls (n = 10) without anti-CD3/CD28 stimulation. (B) The active form of Notch1 (NICD) expression in CD4+ T cells from active BD patients (n = 10) and normal controls (n = 10) with anti-CD3/CD28 stimulation.
target gene Hes-1 (Fig. 3A and B) in association with markedly decreased IL-17 (Fig. 3C) and a reduced frequency of IL-17-expressing CD4+ T cells (Fig. 3D and E). However, inhibition of the Notch pathway showed a small but significant decrease in IFN-γ protein secretion and did not influence the frequency of IFN-γ-expressing cells (Fig. 3C, D and F). These results indicated that Notch pathway blockage may preferentially inhibit the Th17 response in BD patients.

As STAT3 has been shown to be critically involved in Th17 lymphocyte differentiation, our further experiments examined whether Notch pathway blockage could preferentially inhibit the Th17 response through modulating STAT3 phosphorylation. As shown in Fig. 4A, BD patients showed elevated STAT3 phosphorylation as compared with controls. STAT3 phosphorylation in CD4+ T cells from both controls and active BD patients was significantly decreased following treatment with DAPT (Fig. 4B and C). Taken together, these results suggest that increased STAT3 phosphorylation following enhanced activation of the Notch pathway might trigger the Th17 response in BD patients.

Decreased miR-23b may contribute to activation of the Notch pathway and the expansion of Th1/Th17 cells in BD patients

The aforementioned results showed increased activation of the Notch pathway in active BD patients. It has been shown that several miRNA species are able to modulate the Notch pathway [24]. Our further study examined which miRNA was involved in activation of the Notch pathway and whether it could affect the expansion of Th1/Th17 cells in BD patients. Three miRNAs (miR-34a, miR-23b, and miR-27b) were predicted to be capable of targeting

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**Fig. 3** Blocking the Notch pathway preferentially inhibits the Th17 response in BD patients

(A) Representative histogram illustrates the expression of the active form of Notch1 [Notch intracellular domain (NICD)] in CD4+ T cells with and without Notch pathway inhibitor N-[N-(3,5-difluorophenacetyl)-1-alanyl]-S-phenylglycine t-butyl ester (DAPT) treatment. The active form of Notch1 (NICD) was determined by flow cytometry. Shaded histogram indicates isotype control. (B) Expression of the active form of Notch1 (NICD) (n = 10) and Hes-1 mRNA (n = 8) in CD4+ T cells with and without DAPT treatment. (C) The production of IL-17 and IFN-γ by CD4+ T cells from active BD patients with and without DAPT treatment (n = 10). (D) Representative dot plot illustrates the frequency of IL-17-producing and IFN-γ-producing CD4+ T cells from active BD patients with and without DAPT treatment. (E) The frequency of IL-17-producing CD4+ T cells from active BD patients with and without DAPT treatment (n = 10). (F) The frequency of IFN-γ-producing CD4+ T cells from active BD patients with and without DAPT treatment (n = 10).
Notch1, based on bioinformatics analysis (miRanda, TargetScan and PicTar) and previous reports [25–27]. These predicted miRNAs (miR-23b, miR-27b and miR-34a) were screened in CD4+ T cells from active BD patients and controls. The results showed that only miR-23b expression was significantly decreased in active BD patients as compared with normal controls (Fig. 5A). In contrast, the expression of miR-27b showed no substantial changes (Fig. 5B). Moreover, the expression of miR-34a was even undetectable in CD4+ T cells with our method, consistent with a previous report [28]. These data suggest that of the three predicted miRNAs, only a specific association was found for miR-23b in BD patients.

A further study was designed to examine the effect of miR-23b on the expression of the active form of Notch1 (NICD). As shown in supplementary Fig. S1D, available at Rheumatology Online, transfection of miR-23b mimics and inhibitor showed stable expression, with about 50–60% efficiency in CD4+ T cells as indicated by the Cy3-labelled reporter fluorescence. CD4+ T cells transfected with miR-23b mimics were shown to significantly inhibit the expression of NICD compared with those...
transfected with a control sequence (Fig. 5C). CD4+ T cells transfected with miR-23b inhibitor could produce greater amounts of NICD as compared with controls (Fig. 5D). Considering the decreased expression of miR-23b in active BD patients, these results indicated that decreased expression of miR-23b might be involved in activation of the Notch pathway in BD patients.

To test whether miR-23b plays a T cell intrinsic role in regulating the expansion of Th17/Th1 cells, CD4+ T cells transfected with miR-23b mimics or inhibitor were treated by anti-CD3/CD28 stimulation. The results showed that CD4+ T cells transfected with miR-23b mimics significantly inhibited both IL-17-expressing and IFN-γ-expressing T cells compared with those transfected with a control sequence (Fig. 6A and C). In contrast, CD4+ T cells transfected with miR-23b inhibitor significantly promoted both IL-17-expressing and IFN-γ-expressing T cells (Fig. 6B and D). Taken together, our results showed that the decreased miR-23b expression may contribute to activation of the Notch pathway and an increase in Th1/Th17 cells in BD patients.

**Discussion**

In the present study we demonstrated that increased activation of the Notch pathway is associated with an increased Th17 response in active BD patients. We further showed that blocking the Notch pathway can preferentially attenuate the Th17 response. This effect may be mediated by modulating STAT3 phosphorylation. Additionally, we showed that decreased expression of miR-23b may contribute to activation of the Notch pathway due to decreased expression of miR-23b may contribute to the pathogenesis of BD.

The role of the Notch pathway has been reported in many inflammation-related disorders but not yet in clinical uveitis [14, 15, 17–19]. To our knowledge, our study is the first attempt to examine the role of Notch in clinical uveitis. To assess whether the Notch pathway is activated in BD patients, we determined the expression of the Notch target gene Hes-1, which is the best characterized...
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**Fig. 6** miR-23b regulates the increase in Th1 and Th17 cells

(A) Representative dot plot illustrates the frequency of IL-17-expressing and IFN-γ-expressing CD4+ T cells from CD4+ T cells transfected with miR-23 mimics or mimics control at 72 h. (B) Representative dot plot illustrates the frequency of IL-17-expressing and IFN-γ-expressing CD4+ T cells from CD4+ T cells transfected with miR-23 inhibitor or inhibitor control at 72 h. (C) Statistics histogram illustrates the frequency of IL-17-expressing and IFN-γ-expressing CD4+ T cells from CD4+ T cells transfected with miR-23 mimics or mimics control at 72 h (n = 5). (D) Statistics histogram illustrates the frequency of IL-17-expressing and IFN-γ-expressing CD4+ T cells from CD4+ T cells transfected with miR-23 inhibitor or inhibitor control at 72 h (n = 5).

Target gene of the Notch pathway. Up-regulation of Hes-1 suggests that the Notch pathway is activated. As expected, we observed an increased expression of Hes-1 mRNA in PBMCs in active BD patients but not inactive BD patients or normal controls, providing evidence for ongoing activation of the Notch pathway in active BD patients. We next determined which Notch receptor mediates activation of the Notch pathway in active BD patients. We found that only Notch1 expression was significantly increased in CD4+ T cells from active BD patients compared with controls. We further confirmed increased expression of the active form of Notch1 (NICD) using flow cytometry. It is worth noting that CD4+ T cells stimulated with anti-CD3/CD28 showed greater activation of the Notch pathway compared with those without anti-CD3/CD28. Earlier studies have shown that the Notch pathway could be activated not only by TCR stimulation, but also via pattern recognition receptors (PRRs) including TLR4 and NOD2 [29–31]. The increased expression of TLR4 and the NOD2 gene has been reported in BD patients [32, 33]. How these pathways stimulate Notch is not yet known, but it may be due to enhanced expression of Notch ligands on antigen-presenting cells. Actually, we found that the expression of Jagged-1 and DLL-1, but not DLL-4, was significantly increased in BD patients. Further studies are needed to investigate the exact role of Notch receptors and their ligands in the pathogenesis of BD.

We subsequently found that the Th17 response was significantly increased in active BD patients. A small but significant increase was seen for the Th1 protein response, but not for the number of IFN-γ-expressing cells, suggesting that the Notch pathway mainly affected the Th17 population in BD. This was in agreement with our findings that the Th17/Th1 was increased in BD patients. Earlier studies have shown that the ratio of RORC/Foxp3 is increased in BD patients, especially in the neuro-BD subgroup [34]. Taken together, these results highlight the important role of Th17 cells in the pathogenesis of BD [5, 6].

Blocking the Notch pathway with DAPT resulted in markedly reduced IL-17 production by CD4+ T cells from active BD patients. However, IFN-γ secretion was only marginally affected. Moreover, IL-17-expressing
CD4+ T cells were impaired preferentially by Notch blockade but not the IFN-γ-expressing CD4+ T cells. Our results are partially consistent with a previous study describing that both IL-17 and IFN-γ production were inhibited by blocking the Notch pathway in GCA [17]. However, a decreased production of Th2 cytokines in association with an increased production of Th1 cytokines was described in allergic pulmonary inflammation following Notch inhibition [35]. The discrepancies might be attributed to the different immunological mechanisms of various inflammatory diseases. Further studies are needed to confirm whether this finding is specific for BD.

Earlier studies have shown that STAT3 is critical for Th17 differentiation [36]. In the present study we observed an elevated STAT3 phosphorylation in active BD patients compared with controls. STAT3 phosphorylation was significantly attenuated by Notch blockade. These results indicate a crosstalk between the Notch and Janus kinase (JAK)–STAT pathways as described by several studies [37, 38], which is probably one reason for the effect of Notch inhibition on the Th17 response.

Several miRNAs are involved in modulating autoimmune pathogenesis, including miR-146a, miR-155 and miR-326 [39–41]. We can now add miR-23b to the list of miRNAs modulating the immune response. Our results revealed that a decreased expression of miR-23b may be involved in activation of the Notch pathway and the increase in Th1/Th17 cells in BD patients. miR-23b is among the miRNAs involved in the differentiation of Tregs. Low miR-23b expression would be in line with earlier findings reporting a decreased level of Tregs in BD [42]. These results are partially consistent with a recent report that down-regulated miR-23b induced by IL-17 signalling contributed to the pathogenesis of autoimmune disease [43]. Although it was suggested that miR-23b plays an important role in autoimmune disease, many key questions about miR-23b have not yet been elucidated, such as why miR-23b is down-regulated in autoimmune disease. A previous study showed that miR-23b transcription is regulated by specific nuclear factor (NF)-κB family members and that this regulation is dependent on the IL-17 signalling mediator Act1 [43]. Other possible mechanisms for the regulation of miR-23b expression could be due to epigenetic events or to genetic variants in the miR-23b locus. Our results confirmed that the Notch pathway is regulated by miR-23b and that the Notch pathway is involved in the Th17 response. These results suggest that there may be a vicious circle among miR-23b, the Notch pathway and IL-17. Taken together, an abnormal increase in the stimuli of the immune microenvironment in combination with a loss of immunomodulation by decreased miRNA may cooperatively contribute to excess activation of the Notch pathway and the expansion of inflammatory cells in BD patients.

There are several limitations in our study. First, we investigated immunological abnormalities of the eye by testing peripheral immune functions. Whether such findings can be extrapolated to what is happening in an immunoprivileged site such as the eye is debatable. Further studies are needed to examine the Notch pathway in the eye itself by using intraocular fluid or tissue samples. In our clinic, ocular fluid sampling is restricted due to the possible risk of microbial infection of the eyes of our patients. A further limitation is the fact that the enrolled BD patients in our study were all recruited from a department of ophthalmology and suffered from uveitis. BD is a multisystem disease featuring multiple organ involvement, including vascular disease (thromboophlebitis) [44]. The role of the Notch pathway should therefore also be examined in BD patients recruited from other medical departments.

The lack of an effective treatment for BD is partly due to our poor understanding of the pathogenesis of this disease. The current standard treatment in BD, long-term and/or high doses of glucocorticoids, is associated with serious side effects. Our studies suggest that Notch pathway activation due to decreased miR-23b may be involved in the pathogenesis of BD. Therefore manipulation of the Notch pathway may offer a novel therapeutic approach for BD.

Rheumatology key messages

- This is the first report of the Notch pathway in the pathogenesis of Behçet’s disease (BD).
- Manipulation of the Notch pathway may offer a novel therapy for BD.

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Supplementary data

Supplementary data are available at Rheumatology Online.

References

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