

Natural killer cells dominate a Th-1 polarised response in Behçet's disease patients with uveitis

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ABSTRACT

Objective. Behçet's disease (BD) is a systemic inflammatory disorder of unknown aetiology, characterised by recurring relapses and remissions. BD manifestations have been thought to be associated with the immunological abnormalities triggered by environmental factors in genetically susceptible individuals. Natural killer (NK) cells are important members of innate immunity with their cytotoxic activity and also cytokine secretions. They have the capacity to induce or dampen immune responses. Different study groups have reported conflicting results about NK cell activity in the BD pathogenesis, however, contribution of NK cells to BD is still unclear.

Methods. NK cells from BD patients with uveitis (n=11) as well as age- and gender-matched healthy controls (n=9) were purified and intracytoplasmic cytokine levels of TNF- α , IFN- γ , IL-2, IL-4, IL-10, IL-12 and IL-13 were determined.

Results. Increased TNF- α , IFN- γ and IL-2 in relapse period and increased IL-4 as well as a slight increase of IL-10 in remission period were observed.

Conclusion. Our results show that NK cells are the contributors of BD pathogenesis with their NK1 profile in relapse periods, and also with their NK2 profile in remission periods, in BD patients with uveitis. An increase in IL-10 observed in remission periods may be linked to the regulatory potential of NK cells in the recurrent nature of BD manifestations.

Introduction

Behçet's disease (BD) is a systemic inflammatory disorder of unknown aetiology, characterised by recurrent oral and genital aphthous ulcerations, skin lesions, pathergy reaction, uveitis and other manifestations (1). BD is

shown to increase from north to south, whereas a more severe course is observed in patients with early disease onset (2). BD is diagnosed using the diagnosis criteria of the International Study Group (3), however, new studies aim to find candidate biomarkers for BD (4). Uveitis is the involvement of eye in BD patients, and is shown to be present in around 50% of the BD patients in multi-disciplinary setting. Uveitis in BD is shown to be a distinct entity which can be diagnosed in the absence of systemic manifestations. Uveitis runs a course of relapses and remissions, as the recurrent nature of other manifestations of BD, therefore becomes a good indicator of the course of disease activity (5).

The pathogenesis of BD is thought to originate from the immunological abnormalities triggered by environmental factors in genetically susceptible individuals. Spontaneous and/or induced over-expression of pro-inflammatory cytokines from various cellular sources seems to be responsible for the enhanced inflammatory reaction in BD (6). Previous studies on the circulating CD4⁺ T cells and affected lesions of BD patients in relapse period showed elevated levels of T helper 1 (Th1) type cytokines like interferon (IFN)- γ and IL-12, indicating a role for Th1-polarised immune response in the pathogenesis (7, 8). IFN- α , was also shown to be increased in the sera of BD patients (9). Natural killer (NK) cells are members of innate immunity, which mediate the early responses against certain pathogens with their cytotoxic activity and also modulate the activity of other effector cells of immune system via their cytokine productions. (10). It has been shown that distinct NK cell subsets with disparate repertoires, locations and functional origins exist (11). It was demonstrated that NK cells are dif-

ferentiated into cell populations with distinct patterns of cytokine secretions, namely NK1 and NK2, *in vitro*. NK1 cells mainly produce IFN- γ while NK2 cells produce IL-5 and IL-13 (12). The purification of these subsets from peripheral bloods of healthy individuals, showed the *in vivo* existence of NK1 and NK2 (13) and also IL-10 secreting NK cells, with functions similar to T regulatory cells in humans (14).

The contribution of NK cells to BD pathogenesis has been investigated in a number of studies. An increased number of NK cells with decreased cytotoxic activity has been identified in the blood samples of a relapse period of BD patients (15, 16). Activated NK cells were shown to be increased in the relapse period of BD patients in comparison with the remission period of BD patients and healthy controls. An investigation of the gene expression profile of BD remission declared an associated NK2 phenotype (17). However, the functions of NK cells in BD pathogenesis are still not clearly known.

This study aimed to investigate the functional contribution of NK cells to the pathogenesis of BD by an analysis of their cytokine secretions, both in relapse and remission periods of BD patients with uveitis.

Materials and methods

Study population

Eleven patients (4 females, 7 males, mean age: 27.81 ± 8.01 years) fulfilling the International Study Group's diagnostic criteria (3) and presenting with uveitis were enrolled in the study. All but one patient had oral aphthae and genital ulcers together with uveitis. Other manifestations of BD patients are shown in Table I. Heparinised venous blood samples were collected at relapse of uveitis. All patients enrolled in this study were not being treated at the time of uveitis relapse and the therapy was initiated after blood samples were collected. The patients were treated with standard of care and their ocular manifestations were considered at remission by their ophthalmologist. An average time of 28 ± 6 days was required for the remission of patients. All patients were on azathioprine treatment

Table I. Clinical features of BD patients with uveitis.

Symptoms	Patients
Oral aphthae	10
Genital ulcers	10
Arthritis	5
Headache	4
Positive pathergy response	2
Erythema nodosum	3
Oral aphthae in family	5
BD in family	1

(100–150 mg/day) and 7 patients were also receiving prednisolone (various doses ranging between 16–48 mg/day) in addition. Nine patients completed the study by giving repeat blood samples following the diagnosis of remission of uveitis by ophthalmology department. Two patients left the study with no medical reasons, so it was not possible to collect blood samples when they were in uveitis remission. Clinical features of BD patients are shown in Table I. Nine age-matched healthy subjects (4 females, 5 males, mean age: 27.12 ± 6.89 years) were enrolled in the study as healthy controls. All samples were collected after written informed consent was obtained according to the Declaration of Helsinki. The study was approved by the local Ethics Committee of Istanbul University, Istanbul Faculty of Medicine.

Cell purification

Peripheral blood mononuclear cells (PBMCs) were separated with Ficoll-Hypaque (Sigma Chem. Co., St. Louis, MO) density gradient centrifugation and cells were re-suspended in RPMI-1640 (Sigma Chem. Co., St. Louis, MO) supplemented with 10% foetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 mg/ml) (all supplements from Biochrom AG, Berlin, Germany; named as complete RPMI (cRPMI)), at a concentration of 1×10^6 cells/ml.

Isolation of NK cells

NK cells were isolated from PBMCs by a magnet activated cell sorting system (MACS, Miltenyi Biotec, Germany) using a NK cell isolation kit. Non-NK cells were labelled, were applied on a LS-MACS column and NK cells were purified by negative selection strategy,

according to the manufacturer's protocols. NK cell purities detected by flow cytometry were >95% for all cases.

Intracellular cytokine staining

Freshly purified NK cells were stimulated with combination of phorbol myristate acetate (PMA, 50 ng/ml) and Ca^{++} Ionophore (ionomycin, 250 ng/ml) in 37°C, 5% CO_2 incubator for 5 hours, and monensin (2 μM) was added during the last 4 hours of cell culture (all reagents were from Sigma Chem. Co., St. Louis, MO). Following stimulation, NK cells were fixed and permeabilised with Cytofix and Cytoperm kit according to the manufacturer's protocol (BD Bioscience, San Jose, CA). Permeabilised cells were incubated with FITC-labelled anti-IL-2, -IL-4, -IL-12 and -IFN- γ and PE-labelled anti-IL-10, -IL-13 and -TNF- α (all from BD Bioscience, San Jose, CA). All analysis were performed with a FACSCalibur (Becton Dickinson, San Jose, CA) flow cytometry running CELLQuest software (Becton Dickinson, San Jose, CA) and all levels mentioned are NK cell percentages expressing relevant cytokines.

Statistical analysis

Statistical analysis was performed by SPSS 17.0 software. Mann Whitney U-test was used to determine statistics in between patient groups and healthy controls. Wilcoxon Signed Ranks Test was used to evaluate statistics in between patients relapse and remissions. ($p < 0.05$) was accepted as statistical significance level.

Results

A NK1-polarised profile in relapse period, while a non-significant increase of IL-4⁺ NK cells in remission period were observed in BD patients

Expression levels of TNF- α , IFN- γ , IL-2 and IL-12 were investigated as NK1 cytokines, in relevance with Th1 cytokines. Levels of TNF- α^+ NK cells were higher in relapse period when compared to remission period and healthy controls ($15.53 \pm 3.35\%$, $6.29 \pm 2.81\%$ and $3.67 \pm 0.78\%$, respectively). TNF- α^+ NK cell levels of remission period were not statistically different when compared to levels of healthy controls. NK

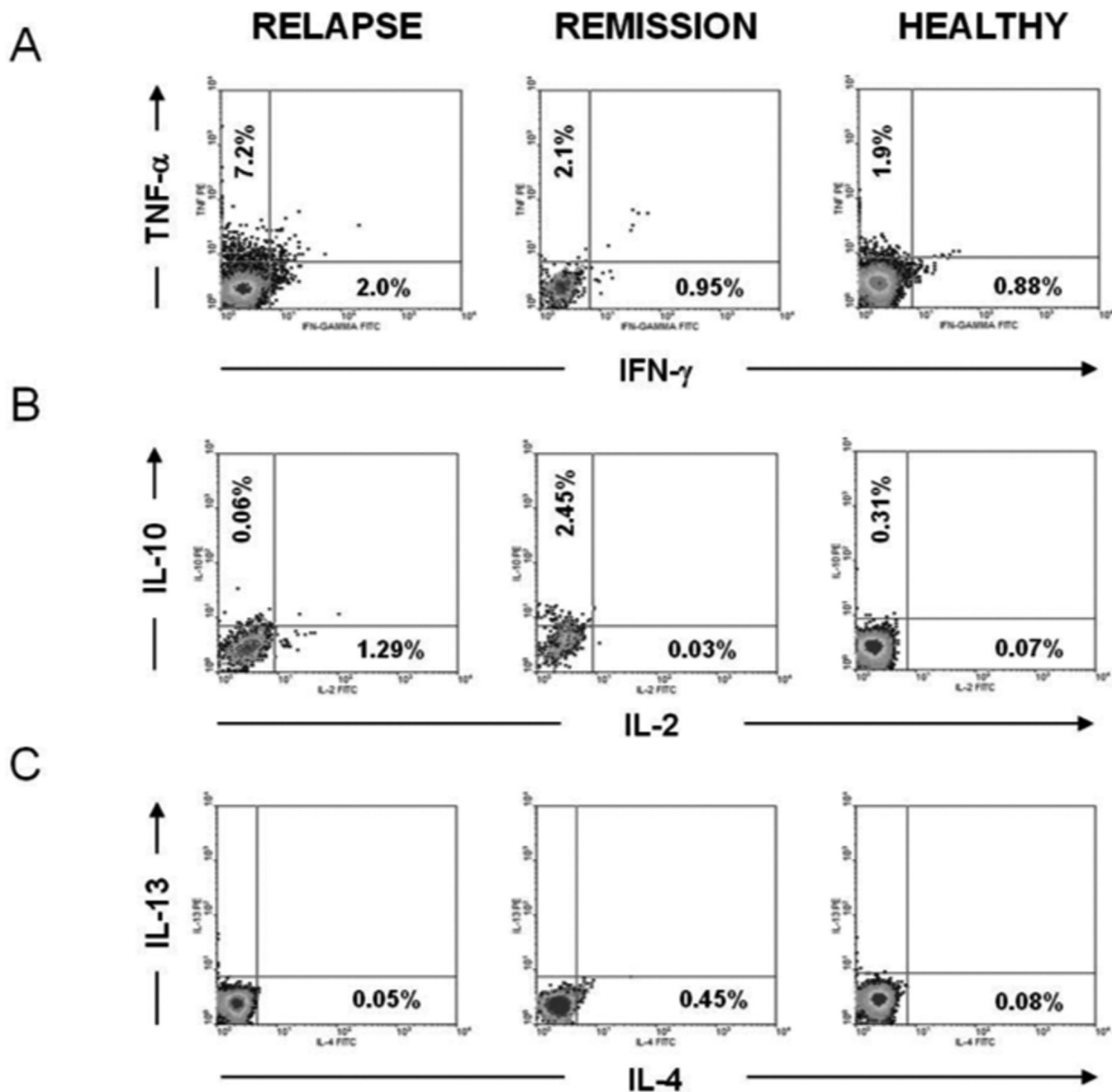


Fig. 1. A demonstrative density plot of a patient in BD relapse and remission periods in comparison with a healthy control. NK cells purified from PBMCs of BD patients in relapse and remission periods and also healthy controls were stained with FITC-labelled IFN- γ , IL-2, IL-4 and PE-labelled TNF- α , IL-10 and IL-13, intracellularly (Fig. 1 A, B and C, respectively). The figure shows demonstrative density plots of a patient and a healthy control. The numbers indicate NK cell percentages expressing the relevant cytokine.

intracellular IFN- γ content of the relapse period was similar to the remission period but was statistically higher ($p=0.045$) when compared to healthy controls ($2.88\pm0.81\%$, $2.02\pm1.45\%$ and $1.12\pm0.20\%$, respectively). IFN- γ^+ NK cell levels in remission were not different from the levels of the controls. The proportion of IL-2-expressing NK cells were statistically increased in the

relapse period when compared to the remission period ($p=0.045$). IL-2 $^+$ NK cell levels in the relapse period were observed to be higher when compared to the controls, although without any statistical significance ($p=0.076$). (Relapse, remission and healthy control levels of IL-2 $^+$ NK cells: $1.50\pm0.42\%$, $0.36\pm0.22\%$ and $0.24\pm0.06\%$, respectively). IL-12 content was not detecta-

ble either in the BD patients or the controls (Fig. 1A-B and 2A). These results collectively demonstrate the dominance of Th1 type cytokines in NK cells of BD patients in the relapse period when compared to the remission period and healthy controls.

Expression levels of IL-4 and IL-13 were investigated as NK2 cytokines, in relevance to Th2 cytokines. The

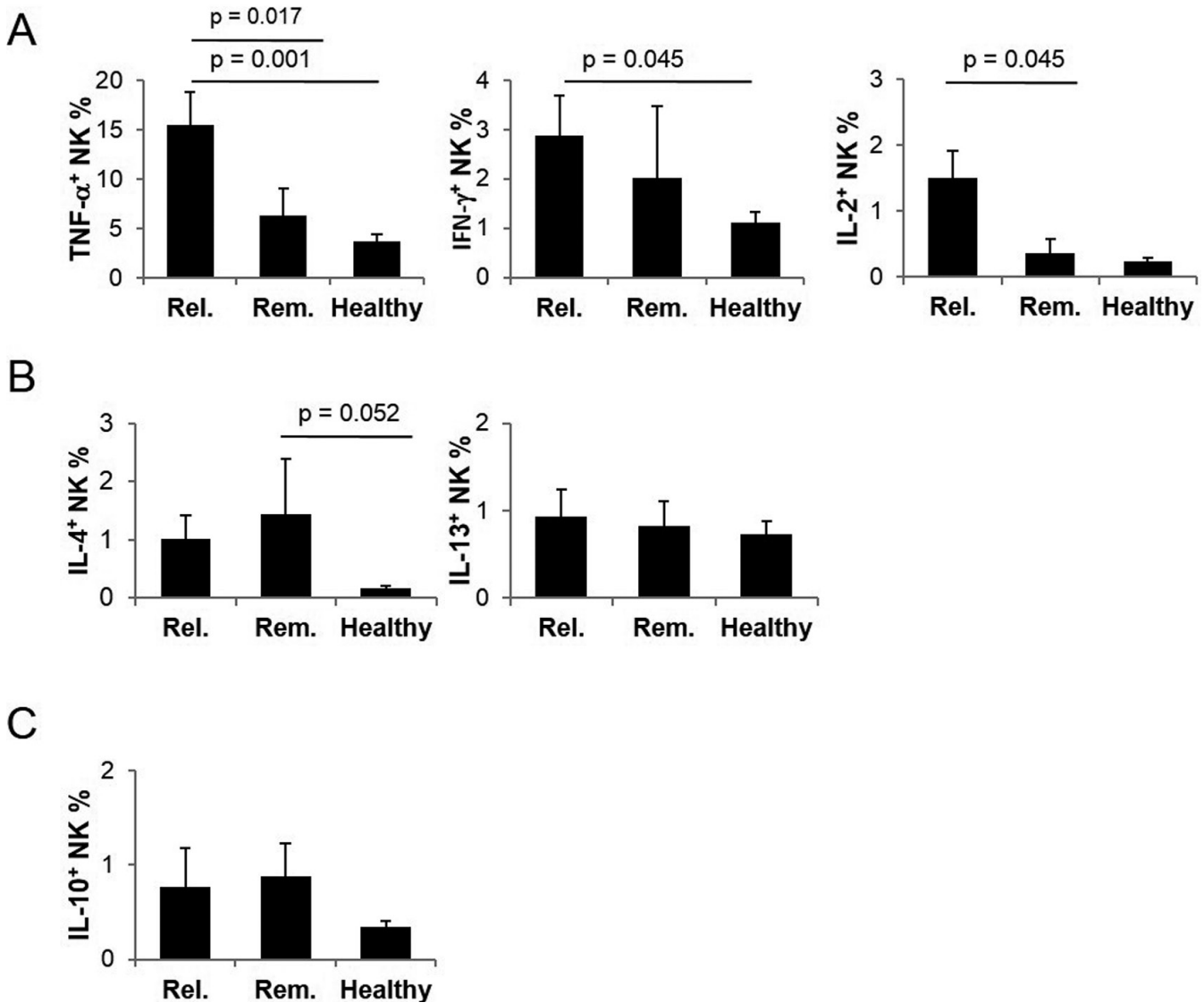


Fig. 2. Intracellular cytokine content of NK cells from BD patients with uveitis. A total of 9 patients were investigated during their relapse and remission periods. TNF- α -expressing NK cells were detectable in 8 patients, IFN- γ -expressing NK cells were detectable in 5 patients and IL-2-expressing NK cells were detectable in 5 patients. 9 healthy controls were enrolled in the study, TNF- α - and IFN- γ -expressing NK cells were detectable in 8 healthy controls while IL-2-expressing NK cells were detectable in 5 healthy controls (Fig. 2A). IL-4-expressing NK cells were detectable in 5 patients and IL-13 expressing NK cells were detectable in 8 patients. IL-4-expressing NK cells were detectable in 6 healthy controls and IL-13-expressing NK cells levels were detectable in 8 of them (Fig. 2B). Among the patients investigated during their relapse and remission periods, IL-10-expressing NK cells were detectable in 7 patients and 6 healthy controls (Fig. 2C). Wilcoxon signed ranks test was used for evaluation of statistical significance between relapse (Rel) and remission (Rem) periods and Mann Whitney U-test was used to evaluate statistical significance between rel, rem and healthy controls. $p < 0.05$ was accepted as statistical significance level. The figures indicate (mean $\pm$ SEM) percentage of NK cells expressing relevant cytokine.

proportion of IL-4⁺ NK cells in relapse and remission periods of BD patients and in healthy controls were as follows; (1.01 $\pm$ 0.40%, 1.43 $\pm$ 0.95%, 0.16 $\pm$ 0.04%, respectively). Although without statistical significance, increased relapse and remission period contents in comparison with healthy controls were observed ($p=0.543$ for comparison of BD relapse and remission, $p=0.052$ for comparison of BD remission and healthy controls). The proportion of IL-13⁺ NK cells was not dif-

ferent between the relapse and remission periods of BD patients and healthy controls (0.94 $\pm$ 0.30%, 0.83 $\pm$ 0.29% and 0.73 $\pm$ 0.15%, respectively) (Fig. 1B-C). These results demonstrate a non-significant increase of Th2-related IL-4 in NK cells during the remission period of BD patients.

Increased IL-10 levels observed in remission period of BD patients

As the main regulatory cytokine, levels of IL-10⁺ NK cells were investi-

gated in the relapse and remission periods of BD patients and healthy controls (0.76 $\pm$ 0.41%, 0.88 $\pm$ 0.35% and 0.34 $\pm$ 0.07%, respectively). There was a tendency for higher IL-10 expression in NK cells during the remission, when compared to relapse period ($p=0.82$) and also to healthy controls ($p=0.83$) (Fig. 1B-C). These results showed a tendency for an increase in levels of IL-10⁺ NK cells in the remission period of BD patients, without statistical significance.

Discussion

Though many studies investigating genetic and immunological aspects of BD, the exact disease mechanism has not yet been clarified. This study aimed to investigate the possible roles of NK-derived cytokines in the pathogenesis of BD with uveitis. It has been claimed that NK cells, in addition to T cells, neutrophils, and NKT cells, contribute to the disease pathogenesis. NK cells have capacity to influence adaptive immune responses and thus may contribute to several autoimmune and inflammatory diseases. NK cells play an important role in the protection of normal tissues and limiting the nonessential triggering of cytokine secretions of adaptive immunity, therefore their functions are considered to be critical in the fine-tuning of innate and adaptive immune responses (18, 19).

Increase of both Th1 and Th2 cytokines, with a dominance of Th1 type, in the sera of relapse period of BD patients, when compared to healthy controls, with an increase of intracytoplasmic IFN- γ in CD4⁺ T cells of BD patients compared to healthy controls were shown (20). Intracytoplasmic cytokine contents of CD3⁺ T cells of relapse period of BD patients were shown to be strongly Th1 type-polarised, and no roles for IL-4 or other Th2-type cytokines were reported to be associated with the remission of the disease (7). Another study showed the increase of Th1-type CD4⁺ T cells together with CD8⁺ T cells in the relapse period and decrease of them with a response to immunosuppressive therapy in the remission period, supporting the possible roles of Th1-type T cells in BD immune pathogenesis. Also, another recently discovered inflammatory subset of Th cells; Th17 cells are shown to be related with BD pathogenesis with high frequency of IL-17 expressing CD4⁺ T cells, and also it was suggested that up-regulated IL-17 expression may be associated with clinical course of BD (21).

NK cells were shown to have two separate subgroups according to their diverse cytokine patterns just like T helper cells; NK1 and NK2 subsets, which may have roles in regulation of immunity and may contribute to inflammatory and

allergic disorders (13). A recent publication also demonstrated the regulatory roles of a NK cell subset secreting IL-10, with regulatory functions in humans (14). In parallel with the previous studies implicating Th1-type polarisation with the disease activity, we herein demonstrated increased TNF- α , IFN- γ and IL-2 levels in NK cells during the relapse period, underlying the important contribution of NK1 cells in the pathogenesis by contributing to the inflammatory milieu of BD. In forming a bridge between innate and adaptive immunity, NK cells may be strengthening adaptive immune responses by the stimulation of T cells. Increased NK-derived IL-2 levels during the relapse period supports the bridging function of NK cells in BD pathogenesis. Our results also show that NK-polarisation tends to switch to NK2-type during the remission period and the decrease in NK1-type cytokines can be a good indicator of remission. On the other hand, since all patients received immunosuppressive therapy following relapse, the exact nature of this type switch could not be determined. Immunosuppressive medications could have affected the polarisation of NK cells. For T helper cells, it is known that corticosteroids can inhibit IL-12 production from antigen presenting cells and thus induce production of Th2-type cytokines (22). None of the patients displayed high levels of NK-derived IL-12 in this study. In addition to corticosteroids, azathioprine may induce production of Th2-type cytokines and may be effective through this polarisation on remission induction. A recent study of our group investigating the cytokine contents of CD3⁺ T cells revealed that BD patients had increased IFN- γ and TNF- α levels. Similar observations of the current study in NK cells in the relapse period of BD patients suggest a common inflammatory pathway with a different type of immune cells working in harmony (23).

These data support the idea that Th1- and NK1-biased inflammation contributes to the pathogenesis of BD. Plasticity of cytokine secretion patterns and the relative increase in Th2/NK2 content during remission supports the importance of cytokine balance in the

recurrent nature of BD manifestations. A slightly increased IL-10 production observed in the remission period may underline possible Immunoregulatory effects of NK cells in remission of BD. Genetic studies implicating the critical role of IL-10 in the BD pathogenesis further supports the importance of IL-10 producing regulatory cells, including NK cells in the induction and control of BD manifestations by affecting the level of IL-10 expression (24). The level of IL-10 expression in NK and other regulatory immune cells may be less than expected and contribute to the ongoing recurrent nature of the disease. However, a slight increase of IL-10 expression in the remission period may also contribute to the general regulatory milieu, which can mainly be maintained by other regulatory cell subsets. Therefore, NK cell may be contributors of regulation of inflammation in BD.

Key message

- NK cells contribute to the pathogenesis of BD with different cytokine profiles in relapse and remission periods.

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