ines and cytokine receptors has gained wide acceptance [12]: NK type 1 (NK1) cells mainly produce IFNγ and IL-10, and express high levels of IL-12 receptor β_2 (IL-12Rβ_2); while NK type 2 (NK2) cells produce IL-5 and/or IL-13, and express low levels of IL-12Rβ_2. This NK1/NK2 paradigm has been shown to control pathogenic Th1-biased or Th2-biased immune response in several human immune-mediated diseases, such as multiple sclerosis [13], asthma [14], and pemphigus vulgaris [15].

In the present study, we investigated the potential regulatory functions of NK cells in the Th1-biased environment of BD by evaluating their activation status, gene expression profiles, and functional properties in association with the disease status.

Materials and methods

Patients and controls

We studied 47 patients with BD (19 men and 28 women, aged 47.3 ± 17.6 years) who fulfilled the criteria proposed by an International Study Group [16]. Twenty-nine healthy individuals (14 men and 15 women, aged 38.2 ± 12.3 years) provided control samples.

The BD of the patients was classified as active (aBD) in 10 cases and inactive (iBD) in 37 cases at the time of blood sampling. Active disease was defined as flare of characteristic BD symptoms, including severe skin, mucosal, and/or ocular involvement that required introduction or increase of systemic corticosteroids (≥ 0.5 mg/kg), cyclosporine, and/or infliximab [6]. Five patients who had aBD at their first examination were re-evaluated after their BD-related symptoms resolved.

All samples were obtained after the patients and control subjects gave their written informed consent, approved by the International Review Boards of Keio University and Yokohama City University.

HLA-B51 typing

The presence or absence of HLA-B51 was determined by PCR of the genomic DNA using sequence-specific primers and sequence-based typing [17].

Cell preparations

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by Lymphoprep (Fresenius Kabi Norge AS, Oslo, Norway) density-gradient centrifugation. NK cells were purified by the MACS cell isolation system (Miltenyi Biotec, Bergisch Gladbach, Germany) as $CD14^-CD3^-CD56^+$ cells [15]. Namely, the $CD14^+$ cells and $CD3^+$ cells were depleted from PBMCs by incubation with anti-CD14 and anti-CD3 mAb-coupled magnetic beads, and then the $CD56^+$ cells were positively selected by incubation with anti-CD56 mAb-coupled magnetic beads, according to the manufacturer's protocol. The sorted fraction contained >99.6 ± 0.2% $CD56^+$ cells, and contamination with $CD3^+$ cells was <0.3 ± 0.3%. In some experiments, T cells were also isolated as $CD56^-CD3^+$ cells using the MACS cell isolation system.

Activated status of natural killer cells

PBMCs were incubated with the following combination of fluorescently labeled mAbs: anti-CD56-fluorescein isothiocyanate, anti-CD69-phycoerythrin-cyanin 5.1, and anti-CD3-allophycocyanin (Beckman-Coulter, Fullerton, CA, USA). Fluorescent cell staining was detected by a FACSCalibur® flow cytometer (Becton Dickinson, San Jose, CA, USA) using CellQuest™ software. Appropriate fluorescently labeled isotype-matched mAbs to irrelevant antigens were used in all analyses. The proportion of activated NK cells was assessed from the cells expressing CD69, an early activation marker of lymphocytes [18], within the $CD56^+CD3^-$ NK cell fraction.

Cytotoxic activity

The nonspecific cytotoxic activity of NK cells was quantified by a flow cytometry-based assay using NKTEST® (Orpegen Pharma, Heidelberg, Germany). Briefly, K562 target cells pre-stained with a lipophilic green fluorescent membrane dye were mixed with freshly isolated effector PBMCs at an effector-to-target ratio of 25:1 and were incubated for 2 hours at 37° C. Dead cells were detected by incubation with a DNA staining solution and subsequent analysis on a flow cytometer. The specific cytotoxicity (%) was determined by subtracting the proportion of dead cells in the mock-treated sample from the proportion in the sample pre-treated with effector cells.

Expression of genes associated with NK1/NK2 phenotype and cytotoxicity

The total RNA was extracted from MACS-sorted NK cells using an RNeasy® mini kit (Qiagen, Hilden, Germany), and was subjected to oligo (dT)-primed reverse transcription to generate first-strand cDNA. The cDNA equivalent to 5 ng total RNA was subjected to semiquantitative PCR to detect IL-12Rβ2, IFNγ, IL-5, IL-10, IL-13, perforin, granzyme B, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), using specific primer sets as described elsewhere [15]. The PCR products were fractionated on agarose gels and visualized by ethidium bromide staining. The intensity of individual bands was semiquantitatively analyzed using the Image/J® software [19]. The relative expression level of individual genes was normalized to the expression of GAPDH.

The mRNA expression of selected genes was further evaluated using a quantitative Taqman® real-time PCR system (Applied Biosystems, Foster City, CA, USA). All primers and probes were purchased from Applied Biosystems. The gene expression was standardized based on serial amounts of cDNA prepared from a healthy donor's

PBMCs that were stimulated with phorbol 12-myristate-13-acetate and ionomycin [15]. The relative expression levels of individual genes were normalized to the expression level of GAPDH.

Phosphorylation status of signal transducer and activator of transduction 4

The phosphorylated signal transducer and activator of transduction 4 (Stat4) and total Stat4 in IL-12-stimulated NK cells was detected by immunoblots as previously described [15]. The antibodies used were rabbit anti-phosphorylated-Stat4 polyclonal antibodies (Zymed Laboratories, South San Francisco, CA, USA) and rabbit anti-Stat4 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The intensity of individual bands with the expected molecular sizes was semiquantitatively analyzed using the image/J® software. The phosphorylation status of Stat4 was expressed as the ratio of the intensity of phosphorylated Stat4 to that of total Stat4.

IFNγ expression in CD4+ T cells co-cultured with natural killer cells

The capacity of NK cells to modulate the expression of IFNγ by T cells was evaluated using a cell-contact-free co-culture system. Briefly, MACS-sorted T cells (2×10^6) obtained from aBD patients were cultured in RPMI1640 supplemented with 7.5% low IgG fetal bovine serum (HyClone, South Logan, UT, USA) with or without sorted NK cells (5×10^5) prepared from iBD patients or healthy controls, applied to the upper chamber of an insert separated by a 0.4 μm pore-size membrane (BD Biosciences, San Jose, CA, USA) on 12-well plastic plates, for 12 hours at 37°C. Leukocyte Activation Cocktail® (5 μl/well; BD Biosciences) was added at the initiation of the culture. The T cells were then fixed and permeabilized using an Intracellular Cytokine Staining Kit Human® (BD Biosciences), and were subsequently stained with anti-IFNγ-phycoerythrin (BD Biosciences) and anti-CD4-phycoerythrin-cyanin 5.1 (Beckman-Coulter), according to the manufacturer's protocols. The appropriate fluorescently labeled control antibodies were used to define the background Immunofluorescence of the cells. Finally, the cells were subjected to flow cytometry, and the IFNγ expression level on the gated $CD4^+$ T cells was calculated as a mean fluorescence intensity using CellQuest™ software. The relative IFNγ expression was calculated as the ratio of IFNγ expression by $CD4^+$ T cells cultured with NK cells to the expression by $CD4^+$ T cells cultured alone.

Statistical analysis

All results are expressed as the mean ± standard deviation. Statistical comparisons between two groups were performed using the Mann-Whitney *U* test. Serial measurements were statistically evaluated by the Wilcoxon *t* test.

Results

Clinical features of Behçet's disease patients

Of 47 patients with BD, 100%, 61%, 96%, and 28% had had oral ulcer, uveitis, skin lesion, and genital ulcer, respectively, during the course of the disease. Only a small proportion of the patients had history of intestinal (6%), vascular (11%), and neurological (6%) involvement. HLA-B51 was detected in 28 patients (60%). Treatment at the time of blood sampling included colchicine (n = 13), low-dose prednisolone (n = 3), cyclosporine (n = 2), etanercept (n = 1), colchicine and low-dose prednisolone (n = 4), low-dose prednisolone and methotrexate (n = 1), colchicine, low-dose prednisolone and cyclosporine (n = 1), colchicine, low-dose prednisolone and azathioprine (n = 1), and colchicine, low-dose prednisolone and infliximab (n = 1). Twenty patients (42%) received no treatment.

Ten patients (21%) were classified as having aBD at the time of blood sampling, based on a major uveitis attack (n = 7), intestinal flare with a minor uveitis attack (n = 2), or exacerbation of mucocutaneous symptoms with high fever (n = 1). None of the aBD patients had concomitant flare of vascular or neurological involvement. There was no difference in the frequency of HLA-B51 or treatment regimens between aBD and iBD. Seven patients with uveitis attack were treated with infliximab (n = 4), cyclosporine (n = 1), or an increased dosage of prednisolone in combination with cyclosporine (n = 2), resulting in resolution of symptoms within 3 months. Two patients with intestinal flare were treated with infliximab, resulting in resolution of all intestinal symptoms within 3 months. The mucocutaneous flare in the remaining patient was improved by high-dose prednisolone in combination with an increase in the dosage of cyclosporine.

Activation status of natural killer cells

We determined the activation status of the circulating NK cells in seven patients with aBD, 22 patients with iBD, and 19 healthy controls by examining the CD69 expression on the NK cells. As shown in Figure 1, the proportion of $CD69^+$-activated NK cells was significantly greater in the aBD patients than in the iBD patients or healthy controls ($P = 0.01$ and $P = 0.003$, respectively). There was a trend toward an increased proportion of activated NK cells in the iBD patients compared with in healthy controls, but the difference did not reach statistical significance ($P = 0.1$). These findings indicate that *in vivo* activation of circulating NK cells is observed in patients with aBD, but is not remarkable in those with iBD.

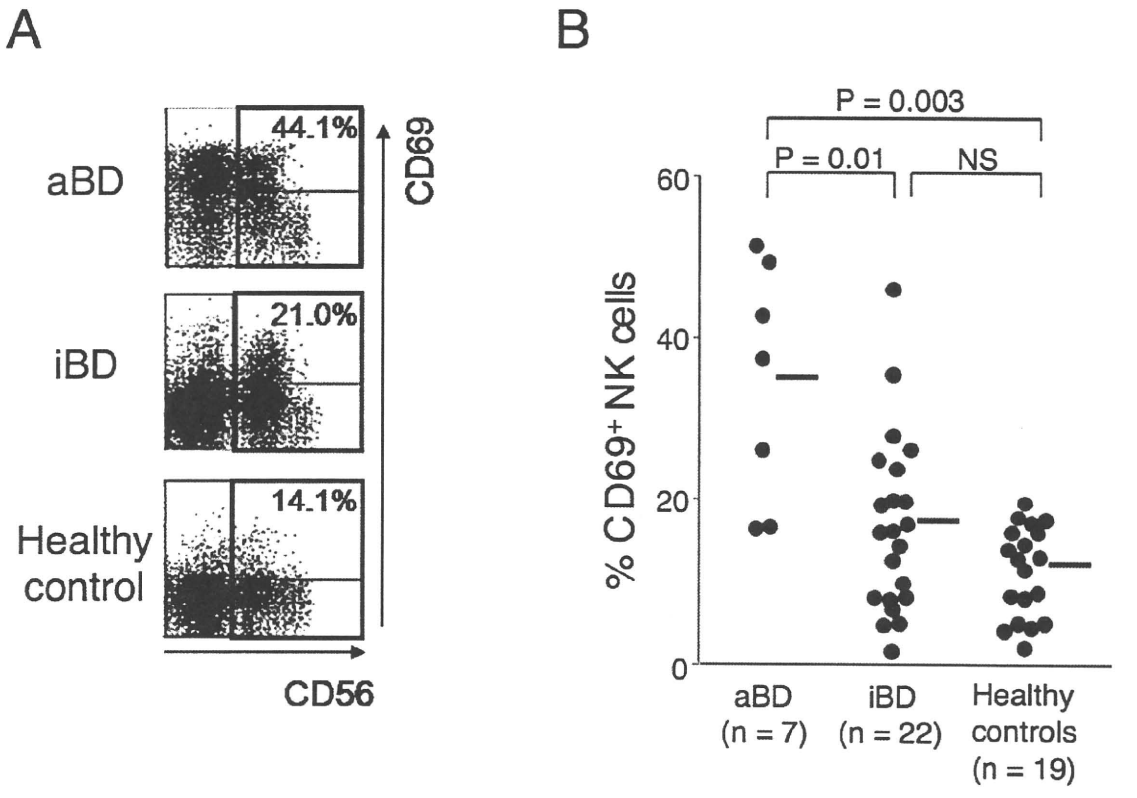

Figure 1 Activation status of natural killer cells in Behçet's disease. Proportion of activated natural killer (NK) cells in active Behçet's disease (aBD) patients, inactive Behçet's disease (iBD) patients, and healthy controls. **(a)** Representative dot-plot analysis for the expression of CD69 and CD56 in the gated $CD3^-$ lymphocytes from a patient with aBD, a patient with iBD, and a healthy control. The numbers indicate the proportion of $CD69^+$-activated cells in total NK cells. **(b)** Proportion of $CD69^+$-activated NK cells in seven aBD patients, 22 iBD patients, and 19 healthy controls. Horizontal bars, mean values. NS, not significant.

Cytotoxic activity of natural killer cells

There was no difference in the nonspecific cytotoxic activity among the NK cells from three patients with aBD, 10 patients with iBD, and 13 healthy controls (14.9 ± 10.3%, 14.3 ± 5.4%, and 14.4 ± 7.4%, respectively).

Gene expression profiles of natural killer cells

NK cells freshly isolated from aBD patients, iBD patients, and healthy controls were first subjected to semiquantitative PCR to measure the expression of genes associated with NK1/NK2 differentiation and cytotoxicity, including those encoding IL-12Rβ2, IL-5, IL-10, IL-13, IFNγ, perforin, and granzyme B. Of these molecules, the mRNA levels of IL-12Rβ2, perforin, and granzyme B were significantly lower, and that of IL-13 was significantly higher, in the iBD patients than in the aBD patients or healthy controls (P <0.05 for all comparisons) (data not shown). No IL-5 expression was detected in any of the samples from BD patients or healthy controls, and there was no statistically significant difference in the expression level of IL-10 or IFNγ.

To confirm the results obtained by semiquantitative PCR, the gene expression levels of IL-12Rβ2, IL-13, perforin, and granzyme B were further evaluated by quantitative TaqMan® real-time PCR (Figure 2). The IL-12Rβ2 expression was significantly lower in the iBD patients than in the aBD patients or healthy controls (P = 0.006 and P = 0.0002, respectively). The increased IL-13 expression was detected in a subset of patients with BD: most of them had iBD. The IL-13 expression level in iBD patients was significantly higher than the level in the healthy controls (P = 0.04), and tended to be higher than the level in aBD patients (P = 0.2). Interestingly, differences in IL-12Rβ2 and IL-13 levels were not detectable between the aBD patients and healthy controls.

These findings indicated that the NK cells from iBD patients have a gene expression profile compatible with NK2; that is, upregulated IL-13 and downregulated IL-

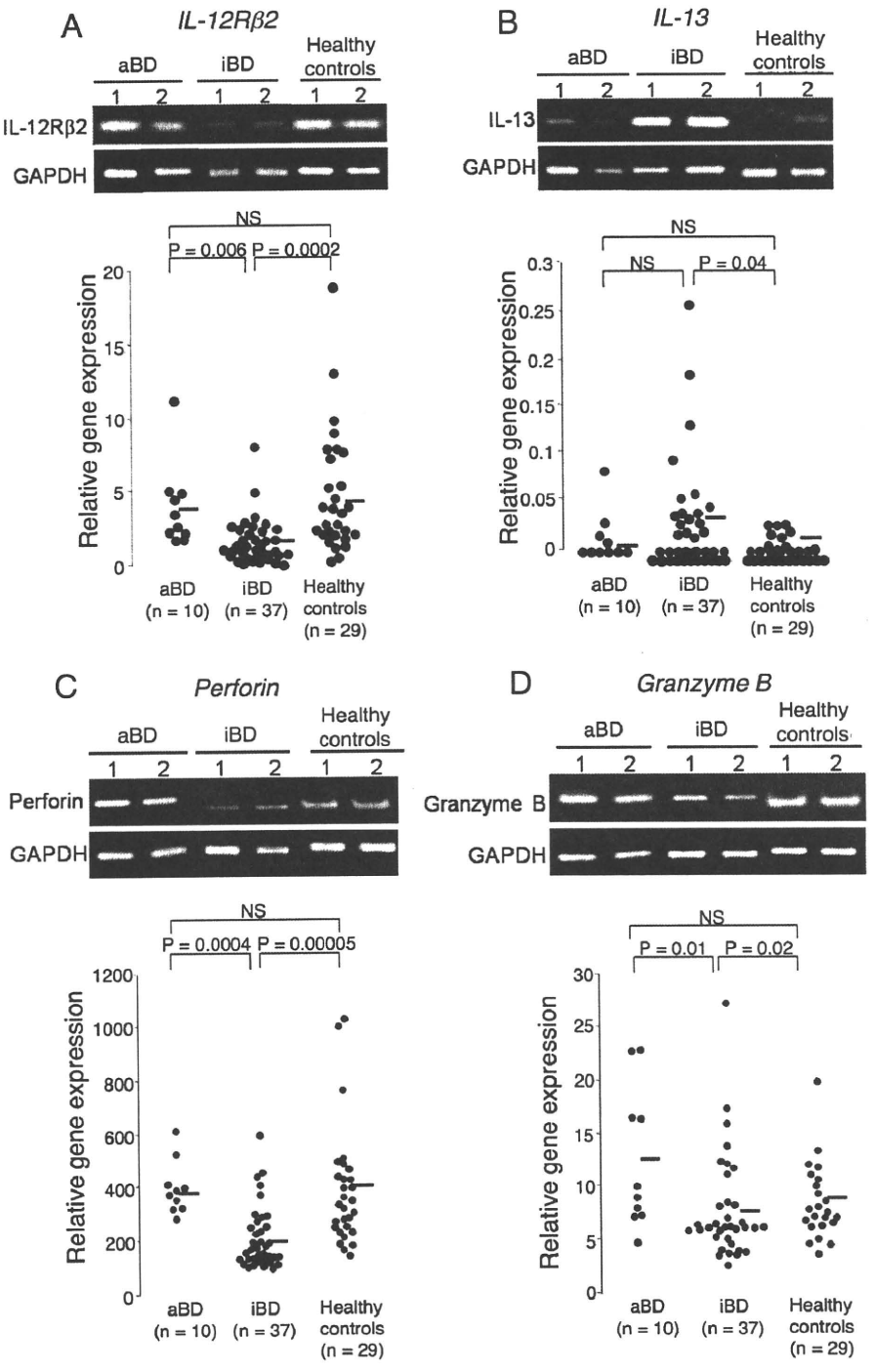

Figure 2 Gene expression levels of natural killer cells in Behçet's disease. Gene expression levels of **(a)** interleukin-12 receptor β_2 (IL-12Rβ2), **(b)** IL-13, **(c)** perforin, and **(d)** granzyme B in natural killer (NK) cells from active Behçet's disease patients (aBD), inactive Behçet's disease (iBD) patients, and healthy controls. The IL-12Rβ2, IL-13, perforin, and granzyme B expression levels in the NK cells were evaluated using semiquantitative PCR: two representative images each from aBD patients, iBD patients, and healthy controls are shown in the upper portion of each panel. The relative gene expression levels were further determined by quantitative Taqman® real-time PCR in 10 aBD patients, 37 iBD patients, and 29 healthy controls: results are shown in the lower portion of each panel. Horizontal bars, mean values. GADPH, glyceraldehyde-3-phosphate dehydrogenase; NS, not significant.

12Rβ2. On the other hand, the expression levels of perforin and granzyme B were significantly lower in the iBD patients than in the aBD patients or healthy controls (P <0.02 for all comparisons), while these expression levels were similar between these aBD patients and healthy controls.

Serial gene expression analysis of natural killer cells

For five aBD patients, additional blood samples were available when their BD symptoms were resolved after the introduction of infliximab (n = 2) or cyclosporine (n = 1), or of an increased dosage of prednisolone in combination with cyclosporine (n = 2). The gene expression level of IL-12Rβ2 was reduced in all five patients as the disease status became quiescent (P = 0.04) (Figure 3). IL-13 expression became detectable in three of the patients, and the change was borderline but did not reach a statistical significance (P = 0.05). These results strongly suggest that an NK2 shift was associated with disease remission. In addition, the expression level of perforin was reduced when the patients' disease status changed to remission (P = 0.02).

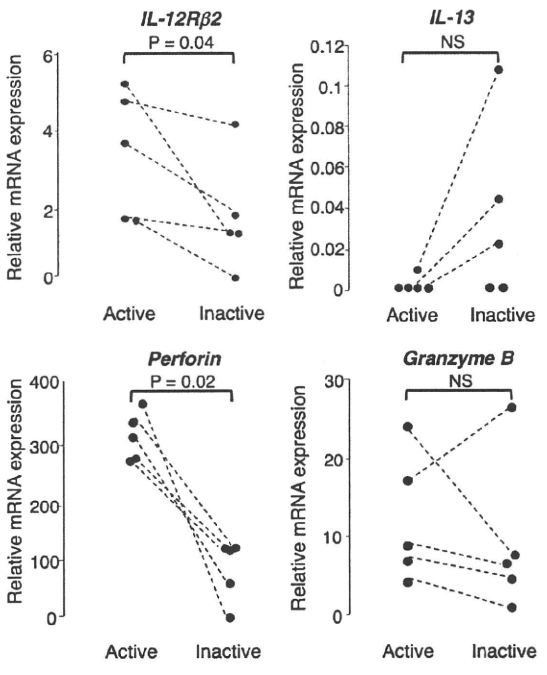

Figure 3 Serial gene expression measurements of natural killer cells in active Behçet's disease. Serial gene expression measurements of interleukin-12 receptor β_2 (IL-12Rβ2), IL-13, perforin, and granzyme B in natural killer (NK) cells from patients with active Behçet's disease at the first evaluation. The relative gene expression levels in NK cells were determined by quantitative PCR in samples obtained at the time of active disease and at a follow-up visit during remission. NS, not significant.

Impaired IL-12 signaling in natural killer cells from inactive Behçet's disease patients

The downregulated IL-12Rβ_2 gene expression observed in the NK cells from iBD patients could lead to impaired IL-12 signaling. To test this possibility, the phosphorylation status of Stat4, which is a downstream component of the IL-12 signaling pathway [20], was evaluated in the NK cells from six iBD patients and five healthy controls. As shown in Figure 4, the IL-12-induced Stat4 phosphorylation was significantly lower in the iBD patients than in the healthy controls (P = 0.02).

Capacity of natural killer cells from inactive Behçet's disease patients to suppress IFNγ expression by Th1 cells

The NK2 bias observed in patients with iBD led us to speculate that NK cells play a role in controlling the pathogenic Th1 response in BD patients. To evaluate this hypothesis, the NK cells from iBD patients or healthy controls were co-cultured with Th1 cells derived from aBD patients in a cell-contact-free system. The intracellular IFNγ expression in the gated $CD4^+$ T cells was then analyzed using flow cytometry (Figure 5). We found that the level of IFNγ expressed by Th1 cells was reduced after

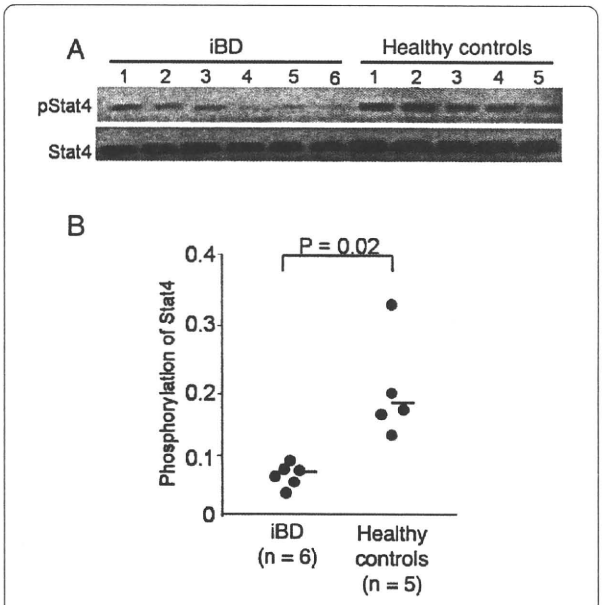

Figure 4 IL-12 signaling of natural killer cells in inactive Behçet's disease patients. IL-12-induced signal transducer and activator of transduction 4 (Stat4) phosphorylation in natural killer (NK) cells from inactive Behçet's disease (iBD) patients and healthy controls. **(a)** NK cells from six iBD patients and five healthy controls were stimulated with IL-12, and were subjected to immunoblotting for the detection of phosphorylated Stat4 (pStat4) and total Stat4. **(b)** Phosphorylation status of Stat4, which is expressed as the ratio of the intensity of pStat4 to that of total Stat4, in NK cells from six iBD patients and five healthy controls. Horizontal bars, mean values.

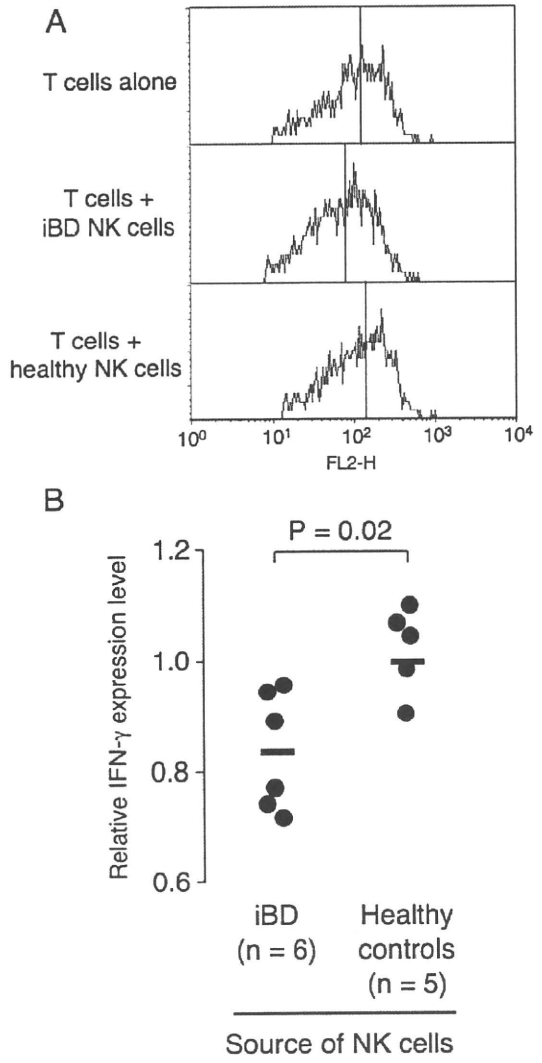

Figure 5 Natural killer cell suppression of IFNγ expression by T-helper 1 cells in inactive Behçet's disease. Suppression of IFNγ expression in T-helper 1 (Th1) cells by cell-contact-free co-culture with natural killer (NK) type 2 cells from inactive Behçet's disease (iBD) patients. T cells from active Behçet's disease patients were cultured alone or in combination with NK cells from healthy controls or iBD patients, and the IFNγ expression level in the $CD4^+$ T cells was evaluated using flow cytometry. **(a)** Representative histogram plots showing expression of IFNγ on gated $CD4^+$ T cells that were cultured alone, or with NK cells from an iBD patient or a healthy control. Vertical line in each histogram indicates the median. **(b)** IFNγ expression levels in Th1 cells cultured with the NK cells from six iBD patients or from five healthy controls. Relative IFNγ expression level calculated as the ratio of IFNγ expression by $CD4^+$ T cells cultured with NK cells to the expression by $CD4^+$ T cells cultured alone. Horizontal bars, mean values.

their co-culture with the NK cells derived from iBD patients. In fact, the relative IFNγ expression level was significantly lower in the Th1 cells co-cultured with iBD patients' NK cells compared with the level in those co-cultured with healthy controls' NK cells ($P = 0.02$). These findings suggest that the NK2 cells from iBD patients can suppress the Th1 response in aBD patients without cognate cell-cell contact.

Discussion

The present study has shown that the NK cells were phenotypically altered in BD patients, especially those in inactive disease status. Features of the circulating NK cells in iBD patients included: downregulated gene expression of IL-12Rβ_2; upregulated gene expression of IL-13 in a subset of the patients; downregulated perforin and granzyme B gene levels; and impaired IL-12-induced Stat4 phosphorylation. Upregulated IL-13 and downregulated IL-12Rβ2 observed in NK cells from iBD patients were compatible with the NK2 phenotype. A serial NK phenotype analysis in aBD patients supported the association between NK2 bias and inactive disease status. Furthermore, NK2 cells obtained from iBD patients directly suppressed the IFNγ expression of Th1 cells derived from aBD patients *in vitro*. These findings together suggest that the NK1/NK2 balance modulates disease flare/remission in BD patients by controlling the pathogenic Th1 response. This situation is analogous to multiple sclerosis, another Th1-mediated disease, in which NK2 bias is associated with disease remission [13].

A major limitation of this study is the small number of patients analyzed, especially those with aBD. During 2 years of the study period, only 10 patients with aBD were enrolled in two major university hospitals in the Tokyo metropolitan area. In addition, there was a limited chance of obtaining peripheral blood samples from patients with aBD, because such patients required immediate introduction of treatment. Further multicenter studies involving a large number of patients with aBD are necessary to confirm our findings. Another limitation is the difficulty in classifying BD patients into those with active disease and those with inactive disease. We used a strict definition to select patients with aBD: flare of characteristic BD symptoms that required introduction of the intensive treatment, such as high-dose corticosteroids, cyclosporine, and infliximab. Patients with mild mucocutaneous manifestations or minor uveitis attack, which did not require intensive therapy, were therefore classified as having iBD. This clinical heterogeneity in the iBD subset may result in variability in the gene and protein expression profiles. Additional analysis according to individual clinical manifestations and/or treatment regimens would clarify these issues, but again the number of patients enrolled was too small to conduct subanalysis. Finally, we should recognize

that a series of experiments involved only a subset of the patients and controls, which potentially bias the results. Our results suggest that the NK2 cells in iBD patients can suppress the Th1 response through at least two distinct mechanisms. First, the NK2 cells in iBD patients were intrinsically hyporesponsive to IL-12 due to their downregulated expression of IL-12Rβ_2 and impaired IL-12 signaling, resulting in deficient IFNγ production even in the Th1 environment. Second, the NK2 cells from iBD patients actively suppressed IFNγ expression in aBD-derived Th1 cells. A similar inhibitory effect of human NK2 cells on the production of IFNγ by T cells was also reported for healthy individuals' NK cells that were induced to express the NK2 phenotype [13], and for NK2 cells obtained from multiple sclerosis patients in remission [21]. Taken together, the NK cells and T cells - two major IFNγ producers - were deficient in IFNγ production in the NK2-biased immune environment observed in iBD patients.

How the NK2 cells from iBD patients suppress the IFNγ expression in Th1 cells, however, remains unclear. One potential soluble mediator in our cell-contact-free culture system is IL-13, a typical T-helper 2 cytokine that inhibits Th1 responses *in vitro* and *in vivo* [22,23], although upregulated IL-13 expression was detected only in one-third of the iBD patients. In addition, this IL-13-mediated inhibitory effect is reported to occur predominantly through the modulation of antigen-presenting cells rather than as a direct effect on T cells [22]. Additional soluble factors secreted from NK2 cells are likely to be involved in this regulation, but the NK cells from iBD patients did not express IL-5, which plays a primary role in Th1 inhibition in multiple sclerosis patients in remission [13]. Furthermore, it has been reported that NK cells modulate Th1 responses also by interacting directly with T cells, B cells, and dendritic cells though cognate cell-cell contact [24,25].

Perforin and granzyme B, major cytoplasmic granule toxins, were downregulated in the NK cells from patients with iBD. Interestingly, this gene expression profile is analogous to that of the NK cells in patients with active pemphigus vulgaris, who also show NK2 bias [15]. This phenomenon could be explained by the reduced IL-12Rβ_2 expression and impaired IL-12 signaling, but the cytotoxic activity was the same among the NK cells of iBD patients, aBD patients, and healthy controls. The reason for this inconsistency is unknown, but the cytotoxic activity of NK cells might be regulated by more complicated mechanisms, involving a balance between activating and inhibitory NK receptors, as well as the expression of the ligands for death receptors on target cells [26].

In aBD patients, the proportion of activated NK cells in the circulation was markedly increased. This is reasonable because IL-12 can activate NK cells in the Th1 environment, even though the nonspecific cytotoxic activity and gene expression profiles were similar between the NK cells from aBD patients and healthy controls. These activated NK cells would migrate to sites of inflammation and contribute to the ongoing tissue damage in aBD patients, but this appears to be just a bystander effect of the Th1 environment of aBD.

Conclusions

The present study is the first demonstrating a novel regulatory role for NK cells in the pathogenic process of BD. Our results have suggested that NK cells are actively involved in the induction and maintenance of disease remission in BD patients, through NK2 polarization. Future studies aimed at elucidating the mechanisms that control the NK1/NK2 paradigm in BD patients may be useful for developing new NK cell-targeted therapeutic strategies for BD.

Abbreviations

aBD: active Behçet's disease; BD: Behçet's disease; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; iBD: inactive Behçet's disease; IFN: interferon; IL: interleukin; IL-12Rβ_2: interleukin-12 receptor β_2; mAb: monoclonal antibody; NK: natural killer; NK1: natural killer type 1; NK2: natural killer type 2; PBMC: peripheral blood mononuclear cell; PCR: polymerase-chain reaction; Stat4: signal transducer and activator of transduction 4; Th1: T-helper 1.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YY performed the acquisition of data, and analysis and interpretation of the data, and wrote the manuscript. HT made a substantial contribution to the acquisition of data. TS and YO performed the acquisition of data. NM, KT, and ZI provided peripheral blood samples and clinical information, and performed analysis of the data. MK designed the experiments, performed data analysis and interpretation, and wrote the manuscript. All authors read and approved the final manuscript.

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Review Article

Interleukin-6 as a Potential Therapeutic Target for Pulmonary Arterial Hypertension

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Interleukin-6 (IL-6) is a pleiotropic cytokine with a wide range of biologic activities in immune regulation, hematopoiesis, inflammation, and oncogenesis. Recent accumulating evidence indicates a pathologic role for IL-6 in promoting proliferation of both smooth muscle and endothelial cells in the pulmonary arterioles, resulting in development of pulmonary arterial hypertension (PAH). Here, we describe a patient with mixed connective tissue disease and severe, refractory PAH. Her functional activity and hemodynamic parameters dramatically responded to tocilizumab, a humanized monoclonal antibody to human IL-6 receptor, which was aimed at treating multicentric Castleman's disease. It appears that IL-6 blockade may hold promise as an adjunct drug in treatment of PAH in idiopathic form as well as in association with connective tissue disease.

1. Introduction

Pulmonary arterial hypertension (PAH) is a cause of significant morbidity and mortality in patients with connective tissue disease (CTD), especially in those with systemic sclerosis (SSc) or mixed connective tissue disease (MCTD) [1]. In fact, a survival study over the past 30 years in consecutive patients evaluated at the University of Pittsburgh has demonstrated that PAH became the primary cause of SSc-related deaths today [2]. PAH is characterized by increased pulmonary vascular resistance due to remodeling of the pulmonary arterioles. Left untreated, PAH leads irremediably to right ventricular hypertrophy, pressure overload and dilation, and impaired cardiac output, resulting in death [3]. Until recently, there was no effective therapy for PAH, a disease with a median survival estimated to be approximately one year following the diagnosis in patients with SSc [4]. However, in the past two decades, novel therapies have been developed, focusing on vasoactive substances derived from the pulmonary vascular endothelium [5]. These substances, such as endothelin-1, nitric oxide, and prostacyclin regulate smooth muscle cell tone and proliferation and were shown to be central to the pathogenesis of PAH [6]. Therefore, current therapeutic agents target these 3 essential biological pathways: the endothelin-1/endothelin receptor, nitric oxide/cGMP, and prostacyclin/cAMP pathways. Improvement of symptoms, functional activity, and quality of life and even prolongation of survival have been partially achieved with currently available therapies, but mostly in patients with idiopathic PAH [5]. Indeed, it has become clearer in the past few years that SSc patients with PAH have a strikingly divergent response to current therapies and overall worse outcome compared with patients with idiopathic PAH in spite of seemingly milder hemodynamic impairment [7, 8]. In a recent multicentre longitudinal study to evaluate 3-year survival in SSc patients, 20 of 47 patients with PAH died during follow-up, giving a 3-year survival of only 56%, despite the fact that they were treated with modern PAH drugs [9]. Even in SSc patients with mildly symptomatic PAH in New York Heart Association (NYHA) functional class II, approximately two-thirds deteriorated to functional class III or IV, and some died during a 5-year period, although they were treated with one or more PAH drugs [10]. While there have been significant

advances in the treatment of PAH, survival of patients with PAH associated with CTD on modern PAH drugs remains unacceptably low. Therefore, novel therapeutic strategies targeting pathways beyond pulmonary vascular endothelium are required to further improve survival of CTD patients with PAH.

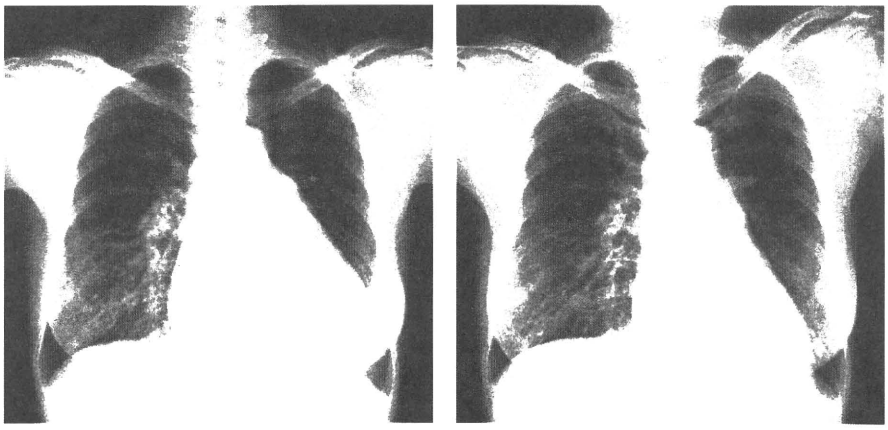
We have recently experienced a rare case of PAH-CTD complicated by multicentric Castleman's disease (MCD) during the course of the disease. MCD was successfully treated with tocilizumab, a humanized antihuman interleukin6 (IL-6) receptor monoclonal antibody, which dramatically improved functional activity and hemodynamic parameters of PAH as well.

2. Case Report

A 45-year-old woman first noticed polyarthralgia and puffy fingers in 1997 and developed slowly progressive dyspnea on exertion, which made her hospitalization in a regional hospital in 2001. Pulmonary hypertension was detected by transthoracic echocardiography, which showed mild right ventricular hypertrophy in conjunction with abnormal contour of the interventricular septum and increased systolic pulmonary arterial pressure (PAP) (100 mmHg) estimated by Doppler echocardiography. Interstitial lung disease (ILD) and pericardial effusion were also detected. Taken together with increased levels of C-reactive protein (CRP), positive antinuclear, and anti-U1RNP antibodies, she was diagnosed as having mixed connective tissue disease (MCTD) complicating pulmonary hypertension. She was treated with corticosteroid pulse therapy followed by high-dose prednisolone (1 mg/kg), resulting in improvement of exertional dyspnea and reduction in estimated systolic PAP to 60 mmHg. In November 2005, she visited a pulmonologist of the referring centre because of worsening dyspnea. She underwent a systematic cardiac evaluation, including right heart catheterization and ventilation-perfusion scan, and a diagnosis of PAH in NYHA functional class III was made based on mean PAP 58 mmHg, pulmonary capillary wedge pressure (PCWP) 10 mmHg, cardiac output 3.4 L/min, and pulmonary vascular resistance (PVR) 14.4 Wood units. The 6-minute walk distance (6MWD) was only 300 meters. Bosentan 250 mg was initiated with oxygen supplementation in January 2006, with subtle improvement of exertional dyspnea. After summer of 2007, her symptom gradually worsened again. In addition, she experienced low-grade fever, loss of appetite, and body weight loss (−5 kg/6 months) with cervical lymphoadenopathy and hepatosplenomegaly, which had worsened despite the use of low-dose prednisolone. She was referred to our hospital for additional evaluation into the etiology of PAH in April 2008.

She had marked limitation of physical activity (NYHA functional class III), and 6MWD was only 310 meters. Physical examination demonstrated jugular venous dilatation, lower extremity edema, and lymphoadenopathy on cervical, axillary, and inguinal lesions. Nailfold capillary changes were found, but sclerodactyly, muscle weakness, arthritis, and butterfly rash were absent. Laboratory data showed marked anemia (hemoglobin 8.1 g/dL), hypoalbuminemia (2.9 g/dL), polyclonal hypergammaglobulinemia with IgG 6451 mg/dL, CRP 9.1 mg/dL, brain natriuretic peptide (BNP) 181 pg/mL, a positive antinuclear antibody at a titer of 1 : 1,280 with pure speckled pattern, and a positive anti-U1 RNP antibody (86 Index; normal range <15). The protrusion of the main pulmonary artery, increased width of the descending branch of the right pulmonary artery, and an increase in the cardiothoracic ratio were noted on chest X-ray (Figure 1). Electrocardiogram showed signs of an increased right heart load. Hemodynamics assessed by right heart catheterization included man PAP 43 mmHg, PCWP 11 mmHg, right atrial pressure (RAP) 12 mmHg, cardiac output 5.5 L/min, and PVR 5.8 Wood units, indicating that 2-year treatment with bosentan partially improved these parameters. High-resolution computed tomography showed ground-glass opacities with minimal honeycomb cysts on bilateral lower lung field, dilatation of the right atrium, right ventricle, and central pulmonary arteries, and multiple mediastinal lymphadenopathy. Histological evaluation of the biopsied axillary lymph node demonstrated an intense plasmacytosis in the interfollicular areas with a prominent increase in capillaries and postcapillary venules, some of which were hyalinized. These findings were compatible with MCD in mixed histological features of plasma cell and hyaline vascular types [11]. Serology for human immunodeficiency virus (HIV) or human herpesvirus type 8 (HHV-8) was negative. Cytomegalovirus antigenemia was undetectable. Gene sequence for HHV-8 or Epstein-Barr virus was not found in the lymph node. A markedly elevated level of serum IL-6 (41.8 pg/mL; normal range <4) was consistent with the diagnosis of MCD [12]. Thus, we decided to first treat concomitant MCD with tocilizumab at a dose of 8 mg/kg every 2 weeks.

Serial functional, hemodynamic, and laboratory parameters before and after the tocilizumab treatment are summarized in Table 1. After 4 infusions of tocilizumab, low-grade fever and loss of appetite were completely gone, and fatigue was prominently improved with normalization of hemoglobin level. A prominent increase in circulating IL-6 concentration after introduction of tocilizumab indicated efficient IL-6 receptor blockade. Lymphadenopathy and hepatosplenomegaly were gradually resolved and were finally undetectable at 3 months after introduction of tocilizumab. Functional activity was gradually improved to NYHA functional class II and 434 meters at 6MWD. BNP was decreased to 48 pg/mL. Right heart catheterization at 3 months revealed that mean PAP was reduced to 31 mmHg and RAP was 5 mmHg. There was no improvement in cardiac output or PVR, but cardiac output was calculated with the Fick method by an equation containing a hemoglobin level, which rose up markedly after the tocilizumab treatment. In fact, systemic venous oxygen saturation had dramatically improved from 52.1% to 69.4%. Her dyspnea has continued to improve and was finally undetectable (NYHA functional class I) at 6 months when oxygen supplementation was discontinued. The increased right heart load findings on chest X-ray were remarkably improved (Figure 1). After 12 months of treatment with tocilizumab, she was in NYHA functional



Before tocilizumab (a)

After 6-month treatment with tocilizumab (b)

FIGURE 1: Chest X-ray before tocilizumab and after 6-month treatment with tocilizumab.

class I and was able to walk 663 meters at 6MWD without reduction of arterial oxygen saturation. The mean PAP was further decreased to 27 mmHg. The patient has been in NYHA functional class I and in remission of MCD on biweekly tocilizumab, as of May 2010. No side effect of the tocilizumab treatment was apparent.

3. IL-6 Overproduction and PAH

MCD is a rare lymphoproliferative disorder characterized by systemic lymphadenopathy and constitutional inflammatory symptoms [11]. Patients with MCD frequently have systemic manifestations, such as low-grade fever, fatigue, loss of appetite, and weight loss. Abnormal laboratory findings include anemia, hypoalbuminemia, hypergammaglobulinemia, and increased acute-phase proteins such as CRP. The etiology of the disease appears to be heterogeneous, but dysregulated overproduction of IL-6 is believed to be responsible for the clinical abnormalities [12]. In fact, IL-6 transgenic mice represented the disease phenotype resembling MCD, which was successfully treated with an anti-IL-6 receptor antibody [13]. In a multicenter prospective study to evaluate the efficacy of tocilizumab in patients with MCD, objective improvement was consistently observed in clinical symptoms, lymphadenopathy and other physical findings, and laboratory parameters [14]. In addition, HHV-8 is reported to be an etiologic agent of MCD, especially in patients infected with HIV [15], since HHV-8 encodes a human IL-6 homolog, which shares functional properties with human IL-6 [16].

PAH is a rare complication of MCD [11], and only 4 cases diagnosed with both of these conditions have been reported previously [17–19]. These case reports raise several hypotheses linking PAH and MCD. One hypothesis includes an association of PAH with HHV-8 infection rather than MCD itself. HHV-8 encodes genes homologous to human genes involved in cell proliferation, antiapoptosis, and angiogenesis [20, 21], and HHV-8 gene sequences have been found in plexiform lesions derived from some patients with idiopathic PAH [22], suggesting the possibility that HHV-8 could be involved in the misguided angiogenesis characteristic of PAH. However, HHV-8 infection was not detected in our case as well as in another 2 reported cases complicating MCD and PAH [17, 19]. One of the reported case infected with HIV and HHV-8 showed an unusual complete reversibility of both MCD and severe PAH, with an immunosuppressive treatment with cyclophosphamide, together with highly active antiretroviral therapy and epoprostenol [18]. In addition, tocilizumab induced partial remission of both MCD and PAH in our case as well as in the other HHV-8-uninfected case [19]. These case reports raise another hypothesis that IL-6 is a common pathogenic factor in both MCD and PAH.

Patients with idiopathic PAH are consistently found to have an increased level of IL-6 in circulation [23, 24] and in lung tissue [25]. In patients with lupus, MCTD, or SSc, a higher serum IL-6 level was reported in patients with PAH than in those without PAH [26–28]. In addition, elevated serum IL-6 was reported in patients with POEMS (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes) syndrome, a rare variant of plasma cell dyscrasia, which sometimes complicates PAH [29]. Several animal models of PAH, including chronic hypoxia [30] and monocrotaline treatment [31], are also associated with increased production of IL-6. Moreover, daily subcutaneous injection of recombinant IL-6 in rats induced the medial thickness of small pulmonary arteries, leading to PAH [32]. These findings together suggest that PAH development is associated with IL-6 overproduction.

4. Roles of IL-6 in Pathogenesis of PAH

IL-6 is a pleiotropic cytokine with a wide range of biologic activities in immune regulation, hematopoiesis, inflammation, and oncogenesis [33]. Accumulating evidence indicates

Table 1: Serial functional, hemodynamic, and laboratory parameters before and after the tocilizumab treatment.

	Pretreatment	3 months	6 months	9 months	12 months
NYHA functional class	III	II	I	I	I
6MWD (m)	310	434	ND	ND	663
Mean PAP (mmHg)	43	31	ND	ND	27
PCWP (mmHg)	11	4	ND	ND	4
RAP (mmHg)	12	2	ND	ND	3
Systemic venous oxygen saturation (%)	52.1	69.4	ND	ND	75.3
Cardiac output (L/min)	5.5	4.5	ND	ND	4.4
PVR (wood unit)	5.8	5.6	ND	ND	5.3
Doppler systolic PAP (mmHg)	100	90	72	51	54
BNP (pg/mL)	181	48	44	46	37
CRP (mg/dL)	9.01	0.54	0.25	0.12	0.04
IgG (mg/dL)	6,451	3,266	2,679	2,433	2,238
Hemoglobin (g/dL)	8.0	12.9	13.9	13.0	12.8
IL-6 (pg/mL)	41.8	1,100	801	806	756

ND, 6MWD and hemodynamic assessment by right heart catheterization were not done at 6 and 9 months.

pathological roles for IL-6 in various disease conditions, such as inflammatory, autoimmune, and neoplastic diseases. Pathologic features in patients with PAH are characterized by muscularization of distal pulmonary arterioles, concentric intimal thickening, and obstruction of the vascular lumen by proliferating endothelial cells to form plexiform lesions [34]. It has been proposed that dysregulated cellular growth and apoptosis are responsible for a typical proliferative cellular phenotype, resulting in pulmonary vascular remodeling in PAH. On the other hand, perivascular infiltration of inflammatory cells, consisting of T cells, B cells, and macrophages, are often present within and around the affected pulmonary arteries of patients with PAH, suggesting that cytokines and growth factors secreted from these inflammatory cells may be involved in uncontrolled proliferation of pulmonary artery smooth muscle and endothelial cells [35].

In this regard, a lung-specific overexpression of IL-6 in mice resulted in increased PVR and pathological lesions similar to that seen in patients with PAH, including distal arteriolar muscularization, plexogenic arteriopathy, and periarteriolar infiltration of T cells [36]. These findings indicate that IL-6 directly or indirectly promotes proliferation of both smooth muscle and endothelial cells, which are potentially mediated through a number of proliferative, prosurvival, and anti-apoptotic processes (Figure 2). In this regard, IL-6 triggers vascular smooth muscle cell proliferation through upregulated expression of vascular endothelial growth factor (VEGF) and its receptor VEGFR2 [36, 37], which was observed in the plexiform lesions of patients with PAH [38].

It has been known that transforming growth factor (TGF)-β/bone morphogenetic protein (BMP) signaling controls growth of vascular smooth muscle and endothelial cells by inhibiting excessive proliferation. Genetic mutations in the gene encoding the type II receptor of BMP (BMPR2) comprise a genetic hallmark of heritable PAH [39], and downregulated protein expression of BMPR2 has also been described in nonheritable PAH [40]. A recent study by Brock et al. demonstrated that IL-6 repressed protein expression of BMPR2 through overexpression of microRNA cluster 17/92 [41]. MicroRNAs regulate posttranslational mechanisms by binding to their target mRNAs and by altering mRNA stability or affect protein translation [42]. Interestingly, protein expression of TGFβR2, another receptor from the identical protein family, was also modulated by the same microRNA cluster [43]. This may explain the lack of TGFβR2 expression in plexiform lesions of patients with PAH [44]. The promoter region of the microRNA 17/92 gene C13 or f25 has a highly conserved binding site for STAT3, a major IL-6 signal transduction pathway [33]. In fact, persistent activation of STAT3 resulted in repressed protein expression of BMPR2 [41].

Angiopoietin-1 (Ang-1)-Tie2 pathway is essential for both embryonic and postnatal angiogenesis and involves in a protective action on endothelial cells by suppressing inflammation and apoptosis [45]. The role of this system in the pathogenesis of PAH has been poorly understood, but a recent study using Tie2-deficient mice found that endothelial survival signaling via the Ang-1-Tie2 pathway is protective in PAH [46]. Exposure of IL-6 decreased expression of Ang-1 in lung vascular smooth muscle cells, leading to reduction of Tie2 activity in endothelial cells and resultant excessive apoptosis.

On the other hand, a constitutive activation of STAT3 was described in vascular smooth muscle cells as well as in endothelial cells from the lung tissue of patients with PAH [47]. Upon stimulation with IL-6, endothelial cells produce CX3CL1/fractalkine, a potent chemokine that recruits monocytes and lymphocytes into the lung [48]. These mononuclear infiltrates are major source of IL-6, but vascular smooth muscle cells and endothelial cells also produce IL-6 upon stimulation with IL-6 [36, 49]. Taken together, IL-6 promotes the development and progression

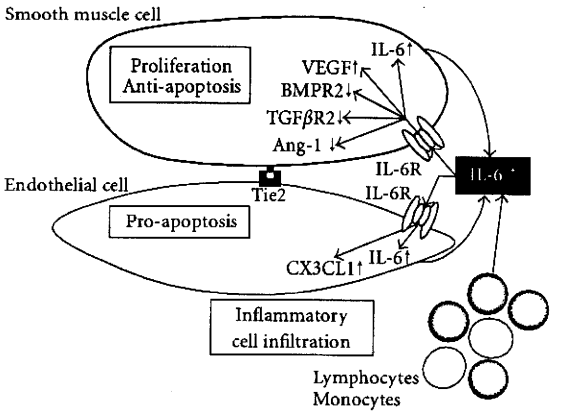


FIGURE 2: Hypothetical mechanism leading to pulmonary vascular remodeling via overexpression of IL-6. IL-6 induced proliferation and antiapoptosis in vascular smooth muscle cells through upregulation of VEGF, and downregulation of BMPR2 and TGFβR2. Upon IL-6 exposure, endothelial cells undergo apoptosis through repressed Tie2 signaling via downregulated Ang-1 expression in smooth muscle cells. Production of CX3CL1 results in recruitment of inflammatory cells, such as lymphocytes and monocytes, which produce enormous amount of IL-6, while vascular smooth muscle and endothelial cells also produce IL-6 upon stimulation with IL-6.

of pulmonary vascular remodeling, leading to PAH, via a variety of mechanisms.

5. The "Second-Hit" Process for Developing PAH

However, PAH observed in mice undergoing IL-6 overexpression was subtle, but was hemodynamically and histologically remarkable upon hypoxia exposure [36, 50]. In contrast, IL-6 knockout mice exposed to hypoxia were resistant to the development of increased PVR [51]. This is consistent with the "second-hit" theory for the pathogenesis of PAH, in which the response to an environmental or endogenous trigger is enhanced in susceptible individuals [52]. For example, exposure to serotonergic or inflammatory stressors produced an enhanced pulmonary hypertensive response in BMPR2 deficient mice [53]. Therefore, it is likely that overproduction of IL-6 alone has minimal impact on development of PAH, but the combination with other factors known to enhance susceptibility to pulmonary vascular remodeling, such as hypoxia and vasculopathy in CTD, appears to have synergistic effects resulting in the development of significant PAH.

The contribution of IL-6 to the pathogenesis of PAH may be different among associated conditions. In this regard, circulating IL-6 level was increased in patients with SSc, and the increased level was associated with the presence of PAH [28, 54]. In addition, the IL-6 level in bronchoalveolar lavage fluid was also increased in patients with SSc irrespective of the presence or absence of interstitial lung disease [55]. IL-6 is abundantly produced *in vitro* by affected skin fibroblasts and alveolar macrophages derived from patients with SSc [56, 57]. These findings together indicate that the lung tissue of SSc patients is always exposed to IL-6. This may explain why prevalence of PAH is the most frequent in patients with SSc among CTDs.

6. Summary and Future Perspectives

Current therapeutic strategies for PAH focus on the pulmonary vascular endothelium and its role in regulating smooth muscle cell tone and proliferation. By using these modern PAH drugs, treatment of PAH has undergone an extraordinary evolution even in patients with CTD, but PAH still remains a chronic intractable disease without a cure. A better understanding of the underlying pathophysiology of the pulmonary vasculature is needed for better therapy. In this regard, the findings of pathologically aberrant proliferation of smooth muscle and endothelial cells as well as increased expression of secreted growth factors, such as VEGF and platelet-derived growth factor (PDGF), in PAH have caused a shift in paradigm in treatment strategies for this disease [58]. The efficacy of imatinib, a prototypical PDGF receptor signaling inhibitor, was reported in patients with severe PAH [59–61], and a phase II multicenter clinical trial of imatinib in patients with PAH has been completed in the United States and Europe and the results are pending. On the other hand, our case report indicates that IL-6 is another potential therapeutic target for PAH.

In summary, this report describes our experience with the use of tocilizumab in a patient with MCTD and PAH. The rationale for such treatment derives from a numerous basic studies showing a critical role of IL-6 in the promotion of pulmonary vascular remodeling and consequent development of PAH. The concept underlying use of IL-6 blockade in PAH is prevention and reversal of lung vascular remodeling rather than prolonged vasodilation of pulmonary arteries. We recognize the limitations of a single case report, but we believe that blockade of IL-6 signaling may be a promising new therapy for PAH, especially in the context of CTD. Further studies of IL-6 blockade in PAH patients with and without CTD are warranted.

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Clinically amyopathic dermatomyositis

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Purpose of review

Clinically amyopathic dermatomyositis (CADM) is a unique subset of dermatomyositis, with typical skin manifestations of dermatomyositis but little or no evidence of myositis. This review focuses on updates on epidemiology, clinical manifestations, and autoantibody profiles in patients with CADM.

Recent findings

A population-based survey of dermatomyositis conducted in the United States revealed that overall age-adjusted and sex-adjusted incidence of CADM was 2.08 per 1 million persons. CADM consisted of approximately 20% of dermatomyositis. In general, late-onset myositis was infrequent. There was no apparent difference in frequency of internal malignancy or interstitial lung disease between CADM and classic dermatomyositis. However, anecdotal and retrospective case reports from eastern Asia showed a relatively high incidence of rapidly progressive interstitial lung disease, which is often fatal, in patients with adult-onset and juvenile-onset CADM. Finally, RNA helicase encoded by melanoma differentiation-associated gene 5 was identified as an autoantigen recognized by anti-CADM-140 antibody, which is associated with CADM and rapidly progressive interstitial lung disease.

Summary

CADM is a distinct clinical entity with unique clinical features and autoantibody profiles different from classic dermatomyositis.

Keywords

autoantibody, clinically amyopathic dermatomyositis, dermatomyositis, interstitial lung disease, malignancy

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Introduction

Idiopathic inflammatory myopathies are a heterogeneous group of disorders characterized by inflammation of skeletal muscles. Clinical manifestations of this clinical entity are diverse with various degrees of muscle, skin, and lung involvement. Dermatomyositis is one of the idiopathic inflammatory myopathies and has muscle manifestations (i.e. proximal muscle weakness, elevated serum levels of enzymes derived from skeletal muscle, myopathic changes by electromyography, and muscle biopsy evidence of inflammation) as well as a variety of cutaneous manifestations, such as heliotrope rashes and Gottron's papules. However, some dermatologists had noticed individuals who displayed hallmark cutaneous lesions of dermatomyositis as an isolated clinical finding and had not developed muscle weakness for a prolonged period. This condition is now termed 'clinically amyopathic dermatomyositis (CADM)', the term proposed by Sontheimer [1]. However, whether CADM is a distinct clinical entity or just an early phase of classic dermatomyositis is still debated. Here, we overview recent updates on epidemiologic, clinical, and immunologic characteristics of CADM.

Definition of clinically amyopathic dermatomyositis

Before the 1990s, the majority of patients with typical dermatomyositis skin manifestations but little or no evidence of myositis had been referred to dermatologists. Such a condition was known especially among dermatologists for many years as 'dermatomyositis sine myositis'. Pearson [2] used the term 'amyopathic dermatomyositis (ADM)' to describe this condition in his writing in 1979. Euwer and Sontheimer [3] also used the same term to describe six patients who manifested typical dermatomyositis rashes but without muscle symptoms for more than 2 years at the time of diagnosis. However, patients with cutaneous manifestations of dermatomyositis without apparent muscle weakness sometimes had subclinical evidence of myositis on laboratory and/or electromyographic evaluation; this condition is referred to as hypomyopathic dermatomyositis (HDM). To avoid confusion

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over the terms to describe similar clinical entities, Sontheimer [1] has proposed strict diagnostic criteria for ADM, HDM, and classic dermatomyositis in 2002. Specifically, patients with ADM show typical skin manifestations of dermatomyositis for 6 months or longer with no clinical evidence of myositis, including proximal muscle weakness and serum muscle enzyme abnormalities. If electromyography and muscle biopsy are carried out, the results should be within normal limits. If such results are suggestive of the presence of subclinical myositis, the patient should be classified as having HDM. However, clinical significance of extensive muscle testing in this condition is unclear. Thus, ADM and HDM are combined into the umbrella designation of CADM mainly for practical reasons. Exclusion criteria include treatment with systemic immunosuppressive therapy for 2 consecutive months or longer within the first 6 months after onset of skin lesions, and use of drugs at the time of skin disease onset that are known to be capable of inducing isolated dermatomyositis-like skin changes, such as hydroxyurea.

Sontheimer [4] recently proposed a modification of the original diagnostic criteria for CADM. In these criteria, CADM is regarded as having hallmark cutaneous manifestations of dermatomyositis for 6 months or longer with no clinically significant muscle involvement or interstitial lung disease (ILD). This modification results in selection of 'pure CADM' without any systemic complications, but clinical significance should be widely discussed. In contrast, Cao *et al.* [5•] have proposed to use the term 'dermatomyositis-like skin disease' instead of CADM, because patients with CADM appear to be at risk for developing ILD, malignancy, and/or delayed onset of myositis, which are systemic complications observed in patients with classic dermatomyositis.

Epidemiology of clinically amyopathic dermatomyositis

The majority of rheumatologists were unaware of CADM because such patients did not satisfy diagnostic criteria for dermatomyositis proposed by Bohan and Peter [6], which require the presence of myositis. In fact, there were significant differences in the severity of muscle disease and frequencies of ILD and internal malignancy between dermatomyositis patients who referred to dermatology clinic and those referred to rheumatology clinic [7]. Therefore, population-based study is necessary to examine true incidence and prevalence of CADM. In this regard, Bendewald *et al.* [8••] have performed a retrospective population-based survey of patients with dermatomyositis, including CADM, by reviewing patient records for all newly diagnosed dermatomyositis in Olmsted County, Minnesota, United States. They identified 29 validated cases of dermatomyositis over the 32-year period, and nine (31%) were classified as CADM. Overall age-adjusted and sex-adjusted incidence of CADM was 2.08 per 1 million persons (95% confidence interval 0.39–3.77). Patients with CADM were predominantly of adult-onset, female, and white race. Interestingly, muscle manifestations occurred at the mean of 6.85 years of follow-up in three patients (33%). The risk to evolve to classic dermatomyositis among CADM patients in this survey was higher than the result in a recent systematic review [9], in which 37 (13%) of 291 patients developed muscle weakness at 15 months to 6 years after onset of their skin disease.

There was an increasing number of case reports describing occurrence of clinical manifestations of dermatomyositis after administration of certain drugs, including lipid-lowering agents, antibiotics, and antitumor drugs [10]. For example, typical dermatomyositis rashes without muscle manifestation were reported in a patient with ovarian cancer who received chemotherapy consisting of carboplatin and paclitaxel [11] and in a patient treated with simvastatin [12].

Clinically amyopathic dermatomyositis and internal malignancy

It is recognized that approximately 20% of patients with adult-onset classic dermatomyositis are at risk for developing internal malignancy. There has been debate concerning the degree of risk for internal malignancy in patients with CADM in comparison with those with classic dermatomyositis. Anecdotal cases of CADM with a variety of internal malignancy were reported [13–16], but the reported frequency for internal malignancy in patients with adult-onset CADM ranged from 8 to 28% [7,8••,17]. This wide range of incidence is probably due to case ascertainment bias upon collecting patients. A systematic review by Gerami *et al.* [9] reported that internal malignancy was found in 41 (14%) of 291 patients with CADM. This may be somewhat lower than the frequency in patients with classic dermatomyositis. In a retrospective study [18••] of 121 patients with adult-onset dermatomyositis including 14 with CADM, the cumulative incidence rate of malignancy was 21 and 28% at 1 and 5 years after the diagnosis of CADM, respectively. In multivariate analysis, independent factors associated with underlying malignancy were an age at diagnosis more than 52 years, rapid onset of skin and/or muscular symptoms, and the presence of skin necrosis or periungual erythema, but CADM was not associated with malignancy. As there was no apparent difference in the risk of internal malignancy between classic dermatomyositis and CADM, all adults presenting with hallmark skin lesions of dermatomyositis should undergo internal malignancy screening at the time of presentation and repeatedly for a minimum of 3 years after diagnosis.

Clinically amyopathic dermatomyositis and interstitial lung disease

ILD has been anecdotally reported in patients with CADM, and many of them have experienced rapidly progressive ILD and poor outcomes. Case series of rapidly progressive ILD in patients with CADM were almost exclusively reported from eastern Asia, including Japan and China. We previously reported that five (33%) of 15 patients with CADM developed rapidly progressive ILD [19]. Mukae *et al.* [20•] compared the disease course of ILD between 11 patients with CADM and 16 with classic dermatomyositis, and found that rapidly progressive ILD was more common and the mortality rate was higher in CADM than in classic dermatomyositis. In a retrospective cohort [21] of dermatomyositis patients conducted in China, 21 (57%) of 37 patients with CADM had ILD. The majority of them showed a rapidly progressive course and 6-month survival rate was only 41%. Another Chinese study showed that three of five patients with CADM and ILD died within 2 months [22•]. In contrast, rapidly progressive ILD appears to be rare in the United States [4]. In a systematic review of adult-onset CADM, only 36 (13%) of 291 patients had ILD [9]. The reason for the discrepancy among studies is unknown, but eastern Asian ethnicity might confer an especially high risk for CADM-associated rapidly progressive ILD. Another possibility includes referral bias as patients with worsening dyspnea are usually referred to pulmonologists or rheumatologists, rather than dermatologists. The true risk of ILD in patients with CADM awaits an international larger population-based study.

Patients with CADM complicating of rapidly progressive ILD were often fatal in spite of high-dose corticosteroids [21]. There is no established treatment regimen for such conditions, but cyclosporine and/or cyclophosphomide in combination with high-dose corticosteroids are recommended in the early phase of the disease when respiratory symptoms are absent or mild [23,24]. Ando *et al.* [25] reported a patient with CADM and ILD who was initially resistant to cyclophosphamide and cyclosporine, but was successfully treated with tacrolimus. Alternative treatment strategies shown to be effective for ILD in patients with CADM included polymyxin B-immobilized fiber column [26] and intravenous immunoglobulin [27]. A recent study by Gono *et al.* [28••] reported that serum ferritin is useful as a predictor of the occurrence of rapidly progressive ILD and correlates with the prognosis in patients with dermatomyositis, especially in those with CADM. In addition, we have identified autoantibodies reactive with a 140-kDa cytoplasmic protein (CADM-140) in sera from patients with CADM and rapidly progressive ILD [19]. Therefore, intensive treatment using combination therapy with immunosuppressive agents with different modes of action should be considered for CADM patients with ILD showing a positive anti-CADM-140 antibody and/or an increased ferritin level (>1500 ng/ml).

Patients with CADM and ILD sometimes develop pneumomediastinum and/or subcutaneous emphysema during the course of the disease [29]. In a retrospective analysis [30] of patients with polymyositis or dermatomyositis who had ILD and pneumomediastinum, a poor survival was associated with the absence of muscle weakness.

Clinically amyopathic dermatomyositis-specific autoantibodies

A variety of serum autoantibodies have been described in patients with dermatomyositis, including those with CADM [31]. The majority of dermatomyositis-specific autoantibodies, including anti-Mi-2, antip155/TIF1-γ [32], and anti-MJ/NXP-2 [33], are detected in patients with classic dermatomyositis or cancer-associated dermatomyositis. Anti-CADM-140 antibody is highly specific to CADM and is associated with rapidly progressive ILD in Japanese population [19,34]. Another target recognized by autoantibodies in sera from CADM patients is small ubiquitin-like modifier activating enzyme (SAE) [35]. Interestingly, all CADM patients positive for anti-SAE antibodies evolved to classic dermatomyositis with a high frequency of systemic features including dysphagia, but a low frequency of ILD [31]. Finally, autoantibodies against aminoacyl-tRNA synthetases, including anti-Jo-1, are also detected in some patients with CADM [8••], although the frequency appears to be low [9].

Recently, we have identified RNA helicase encoded by melanoma differentiation-associated gene 5 (MDA5) as the CADM-140 antigen by cDNA library screening [36••]. Later, another group [37] confirmed the identity of CADM-140 antigen with MDA5 using partial purification by immunoabsorbent column chromatography followed by peptide mass fingerprinting. We have developed a convenient enzyme-linked immunosorbent assay (ELISA) system using recombinant MDA5 as antigen to detect anti-CADM-140 antibody [36••]. This ELISA was highly specific, and thus makes it possible to measure anti-CADM-140 antibody easily and rapidly in clinical settings, but it is not yet commercially available. MDA5, also termed interferon induced with helicase C domain 1 (IFIH1), is involved in innate immune defense against viruses [38], suggesting a possible association between pathogenesis of CADM and virus infection. As picorna virus species that contain *Coxsackie* virus is specifically recognized by MDA5, infection of the skin and lung epithelium by such virus may result in the release of a large amount of proteolytic fragments of MDA5 and a complex of virus RNA and MDA5 upon cytotoxicity against the infected cells. This may subsequently trigger the autoantibody response to MDA5, which facilitates

ongoing tissue damage through autoantibody-dependent cytotoxic mechanisms.

Juvenile-onset clinically amyopathic dermatomyositis

CADM can also occur in children. In a systematic review of juvenile-onset CADM, Gerami *et al.* [39] identified 68 patients published during 1963–2006. The mean age at diagnosis was 10.8 years, ranging from 2 to 17 years. None of the cases had severe vasculopathy, ILD, or internal malignancy, and thus had good prognosis, although three patients had severe subcutaneous calcinosis. It is of note that 18 (26%) patients developed muscle symptoms during the disease course and made a diagnosis of classic dermatomyositis. A retrospective review [40] at Mayo Clinic revealed that none of seven patients with juvenile-onset CADM had evolved to classic dermatomyositis . A case series [41] in Taiwan showed that two cases with juvenile-onset CADM did not show any clinical evidence of myositis during 6 and 11 years of follow-up. Another case report [42] from Iran showed a 5-year-old boy with juvenile-onset CADM who did not show any muscle symptoms for 2 years. A recent review [43] of juvenile-onset CADM demonstrated that approximately 25% of the patients evolved to having classic dermatomyositis.

In general, juvenile-onset CADM seems to have low risk to develop systemic involvement and good prognosis. Use of systemic corticosteroids is controversial in the treatment of juvenile-onset CADM as opposed to juvenile-onset classic dermatomyositis. Improvement of skin manifestations after treatment with prednisolone, methotrexate, or hydroxychloroquine was reported in case series [41,42], although randomized controlled trials are not available to guide the management of this disease. Proponents for early aggressive systemic corticosteroids for CADM advocate that this intervention may decrease the likelihood of progression to classic dermatomyositis, but we should notice predictable adverse effects of systemic corticosteroids in the face of uncertain benefit. However, there is a case report [44] of a 16-year-old Japanese boy with CADM who died of rapidly progressive ILD at 4 months after onset of skin rashes, despite intensive immunosuppressive treatment. Another case series [45] from Japan reported five children with classic dermatomyositis or CADM and rapidly progressive ILD resistant to high-dose corticosteroids. All except one responded to cyclosporine, but one died of respiratory failure. These reports suggest that in eastern Asia, rapidly progressive ILD can occur even in children with CADM.

Conclusion

The disease entity of CADM is now widely accepted in a community of dermatology and rheumatology. CADM consists of approximately 20% of dermatomyositis, and the progression of CADM to classic dermatomyositis after a long period of skin manifestation alone was relatively rare. A systematic review [9] of adult-onset CADM found that 14% had internal malignancy and only 2% died of rapidly progressive ILD. Therefore, prognosis in patients with CADM seems to be favorable. However, current literature indicates that frequency of rapidly progressive ILD is largely different among ethnic groups: higher incidence in eastern Asia than in North America. Anti-CADM-140 antibody and an increased serum ferritin level are useful to identify patients with high risk for developing rapidly progressive ILD. Therefore, we should be aware of delayed onset of myositis, internal malignancy, and rapidly progressive ILD in patients who were initially presented as CADM.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 706).

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