

# Expression and role of interleukin-9 in Vogt-Koyanagi-Harada disease

Zhixi Peng, Shaoqiu Jiang, Mingxing Wu, Xiyuan Zhou, Qian Wang

*The Second Affiliated Hospital of Chongqing Medical University, Chongqing, China*

**Purpose:** Vogt-Koyanagi-Harada (VKH) disease is a systemic autoimmune disease that can lead to blindness. This study was designed to investigate whether interleukin (IL)-9 plays a role in the development of VKH disease.

**Methods:** IL-9, IL-17, and interferon (IFN)- $\gamma$  levels, present in the supernatants of cultured peripheral blood mononuclear cells (PBMCs) and CD4+T cells, were assessed with enzyme-linked immunosorbent assay. IL-9 mRNA expression in PBMCs was measured with real-time quantitative PCR. The proliferation of PBMCs in response to different doses of recombinant human IL-9 (rIL-9) was measured using the Cell Counting Kit-8 assay.

**Results:** IL-9 mRNA levels in PBMCs were statistically significantly elevated in patients with active VKH disease compared to those in patients with inactive VKH disease ( $p < 0.05$ ) and normal controls ( $p < 0.05$ ). Statistically significantly higher expression of IL-9 was observed in the supernatants of stimulated PBMCs ( $p < 0.01$ ) and CD4+ T cells ( $p < 0.01$ ) from patients with active VKH disease compared to that in cells from patients with inactive VKH disease and normal controls. rIL-9 at a concentration of 100 ng/ml did not induce proliferation of PBMCs ( $p > 0.05$ ). After the PBMCs and CD4+ T cells were stimulated with rIL-9 (100 ng/ml), the secretion of IL-17 was increased statistically significantly ( $p < 0.05$ ), whereas the level of IFN- $\gamma$  was not statistically significantly altered ( $p > 0.05$ ).

**Conclusions:** These findings suggest that IL-9 is involved in the pathogenesis of VKH disease, and that IL-9 might also enhance the inflammatory response by increasing the secretion of IL-17, an established proinflammatory cytokine in VKH disease. Manipulation of IL-9 could represent a novel option for the treatment of VKH disease.

Interleukin (IL)-9-producing T cells were initially thought to be associated with Th2-type responses *in vivo*; however, IL-9 has not been as extensively studied as many other cytokines. With the discovery of T-helper type 9 (Th9) cells, IL-9 has now received considerably more attention. Th9 cells are classified as a novel subset of CD4+ T-helper cells, primarily driven by the combination of transforming growth factor (TGF)- $\beta$  and interleukin (IL)-4 [1], and are characterized by high levels of IL-9 secretion in humans. In addition to Th9 cells, IL-9 is produced by a variety of other cells, including Th2 [2], Th17 [3,4], Treg [3,5], mast [6,7], and natural killer cells [8,9]. IL-9 has been shown to play a pivotal role in the pathophysiological processes of many autoimmune diseases, including rheumatoid arthritis [10], psoriasis [11], atopic dermatitis [12-14], colitis [15,16], systemic lupus erythematosus (SLE) [17], lupus nephritis [18], systemic sclerosis [19], allergic inflammation [20], type 1 diabetes [21], and multiple sclerosis [22]. In addition, IL-9 has been studied in various animal models of autoimmune disease, such as lupus-prone mice [23], experimental autoimmune encephalomyelitis (EAE) [24,25], experimental autoimmune uveitis

(EAU) [26], and experimental autoimmune myasthenia gravis (EAMG) [27].

Vogt-Koyanagi-Harada (VKH) disease is a systemic autoimmune disease that usually causes bilateral granulomatous panuveitis and results in decreased visual acuity [28-30]. If not treated in a timely manner, this disease can lead to blindness. VKH disease is principally caused by an autoimmune response to melanocytes; however, the pathogenesis of VKH disease is unclear [29,31-33]. Several studies have indicated that a Th1/Th17-weighted immune response plays a predominant role in the pathogenesis of VKH disease, with Th1-derived IFN- $\gamma$ , IL-12, TNF- $\alpha$ , and Th17-derived IL-17, IL-23, and ROR $\gamma$ t all being involved. Based on the involvement of IL-9 in various autoimmune diseases, we investigated whether IL-9 is involved in VKH disease. The data showed that increased IL-9 expression is associated with this disease and that IL-9 can promote IL-17 secretion. These results suggest that manipulation of IL-9 might represent a novel option for the treatment of VKH disease.

## METHODS

**Patients and controls:** One hundred and thirty-five patients with VKH disease (71 men and 64 women, mean age of  $36.7 \pm 15.7$  years  $\pm$  standard deviation, SD) and 51 healthy individuals (28 men and 23 women, mean age of  $38.5 \pm 13.4$

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Correspondence to: Qian Wang, The Second Affiliated Hospital of Chongqing Medical University, Chongqing, China, Phone: 023-63693601; FAX: 023-63711527; email: 37527860@qq.com

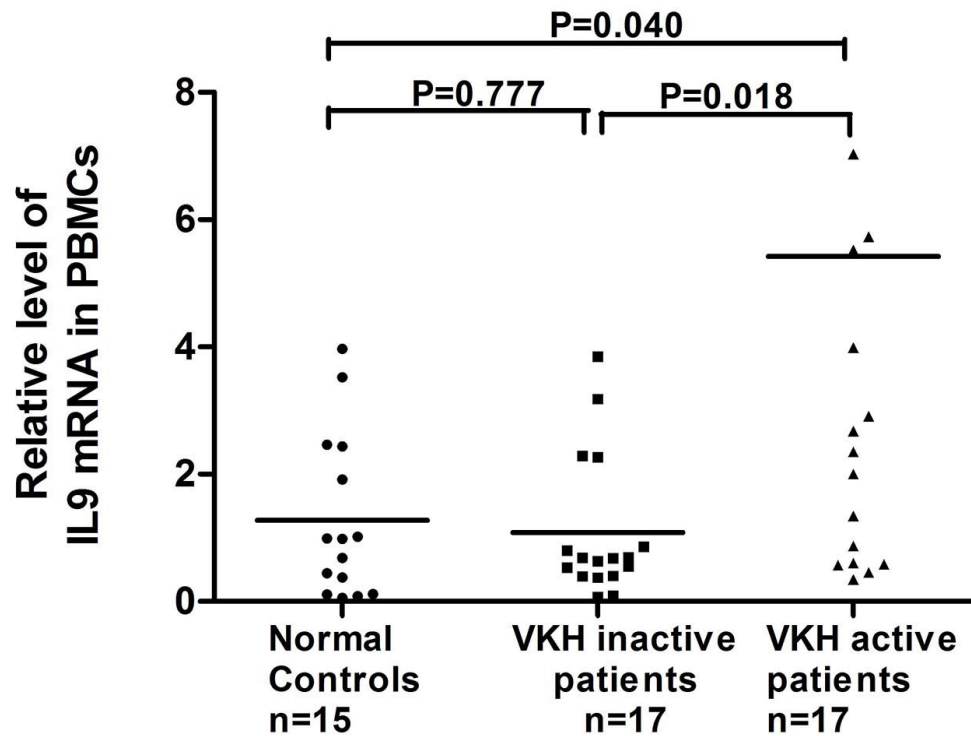


Figure 1. Expression of IL-9 mRNA in VKH disease. Expression of interleukin (IL)-9 mRNA in peripheral blood mononuclear cells (PBMCs) derived from normal controls (n = 15), patients with inactive Vogt-Koyanagi-Harada (VKH; n = 17) disease, and patients with active VKH disease (n = 17) as measured with real-time quantitative PCR.

years) were included in this study. The diagnosis of VKH disease was made according to the revised diagnostic criteria for Vogt-Koyanagi-Harada disease: report of an international committee on nomenclature [34]. Sixty-eight of the patients with active VKH disease had diffuse bilateral choroiditis in association with exudative retinal detachment after the first uveitis attack or mutton-fat keratic precipitates, had cells in the anterior chamber, and sunset glow fundus was apparent in patients with VKH disease with recurrent episodes. The patients included in the study had not used immunosuppressive agents for at least 1 week or had used a low dose of corticosteroids (20 mg/day) before blood sampling. The 67 patients with inactive VKH disease did not have any evidence of disease activity for at least 3 months after being treated. This study was approved by the Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University, Chongqing, China, and was conducted in agreement with the guidelines of the Declaration of Helsinki. This study was adhered to the ARVO statement of human subjects. Consent was obtained from all individuals.

**Cell isolation and culture:** Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized blood using Ficoll-Hypaque density-gradient centrifugation (TBD Science, Tianjin, China). Anticoagulated blood samples were obtained using vacuum tubes containing EDTA. Peripheral CD4+ T cells were extracted using human CD4+ T microbeads

(Miltenyi Biotec, San Diego, CA). PBMCs and CD4+ T cells were resuspended in RPMI 1640 medium (Gibco, Carlsbad, CA) at a concentration of  $1 \times 10^6$  cells/ml. PBMCs and CD4+ T cells were cultured in the absence or presence of recombinant human IL-9 (rIL-9; eBioscience, San Diego, CA), in the presence of anti-CD3 antibody (OKT3, 5  $\mu$ g/ml; eBioscience), or in the presence of an anti-CD28 antibody (1  $\mu$ g/ml; eBioscience).

**RNA preparation and real-time quantitative PCR:** Total RNA was extracted from PBMCs using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. PCR protocol has been stated as: denaturation at 95 °C for 30 s for one cycle; denaturation at 95 °C for 5 s and extension at 60 °C for 34 s for 40 cycles. RNA was quantified using the SYBR green chemistry (TaKaRa, Beijing, China). Real-time quantitative PCR was performed using SYBR green chemistry with a real-time PCR system (Applied Biosystems 7500, Applied Biosystems, Foster City, CA). The sense and antisense primers used in this experiment were as follows: IL-9 sense: 5'-TCA AGA TGC TTC TGG CCA TG-3'; IL-9 antisense: 5'-AGG GAA TGC CCA AAC AGA GA-3'; beta-actin sense: 5'-GGA TGC AGA AGG AGA TCA CTG-3'; beta-actin antisense: 5'-CGA TCC ACA CGG AGT ACT TG-3'. The mRNA expression levels are presented as the relative fold change.

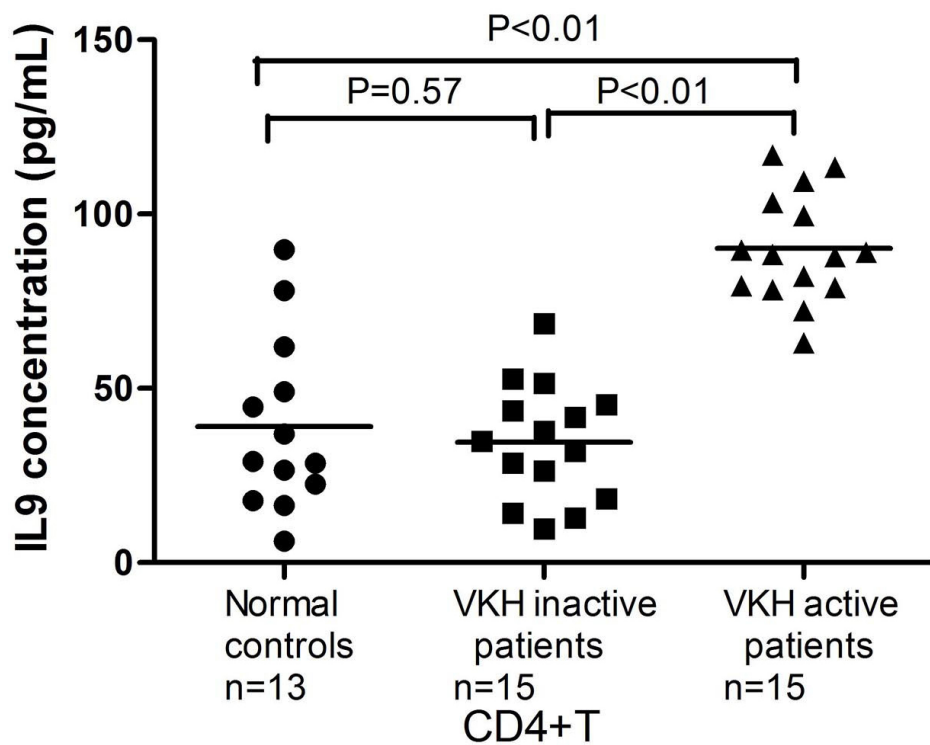
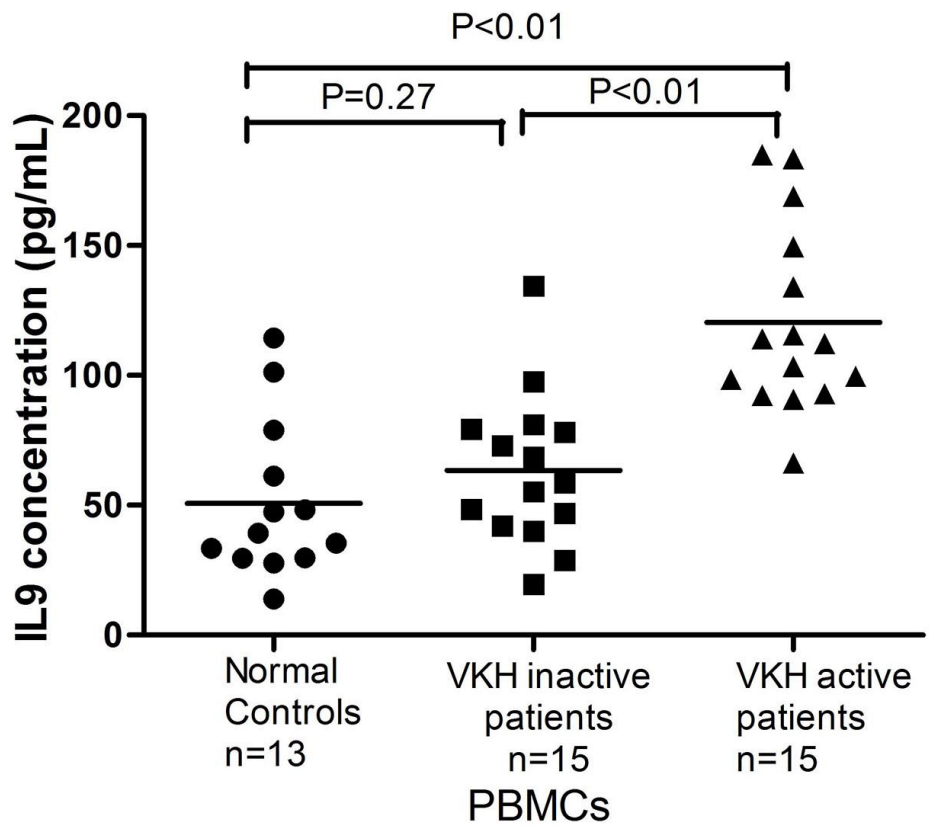


Figure 2. Production of IL-9 in VKH disease. Production of IL-9 by peripheral blood mononuclear cells and CD4+ T cells from normal controls (n = 13), patients with inactive Vogt-Koyanagi-Harada (VKH; n = 15) disease, and patients with active VKH disease (n = 15), as measured with enzyme-linked immunosorbent assay.

**Cell proliferation in response to rIL-9:** PBMCs and CD4+ T cells from healthy individuals and patients with VKH disease were treated with different doses of recombinant human IL-9 (0, 1, 10, 100, and 500 ng/ml; R&D Systems, Minneapolis, MN). After 3 days of stimulation, proliferation of these cells was evaluated using Cell Counting Kit-8 (CCK-8; Sigma-Aldrich, St. Louis, MO). Briefly, 20 µl of CCK-8 reagent in 200 µl of complete culture medium was added to the cells. The absorbance was then measured 1 h later, using an enzyme-linked immunosorbent assay (ELISA) plate reader (SpectraMax M2e, Molecular Devices, Sunnyvale, CA) at 450 nm.

**Measurement of IL-9, IL-17, and IFN-γ:** Levels of IL-9 were measured using a commercially available ELISA kit

(eBioscience) based on the manufacturer’s instructions, with a detection limit of 0.5 pg/ml. IL-9 levels were measured in supernatants derived from PBMCs and CD4+ T cells from patients with active VKH disease (n = 15), patients with inactive VKH disease (n = 15), and normal controls (n = 13). Randomly selected patients were matched with controls according to age and gender.

The expression levels of IL-17 and IFN-γ in these same cell culture supernatants were measured using commercially available ELISA kits (R&D Systems), with detection limits of 15.6 pg/ml and 31.3 pg/ml, respectively. For the determination of IL-17 and IFN-γ production, PBMCs and CD4+ T cells were stimulated with or without anti-CD3 (OKT3, 5 µg/ml) or

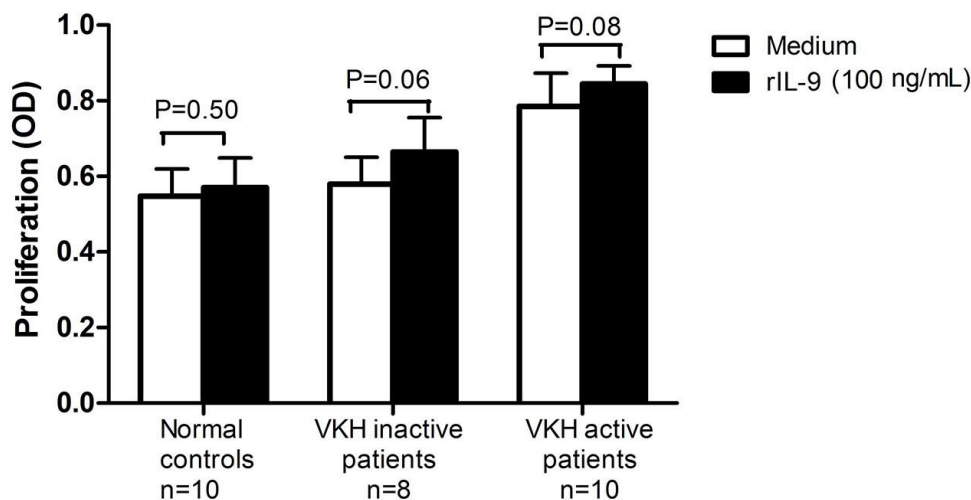
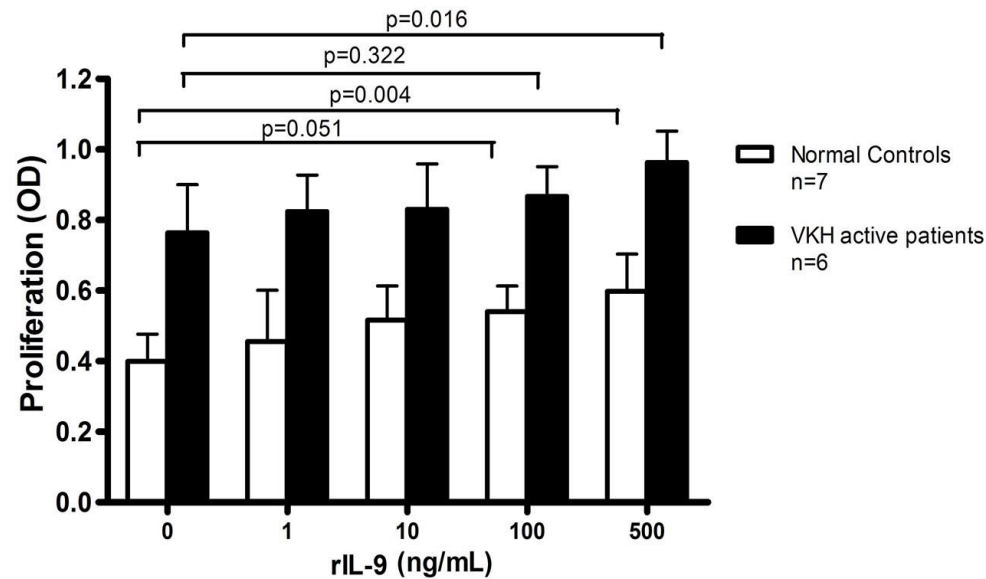


Figure 3. Effect of rIL-9 on PBMC proliferation. Effect of various concentrations of recombinant human interleukin-9 (rIL-9) on the proliferation of peripheral blood mononuclear cells (PBMCs) from normal controls (n = 7) and patients with active Vogt-Koyanagi-Harada disease (VKH; n = 6) disease as measured with Cell Counting Kit-8 (CCK-8) assays. The effect of rIL-9 (100 ng/ml) on the proliferation of PBMCs from normal controls (n = 10), patients with inactive VKH disease (n = 8), patients with active VKH disease (n = 10) was measured with CCK-8 assays.

anti-CD28 antibodies (1 µg/ml; eBioscience) in the presence or absence of rIL-9 (100 ng/ml) for 72 h.

**Statistical analyses:** All statistical analyses were performed using SPSS 20.0. Data were expressed as the mean ± SD. Multiple groups were analyzed with one-way ANOVA and pairwise comparisons by adopting the Mann–Whitney U-tests followed by Bonferroni’s correction to adjust significance levels for multiple comparisons where appropriate. A p value of less than 0.05 was considered statistically significant.

**RESULTS**

*Increased expression of IL-9 mRNA in PBMCs derived from patients with active VKH disease:* The expression of IL-9 mRNA in PBMCs was measured with real-time quantitative PCR, comparing those from active and patients with inactive VKH disease and normal controls. The data showed that IL-9 mRNA expression was higher in patients with active VKH disease than in inactive patients (p = 0.018) or normal controls (p = 0.040; Figure 1).

*IL-9 levels were elevated in the supernatants of PBMCs and CD4+ T cells derived from patients with active VKH disease:* IL-9 levels were statistically significantly higher in the supernatants of PBMCs from patients with active

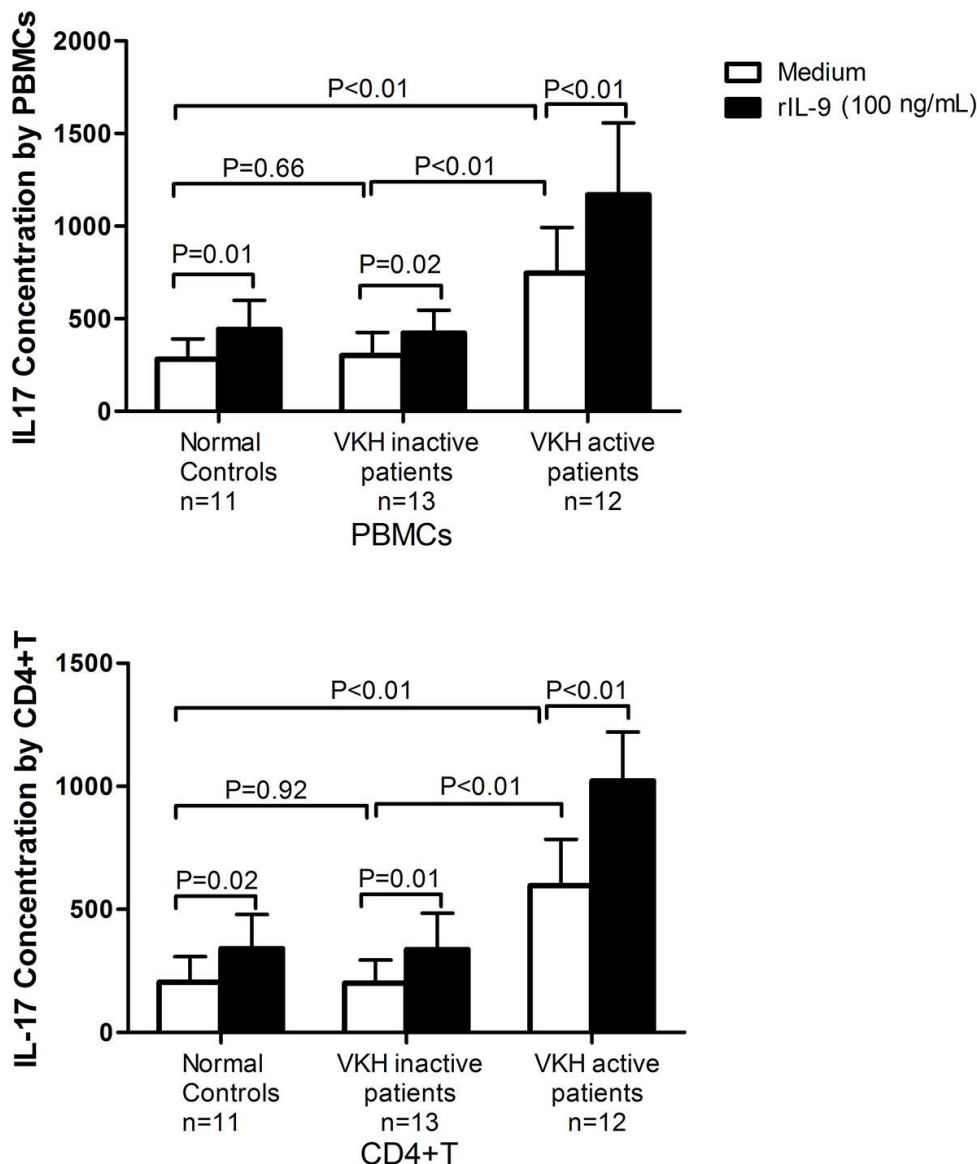


Figure 4. Effect of rIL-9 on IL-17 production. Effect of recombinant human interleukin-9 (rIL-9, 100 ng/ml) on IL-17 production by peripheral blood mononuclear cells (PBMCs) and CD4+ T cells from patients with active Vogt-Koyanagi-Harada (VKH; n = 12) disease, patients with inactive VKH disease (n = 13), and normal controls (n = 11). Cells were cultured with or without rIL-9 in the presence of anti-CD3 and anti-CD28 antibodies for 3 days, and IL-17 was measured with enzyme-linked immunosorbent assays.



VKH disease ( $120.44 \pm 36.07$  pg/ml), compared to levels in those patients with inactive VKH disease ( $63.43 \pm 29.07$  pg/ml,  $p < 0.01$ ) or normal controls ( $50.82 \pm 30.21$  pg/ml,  $p < 0.01$ ). Similar results were found for IL-9 levels; specifically, the supernatant from CD4+T cells from patients with active VKH disease contained higher levels ( $90.13 \pm 15.58$  pg/ml), compared to those from in patients with active VKH disease ( $34.52 \pm 16.71$  pg/ml,  $p < 0.01$ ) or normal controls ( $39.05 \pm 24.90$  pg/ml,  $p < 0.01$ ; Figure 2). Serum levels of IL-9 could not be detected directly in this study.

*Effect of rIL-9 on PBMC proliferation:* PBMCs isolated from healthy controls, as well as patients with active VKH disease, were cultured for 3 days with different concentrations of rIL-9 (0–500 ng/ml). Based on the data, rIL-9 at 500 ng/ml caused a statistically significant increase in PBMC proliferation in cells from patients with active VKH disease and controls ( $p < 0.05$ ). At concentrations of rIL-9 below 100 ng/ml, PBMCs from either patients with active VKH disease or controls did not proliferate ( $p > 0.05$ ). Next, we carefully assessed whether 100 ng/ml rIL-9 could induce the proliferation of PBMCs derived from patients with active VKH disease, patients with inactive

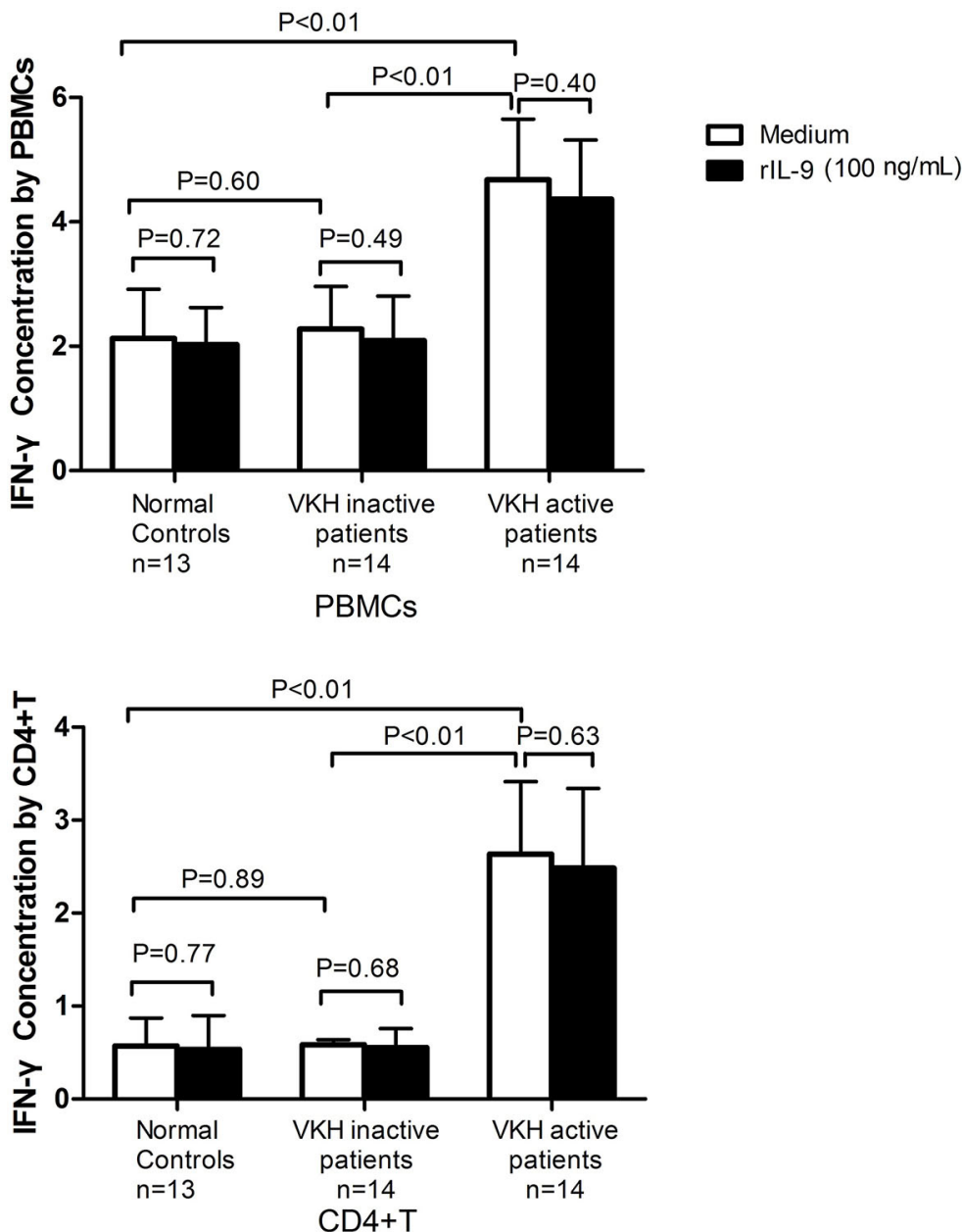


Figure 5. Effect of rIL-9 on IFN-γ production. The effect of recombinant human interleukin-9 (rIL-9; 100 ng/ml) on interferon (IFN)-γ production by peripheral blood mononuclear cells (PBMCs) and CD4+ T cells from patients with active Vogt-Koyanagi-Harada (VKH) (n = 14) disease, patients with inactive VKH disease (n = 14), and normal controls (n = 13). Cells were cultured with or without rIL-9 in the presence of anti-CD3 and anti-CD28 antibodies for 3 days, and IFN-γ was measured with enzyme-linked immunosorbent assays.

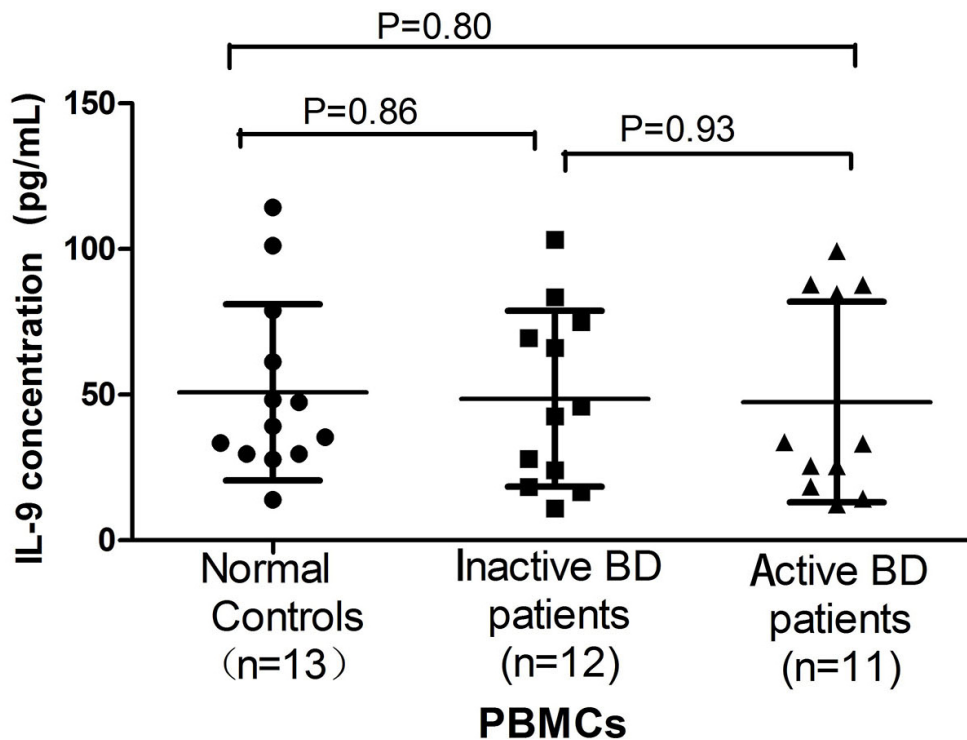


Figure 6. Production of IL-9 in BD. The production of IL-9 by peripheral blood mononuclear cells from normal controls (n = 13), patients with inactive Behcet's disease (BD; n = 12), and patients with active BD (n = 11), as measured with enzyme-linked immunosorbent assay.

VKH disease, and controls. rIL-9 at 100 ng/ml was unable to statistically significantly induce the proliferation of cells from any of these three groups ( $p > 0.05$ ; Figure 3). Based on this, 100 ng/ml rIL-9 was used for subsequent experiments.

*rIL-9 enhances IL-17 production:* In PBMCs from patients with active VKH disease, patients with inactive VKH disease, and controls, the production of IL-17 was found to be statistically significantly higher following stimulation with 100 ng/ml rIL-9 ( $p < 0.01$ ,  $p < 0.05$ , and  $p < 0.05$ , respectively). Consistent with these data, the production of IL-17 by CD4+ T cells was also increased after stimulation with rIL-9. The levels of statistical significance were  $p < 0.01$  for cells from patients with active VKH disease,  $p < 0.05$  for those from patients with inactive VKH disease, and  $p < 0.05$  for those from control individuals (Figure 4). Thus, IL-9 plays a role in the development of VKH disease through IL-17-associated inflammation.

*rIL-9 does not affect IFN- $\gamma$  production:* In contrast to the statistically significant induction of IL-17, levels of IFN- $\gamma$  were not statistically significantly different in either PBMCs or CD4+ T cells derived from patients with active VKH disease ( $p > 0.05$  for PBMCs and CD4+ T cells), patients with inactive VKH disease ( $p > 0.05$  for PBMCs and CD4+ T cells), or normal controls ( $p > 0.05$  in PBMCs and CD4+ T cells) treated with rIL-9. Based on these data, rIL-9 does not have

a statistically significant effect on IFN- $\gamma$  secretion in VKH disease (Figure 5).

## DISCUSSION

The aim of this study was to assess the importance of IL-9 in VKH disease. The data clearly showed that there was an increase in the level of the mRNA encoding IL-9 in PBMCs derived from patients with active VKH disease, compared to that in PBMCs from patients with inactive VKH disease or controls. In support of this finding, the levels of IL-9 in PBMCs and CD4+ T cell supernatants were also higher in active VKH disease-derived cells compared to those in inactive VKH disease cells or controls. In vitro, treatment with 100 ng/ml rIL-9 promoted the secretion of IL-17 by PBMCs and CD4+ T cells, without affecting the production of IFN- $\gamma$ .

VKH disease is considered to be a Th1/IFN- $\gamma$ - and Th17/IL-17-mediated disease [35-37]. Based on the expression profile of IL-9 identified in this study and other studies on IL-9 in autoimmune diseases, it is suggested that IL-9 plays a statistically significant role in this autoimmune disease. The expression of IL-9 has already been assessed in several other autoimmune diseases and in animal models. In Behcet's disease, the expression of IL-9 in PBMCs is not statistically significantly different between individuals (Figure 6). In SLE, it has been demonstrated that serum levels of IL-9 and the percentage of CD4 + IL-9+ T cells are higher compared to

those in healthy controls [17]. Another study on autoimmune diabetes [38] demonstrated a statistically significant increase in the number of IL-17 + IL-9 + CD4 cells, and furthermore, serum IL-9 levels were previously reported to be statistically significantly elevated with systemic sclerosis [19]. Similarly, in atopic dermatitis (AD), increased expression of IL-9 and the IL-9 receptor has been reported in skin lesions [12]; in addition, the percentage of Th9 cells and IL-9 expression levels were increased statistically significantly [14]. Another study showed that children with AD have higher serum IL-9 levels than controls, and that clinical severity is positively and statistically significantly related to IL-9 levels [13]. With respect to allergic contact dermatitis (ACD), IL-9 production has also been shown to increase in nickel-treated PBMCs from nickel-allergic patients [39]. In rheumatoid arthritis, which is an extremely significant autoimmune disease, it has also been shown that IL-9 expression is directly correlated with the degree of inflammatory infiltration and lymphoid organization [10]. Likewise, in ulcerative colitis, the levels of IL-9 mRNA were found to be high in mucosal biopsies of the inflamed gut [16]. In the cerebrospinal fluid of multiple sclerosis (MS) subjects, IL-9 was shown to be inversely correlated with disease severity [22]. IL-9 receptor and IL-9 expression was found to be increased in the skin in a model of psoriasis [11]. In EAMG, the percentage of IL-9-producing CD4+ T cells increased during EAMG progression, and treatment with an anti-IL-9 antibody has been shown to ameliorate EAMG symptoms [27]. Finally, in an experimental animal model of EAU, the production of IL-9 was shown to be upregulated during the active phase [26]. Taken together, these data are consistent with the present data demonstrating the upregulation of IL-9 in VKH disease.

With regard to the role of IL-9 in VKH disease, these results confirm the association between IL-9 and IL-17. It has been demonstrated that IL-9, together with TGF- $\beta$ , can induce differentiation of naive CD4+ T cells into Th17 cells in vitro, and that IL-9 produced by Th17 cells amplifies Th17 development by propagating a positive autocrine loop [4]. Similar results were obtained in patients with psoriasis, in which IL-9 statistically significantly enhanced IL-17A production by cultured PBMCs and CD4+ T cells [11]; this result is in accordance with our data. Furthermore, studies on EAE suggest that IL-9, as a Th17-derived cytokine, can contribute to inflammatory disease and that blocking IL-9 might preferentially attenuate Th17 responses [3,24]. This outcome is in contrast to another study reporting that IL-9 inhibits IL-17 production by Th17 cells, and that this cytokine can downregulate IL-17 mRNA levels in MS [22]. It is possible that these differing results arise due to different methodologies and different mechanisms of pathogenesis.

Several studies have described the relationship between IL-9 and IFN- $\gamma$ . In EAE, blocking IL-9 using an anti-IL-9 monoclonal antibody inhibited disease development and reduced central nervous system mRNA expression of IFN- $\gamma$  [24]; IFN- $\gamma$  also inhibited Th17-mediated IL-9 production [40]. In ACD, blocking either IL-9 or IL-4 enhances allergen-specific IFN- $\gamma$  production [39]. A study using an animal model of psoriasis demonstrated that rIL-9 had no effect on IFN- $\gamma$  mRNA expression in the skin of K5.hTGF- $\beta$ 1 transgenic mice [11]. Whether IL-9 is a pathogenic or protective cytokine is still unknown with respect to immune responses. Some researchers have suggested that IL-9 is a pleiotropic cytokine, and that Th9 cells might contribute to protective immunity and immunopathological diseases through a myriad of pathways [41,42].

The present study raises the possibility that IL-9, which was increased during VKH disease onset and progression, might influence IL-17 production. In addition to being produced in Th9 cells, IL-9 is manufactured by a variety of other cells, including Th2 [2], Th17 [3,4], Treg [3,5], mast [6,7], and NK cells [8,9]. Th9 cells in a T cell transfer model of colitis also showed increased production of IFN- $\gamma$  and IL-17 in vivo [43]. The expression levels of IL-9, IL-17, and IFN- $\gamma$  are under the control of numerous effectors, and thus, the function of IL-9 varies according to the disease and in vivo microenvironment. Further work is needed to fully assess the levels of IL-9 produced by these various cell types, and it is anticipated that this work will reveal how IL-9, along with other cytokines, affects VKH disease progression.

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