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Genetic analysis of innate immunity in Behcet's disease identifies an association with IL-37 and IL-18RAP

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Interleukin-1 (IL-1) and the IL-1 receptor (IL-1R) family play an important role in the pathogenesis of inflammatory diseases. This study aimed to investigate the association between single nucleotide polymorphisms (SNP) of IL-1 and IL-1R family genes with Vogt-Koyanagi-Harada (VKH) and Behcet's disease (BD) in Han Chinese. The case-control study was divided into two stages and included 419 VKH cases, 1063 BD cases and 1872 healthy controls. The MassARRAY platform (Sequenom), iPLEX Gold Assay and TaqMan SNP assays were used to score genotypes of 24 SNPs. The expression of IL-37 and IL-18Rap was measured by ELISA and real-time PCR in genotyped healthy individuals. A significantly lower frequency of the AG genotype, and a higher frequency of the GG genotype and G allele of IL-37/rs3811047 were observed in BD as compared to controls. AA genotype and A allele frequency of IL-18RAP/rs2058660 was significantly decreased in BD as compared to controls. Functional studies performed in healthy controls showed that rs3811047 AG genotype carriers had a higher IL-37 gene expression in peripheral blood mononuclear cells (PBMCs) than GG carriers. GG carriers showed a higher cytokine expression as compared to AG carriers. No association was detected between the tested SNPs and VKH.

Uveitis is a leading cause of visual impairment and blindness, with an estimated prevalence of 38 per 100,000[1]. It can be caused by infectious or non-infectious mechanisms and classification of the disease is mainly based on the anatomical site within the eye[2,3]. The non-infectious uveitis entities are generally considered to be immune mediated and treatment of the disease often includes the use of corticosteroids and immunosuppressive drugs[3,4]. Environmental as well as hereditary factors are thought to be involved in the development of immune mediated uveitis[5,6], whereby analysis of the genetic predisposition has unraveled the involvement of inflammatory pathways, leading to potential targets for treatment. Although the strongest genetic association is observed between the human leukocyte antigen (HLA) system and various uveitis entities[7], this has not yet led to a breakthrough in the management of uveitis. Attention has therefore shifted towards analysis of genetic polymorphisms of factors that are involved in immune regulation and inflammation and various associations with uveitis have been reported in the last decade[7–13].

Interaction of Interleukin-1 (IL-1) cytokine family members with their receptors are the initial steps during inflammation and dysregulated production or activity of the various members of these factors has been shown to be associated with severe immune mediated diseases like psoriatic arthritis (PsA), systemic lupus erythematosus (SLE), multiple sclerosis (MS), ankylosing spondylitis (AS), rheumatoid arthritis (RA) and type 1 diabetes (T1DM)[14–23], but little is known about their role in the pathogenesis of clinical uveitis. In this study we therefore decided to investigate the association of these genes with two well defined immune mediated uveitis entities,

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	Total	%
Patients with BD	1063	
Mean age ± SD	33.9 ± 9.1	
Male	884	83.2
Female	179	16.8
Uveitis	1063	100
Oral ulcer	1063	100
Genital ulcer	590	55.5
Skin lesions	796	74.8
Arthritis	213	20.0
Positive pathergy test	171	16.1
Patients with VKH	419	
Mean age ± SD	39.9 ± 14.0	
Male	233	55.6
Female	186	44.4
Uveitis	419	100
Headache	176	42.0
Tinnitus	181	43.2
Alopecia	139	33.2
Vitiligo	68	16.2
Poliosis	148	35.3
Healthy controls	1872	
Mean age ± SD	39.4 ± 10.6	
Male	1136	60.7
Female	736	39.3

Table 1. Clinical features, age, and sex distribution of patients and controls. SD = standard deviation. BD = Behcet's disease. VKH = Vogt-Koyanagi-Harada disease.

Vogt-Koyanagi-Harada disease (VKH) and Behcet's disease (BD)[8]. These two entities are relatively common in China, which allows the collection of large numbers of patients, providing sufficient statistical power to make reliable conclusions concerning possible associations. The IL-1 gene cluster contains 11 cytokines including IL-1α, IL-1β, IL-1Ra, IL-18, IL-33, IL-36Ra, IL-36α, IL-36β, IL- 36γ, IL-37 and IL-38[24–27]. The IL-1 receptor (IL-1R) family members include IL-1R1 (IL1RI), IL-1R2 (IL1RII), IL-1R3 (IL1RAP), IL-1R4 (IL1RL1), IL-1R5 (IL18R1), IL-1R6 (IL1RL2), IL-1R7 (IL18RAP), IL-1R8 (SIGIRR), IL-1R9 (IL1RAPL1), IL-1R10 (IL1RAPL2)[26]. Most of the genes of the IL-1 and IL-1R family are located in a region on chromosome 2 (2q12 and 2q13) and a coordinated regulation of these genes has been proposed[28].

To test the association between uveitis and genetic polymorphisms in the IL-1 and IL-1R family genes, a set of single nucleotide polymorphisms (SNP) was selected. Selection was based on earlier reports on disease association, whereby linkage disequilibrium (LD) data from the Han Chinese Hap Map database were taken into account. This resulted in a selection of twenty-four SNPs, with a minor allele frequency (MAF) that was higher than 0.05 in Han Chinese. This resulted in the following ten genes: IL1A, IL1B, IL1RN, IL18, IL33, IL37, IL38, IL18RAP, IL1RL1 and IL-1 ligand cluster. Using a two stage set up we found a significant association between IL-37/rs3811047 and IL-18Rap/rs2058660 polymorphisms with BD but not with VKH.

Results

Clinical characteristics of VKH and BD. The genotype frequencies of the twenty-four SNPs tested, did not deviate from the Hardy-Weinberg equilibrium in healthy controls. Clinical features, age, and sex distribution in the BD, VKH and healthy controls are shown in Table 1. The clinical characteristics of the BD patients included five different features such as ulcers of the oral cavity and genital region, positive pathergy test, skin lesions, and arthritis. VKH patients had six primary features including uveitis, headache, tinnitus, alopecia, poliosis and vitiligo. The details are shown in Table 1.

Genotype and allele frequencies of the examined SNPs in cases and controls in the first-phase study. In the first phase study, we tested the association between 24 SNPs of 10 IL-1 and IL-1R family genes with BD (n = 416) or VKH (n = 419) as compared to healthy controls (n = 627). The results demonstrated significant differences between BD cases and healthy controls for only two SNPs (rs2058660 and rs3811047) in two genes (IL-18Rap and IL-37) (Table 2). No association was observed for VKH for any of the 24 SNPs tested (Supplemental Tables 1 and 2).

Compared to controls, the frequency of the IL-37/rs3811047 AG genotype was significantly lower in BD ($P_c = 4.32 \times 10^{-4}$, OR = 0.522). A significant increase in the frequency of the GG genotype and G allele was observed ($P_c = 1.66 \times 10^{-3}$, OR = 1.800 and Pc = 2.19 × 10^{-2}, OR = 1.529, respectively). The frequency of the IL-18RAP /rs2058660 AA genotype and A allele was significantly decreased in BD as compared to healthy

Gene	SNPs	Stage	Genotype Allele	BD		Controls		P Value	Pc Value	OR(95% Cl)
				N	%	N	%			
IL-37	rs3811047	First	AA	13	3.1	16	2.6	0.581	NS	1.232(0.586–2.589)
			AG	92	22.1	221	35.2	6.00×10^{-6}	4.32×10^{-4}	0.522(0.393–0.693)
			GG	311	74.8	390	62.2	2.30×10^{-5}	1.66×10^{-3}	1.800(1.369–2.367)
			G	714	85.8	1001	79.8	4.57×10^{-4}	2.19×10^{-2}	1.529(1.205–1.942)
			A	118	14.2	253	20.2	4.57×10^{-4}	2.19×10^{-2}	0.654(0.515–0.830)
		Second	AA	9	1.4	50	4	0.002	NS	0.337(0.165–0.690)
			AG	141	21.8	365	29.3	4.52×10^{-4}	3.25×10^{-2}	0.672(0.528–0.840)
			GG	497	76.8	830	66.7	5.00×10^{-6}	3.60×10^{-4}	1.657(1.333–2.059)
			G	1135	87.7	2025	81.3	5.10×10^{-7}	2.45×10^{-5}	1.639(1.350–1.990)
			A	159	12.3	465	18.7	5.10×10^{-7}	2.45×10^{-5}	0.610(0.502–0.741)
		Combined	AA	22	2.1	66	3.5	0.026	NS	0.578(0.355–0.943)
			AG	233	21.9	586	31.3	5.10×10^{-8}	3.67×10^{-6}	0.616(0.517–0.734)
			GG	808	76	1220	65.2	1.01×10^{-9}	7.27×10^{-8}	1.693(1429–2.007)
			G	1849	87	3026	80.8	1.60×10^{-9}	7.68×10^{-8}	1.584(1.363–1.841)
			A	277	13	718	19.2	1.60×10^{-9}	7.68×10^{-8}	0.631(0.543–0.734)
IL-18RAP	rs2058660	First	AA	89	21.4	195	31.1	5.64×10^{-4}	2.08×10^{-2}	0.603(0.452–0.805)
			AG	194	46.6	289	46.1	0.863	NS	1.022(0.797–1.310)
			GG	133	32	143	22.8	0.001	NS	1.591(1.205–2.100)
			A	372	44.7	679	54.1	2.44×10^{-5}	1.17×10^{-3}	0.685(0.574–0.817)
			G	460	55.3	575	45.9	2.44×10^{-5}	1.17×10^{-3}	1.460(1.224–1.741)
		Second	AA	124	19.2	344	27.6	5.20×10^{-5}	3.74×10^{-3}	0.621(0.492–0.783)
			AG	346	53.5	603	48.4	0.037	NS	1.224(1.012–1.480)
			GG	177	27.3	298	24.0	0.104	NS	1.197(0.964–1.486)
			A	594	45.9	1291	51.8	5.23×10^{-4}	2.51×10^{-2}	0.788(0.689–0.902)
			G	700	54.1	1199	48.2	5.23×10^{-4}	2.51×10^{-2}	1.269(1.109–1.452)
		Combined	AA	213	20	539	28.8	1.77×10^{-7}	1.27×10^{-5}	0.620(0.517–0.742)
			AG	540	50.8	892	47.6	0.101	NS	1.134(0.976–1.319)
			GG	310	29.2	441	23.6	8.24×10^{-4}	NS	1.336(1.127–1.583)
			A	966	45.4	1970	52.6	1.24×10^{-7}	5.94×10^{-6}	0.750(0.674–0.834)
			G	1160	54.6	1774	47.4	1.24×10^{-7}	5.94×10^{-6}	1.334(1.198–1.484)

Table 2. Genotype and allele frequencies of IL-18Rap and IL-37 polymorphisms in BD and healthy controls. SNP = single-nucleotide polymorphism; BD = 5% confidence interval; Pc = Bonferroni corrected p value.

controls (Pc = 2.08×10^{-2}, OR = 0.603 and Pc = 1.17×10^{-3}, OR = 0.685, respectively). The frequencies of the IL-37/rs3811047 AA genotypes and the frequencies of the IL-18RAP/rs2058660 AG and GG genotypes were not significantly different between BD cases and healthy controls (Table 2).

Genotype and allele frequency of the examined SNPs in cases and healthy controls in the second phase and combined study. Since IL-37/rs3811047 and IL-18RAP/rs2058660 displayed a significant association with BD in the first phase exploratory study, a confirmatory second phase study was performed for these two SNPs in a separate set of BD patients (n = 647) and healthy controls (n = 1245). The data again showed that the frequency of the IL-37/rs3811047 GG genotype and G allele was significantly higher in BD as compared to controls (Pc = 3.60×10^{-4}, OR = 1.657; $P_c = 2.45 \times 10^{-5}$, OR = 1.639). The AG genotype was significantly lower in BD ($P_c = 3.25 \times 10^{-2}$, OR = 0.672). The frequency of the AA genotype and A allele of IL-18RAP/rs2058660 was significantly decreased in BD cases as compared to controls (Pc = 3.74×10^{-3}, OR = 0.621 and Pc = 2.51×10^{-2}, OR = 0.788, respectively) (Table 2). Combination of the data from the first and second phase studies further strengthened the association between IL-37/rs3811047 and BD (GG genotype: Pc = 7.27×10^{-8}, OR = 1.693; AG genotype: Pc = 3.67×10^{-6}, OR = 0.616; G allele: Pc = 7.68×10^{-8}, OR = 1.584) and the observed association between IL-18RAP/rs2058660 and BD (AA genotype: Pc = 1.27×10^{-5}, OR = 0.620; A allele: Pc = 5.94×10^{-6}, OR = 0.750) (Table 2).

The Influence of IL/37rs3811047 and IL-18RAP/rs2058660 genotypes on mRNA and cytokine expression. To study the effect of genotype on mRNA or cytokine expression, we performed experiments with peripheral blood mononuclear cells (PBMCs) taken from healthy genotyped individuals instead of using patients. This approach was chosen in view of the ongoing inflammation in these patients and the fact that they often were receiving immunosuppressive drug therapy.

Real-time PCR analysis was applied to determine whether rs3811047 and rs2058660 polymorphisms altered the mRNA expression of the IL-37 or IL-18RAP gene, respectively. Functional studies using PBMCs from healthy
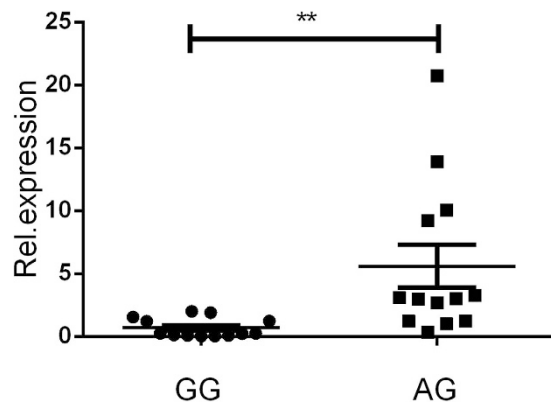
Figure 1. The influence of IL37/rs3811047 genotypes (GG: N = 13, AG: N = 13) on the mRNA expression of IL-37 by PBMCs obtained from healthy genotyped individuals. A statistically significant higher mRNA expression of IL-37 was observed in AG as compared to GG carriers. **P < 0.01.
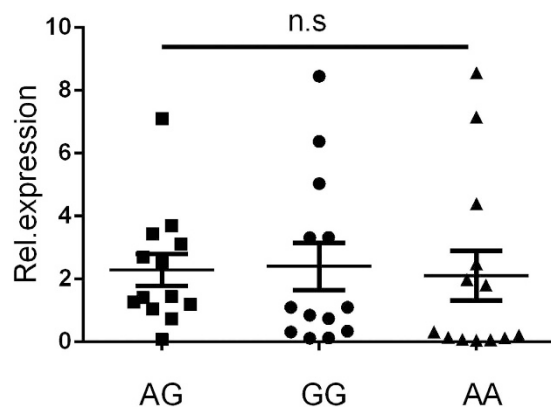

Figure 2. The influence of IL-18RAP/rs2058660 genotypes (GG: N = 13, AG: N = 13, AA: N = 13) on the mRNA expression of IL-18RAP in PBMCs. No statistically significant difference concerning IL-18RAP mRNA expression was detected between the different genotypes.
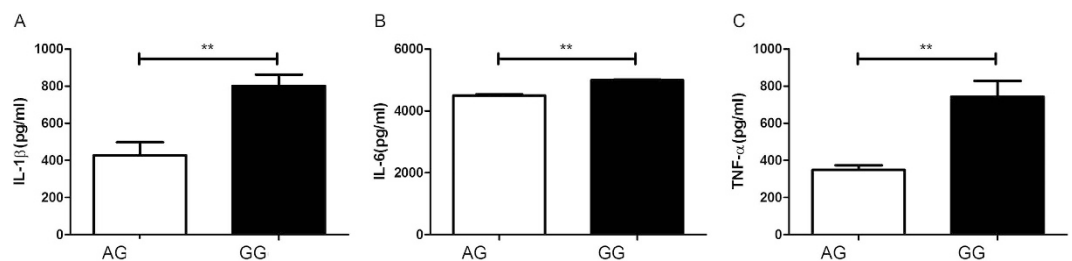
Figure 3. Effect of IL37/rs3811047 genotype on cytokine production by LPS stimulated PBMCs from healthy genotyped individuals (GG: N = 10, and AG: N = 10). Data are expressed as the mean ± SD. **P < 0.01.

genotyped donors indicated that rs3811047/AG carriers had a higher expression of the IL-37 gene as compared to GG carriers (Fig. 1). Polymorphisms of rs2058660 did not significantly affect the IL-18RAP expression in PBMCs from healthy individuals (Fig. 2).

The effect of IL-37/rs3811047 genotype on cytokine production was tested in lipopolysaccharide (LPS) stimulated PBMCs taken from genotyped healthy controls. An enzyme-linked immunosorbent assay (ELISA) was applied to examine the level of tumor necrosis factor (TNF-α), IL-1β and IL-6 in 72h cell culture supernatants. GG carriers showed a two-fold higher level of IL-1β and TNF-α as compared to that detected in AG carriers, whereas IL-6 production was only modestly increased (Fig. 3). We did not test AA carriers since the frequency of this genotype is very low (3.5%; see data of controls in Table 2).

Discussion

This study shows that IL-37 and IL-18RAP gene polymorphisms are associated with BD but not with VKH uveitis in Han Chinese. No association could be found for gene polymorphisms in other members of the IL-1 and IL-1R family with either BD or VKH. Functional studies showed that carriers of the GG risk genotype of IL-37/rs3811047 had a higher mRNA expression of the gene and that PBMCs from such carriers showed a higher pro-inflammatory cytokine response compared to carriers of the AG genotype. An explanation for the novel association of BD with the IL-18RAP/rs2058660 gene polymorphism could not yet be supported by functional assays with PBMCs, although a recent report did show that this polymorphism was linked to a low expression of the β-chain of this factor, causing a lower response to IL-18, as detected both on the RNA and protein level of CCL3, CCL20, TNF-α and CXCL8 in granulocytes[29]. Another study that used human monocyte-derived macrophages showed that polymorphisms in this region had a marked effect on the expression of cell-surface IL-18RAP protein, leading to an altered cytokine secretion following stimulation of a wide range of pattern-recognition receptors[30]. The IL-18RAP/rs2058660 tag SNP belongs to a linkage block on chromosome 2.q12 that has a strong association with leprosy and Crohn's disease(CD)[31].

Our study confirms earlier reports on the association of IL-37/rs3811047 with immune mediated diseases such as PsA[14], RA[31] and Graves' disease (GD)[32]. To our knowledge this is the first report addressing the association of IL-37 gene polymorphisms with uveitis. The fact that only an association was found with ocular BD and not with VKH uveitis suggests that our finding cannot be generalized for all uveitis entities and that further research is necessary to study the association of IL-37 with other uveitis types. Why only an association was found with BD and not with VKH may be caused by differences in disease causing mechanisms. BD is considered an autoinflammatory disorder caused by an aberrant response against microbial antigens, whereas VKH is mediated by a loss of immunological tolerance against melanin associated antigens. An association with factors regulating innate immunity is therefore more plausible for BD than for VKH. Earlier studies from our group however showed that IL-37 expression in PBMCs was decreased in both VKH and BD patients and that it was related to disease activity[33,34]. Further study in VKH patients showed that treatment with corticosteroids and cyclosporine A was associated with an increased expression of IL-37[34]. A decreased IL-37 expression in BD was confirmed recently[35], although other groups reported that IL-37 expression was increased in immune-mediated inflammatory conditions such as RA, PsA, AS, GD and SLE[36–41]. The reasons for these discrepancies concerning IL-37 expression between uveitis and other autoimmune diseases is not clear and deserves further study.

IL-37 is coded by a gene that belongs to the so called IL-1 gene cluster that is located in a 360 kb region on human chromosome 2q13[42,43]. Other proteins coded by this region include IL-1α, IL-1β, IL-1 receptor antagonist, IL-36α, IL-36β and IL-36γ, IL-36 receptor antagonist and IL-38. We were not able to find an association between BD with other genes than IL-37 in this cluster, although others have reported an association between BD and rs16944 in the IL-1β locus[43]. The rs16944 locus is in strong linkage disequilibrium (LD) with the rs1143627 locus used in our study. The discrepancy may be due to a different BD patient subgroup, since only 54.6% of these Tunisian patients showed ocular involvement, whereas all our patients had intraocular inflammation.

There is ample evidence that IL-37 suppresses both innate as well as adaptive immune responses[44]. It is expressed in PBMCs and can be induced by Toll-like receptor agonists. IL-37 interacts with a heterologous receptor combination comprised of IL-18Ra and IL-1R8 which leads to an inhibition of the activation of inflammatory pathways in the cell[21].

An earlier Genome-wide analysis (GWAS) in both BD and VKH did not reveal an association with IL-37 or IL-18RAP, but showed MHC class I, IL10, IL23R/IL12RB2, HLA-B*51 and ERAP1 associations with BD[45,46]. This may be due to the strong p value threshold used in GWAS, sample size or the use of insensitive tag SNPs. It should be noted that the observed associations were present in common variants of these genes and that the odds ratios (OR) were modest. The data are nevertheless supportive of an inflammatory pathway that involves triggering of toll like receptors (TLRs)[47] followed by the activation of the IL-1 family. A pro-inflammatory genotype involving a myriad of cytokines and their receptors may render an individual at risk for a disease like BD, despite the fact that the OR for each individual cytokine might be low[48].

The current study has several limitations. Although great effort was made to exactly match patients and controls for gender this was not completely achieved due to the large male preponderance in our BD group. On the other hand, we did not detect differences in genotype frequencies after stratification for gender (data not shown). Gender differences have been noted for certain geographical regions and males often suffer from the more severe forms of BD uveitis[49]. Validation of our findings is therefore necessary in other ethnic samples. All our BD patients had uveitis and it is not known whether our findings are restricted to patients with ocular BD. Further studies are therefore needed in BD patients recruited via other disciplines than ophthalmology, such as dermatology or rheumatology. A further restriction of our findings concerns the functional assays we performed. These were restricted to the testing of PBMCs, whereas recent studies suggest that addressing the response in neutrophils or macrophages might be more suitable[50,51].

In conclusion, this study reports a novel association between IL-37/rs3811047 and IL-18RAP/ rs2058660 polymorphisms with BD in Han Chinese, which supports the important role of the IL-1 pathway in the pathogenesis of this disease and may provide a future target for its treatment.

Materials and Methods

Study Population. The study was divided into two phases: an exploratory and a confirmatory phase. A total of 3354 unrelated out-patients including 419 VKH cases, 1063 BD cases and 1872 healthy controls seen at the First Affiliated Hospital of Chongqing Medical University between April 2008 and August 2015 were included. For the first phase, 419 VKH patients and 416 BD patients as well as 627 healthy controls were tested. For the second phase, a different set of 647 BD patients and 1245 healthy controls were investigated. All subjects, either patients or controls, were Han Chinese. The controls were randomly selected among normal unrelated individuals

with suitable age, ethnicity and geographic origin. Diagnostic criteria for BD and VKH disease strictly followed the International Study Group for BD and the modified diagnostic standards for VKH disease[51–53]. The prominent features of the BD and VKH cases in this study are shown in Table 1.

Ethical considerations.   Before the collection of blood, all the investigated individuals had signed the informed consent. The investigation protocols obtained the approval of the Clinical Research Ethics Committee of the First Affiliated Hospital of Chongqing Medical University. All experiments were conducted in accordance with the approved guidelines and regulations and the study was conducted according to the tenets of the Declaration of Helsinki.

SNP selection and genotyping.   Based on the data of Han Chinese in the HapMap database, this study utilized HaploView 4.2 to screen the candidate tag SNPs through a r2 critical value of 0.8 as well as a minor allele frequency (MAF) larger than 0.05. According to the criteria and previous reported associations of the ten IL-1 and IL-1R related genes (such as IL1A, IL1B, IL1RN, IL18, IL18RAP, IL33, IL37, IL38, IL18R1 and L1 ligand cluster) with autoimmune disease, this study selected five SNPs of IL1A (rs2071374, rs3783526, rs2856836, rs1894399, rs1800587), three SNPs of IL1B (rs1143627, rs1143643, rs2853550), five SNPs of IL1RN/ (rs1688075, rs30735, rs2234650, rs928940, rs315952), one SNP of IL18 (rs1946518), one SNP of IL18RAP (rs2058660), one SNP of IL18R1 (rs13015714), one SNP of IL37 (rs3811047), two SNPs of IL38 (rs7570267, rs3811058), four SNPs for IL33 (rs10118795, rs1929992, rs10975519, rs1048274), and one SNP of IL-1 ligand cluster (rs6712572).

All blood samples were stored in 3.2% sodium citrate-treated tubes at $-80\,^{\circ}$C, from which the DNA of BD and VKH patients and controls was extracted by utilizing the QIA amp DNA Blood Mini Kit (QIAGEN, CA). Subsequently, the collected DNA specimens were kept at $-20\,^{\circ}$C. The polymerase chain reaction (PCR) was performed on a 9700 thermocycler of the ABI Gene Amp PCR System according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). The MassARRAY platform (Sequenom, San Diego, CA) and iPLEX Gold Assay were used to score genotypes. The MassArray Designer of Sequenom was utilized to design the PCR as well as extended primers of the respective SNPs. Experimental data were analyzed through TYPER software version 4.0. In the confirmatory experiment, rs3811047 and rs2058660 genotypes were tested by the TaqMan® SNP Genotyping Assay (Applied Biosystems, Foster City, CA) in the 7500 Real-Time PCR system of the Applied Biosystems. Data were analyzed with TaqMan® Genotyper Software.

Cells isolation and culture, RNA preparation and real-time quantitative PCR.   Peripheral blood mononuclear cells (PBMCs) were isolated from blood obtained from healthy controls using Ficoll-Hypaque density-gradient centrifugation. TRIzol reagent (Invitrogen, Carlsbad, CA)) was used to extract RNA from PBMCs. The PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Japan) was used to synthesize cDNA according to the manufacturer's instructions. The Power SYBR Green PCR Master MIX (Biosystems, UK) was applied to test IL-37/IL-18RAP expression with the ABI 7500 Real-Time PCR System. The following primers of IL-37/IL-18RAP and ß-actin were used: IL-37 (forward: 5′-AGTCATCCATCCCTTCAGC-3′ and reverse: 5′-CCCAACAGGCTCATTACAA-3′), IL-18RAP (forward: 5′-CAGATATTCTGGATCCTGTCGAG-3′ and reverse: 5′-TGCTTTGCAGCTAATAGTTAAAGG-3′), β-actin (forward: 5′-CGAGAAGATGACCCAGATCATG-3′ and reverse: 5′-CAGAGGCGTACAGGGATAGCA-3′). Expressions of IL-37/IL-18RAP were calculated and compared with the expression of β-actin through the 2-$\Delta\Delta$CT approach.

Measurement of cytokines by ELISA.   Human Duoset ELISA kits (R&D Systems) were used to analyze the level of IL-6, IL-1β and TNF-α in the culture supernatants of PBMCs following stimulation with LPS (100ng/ml; Sigma-Aldrich, USA) for 72 hours.

Statistical analysis.   The Chi-square (χ^2) test was adopted to examine the Hardy-Weinberg equilibrium(HWE) in healthy samples while the genotype frequency was estimated by direct counting. The χ^2 test was used to examine the differences between patients and healthy controls with regard to allele and genotype frequencies with SPSS17.0 statistical software package (version17.0, SPSS, Chicago, IL). Correction for multiple comparisons was performed using the Bonferroni method whereby the p value was multiplied with the number of comparisons (P corrected (P$_c$)). A P$_c$ < 0.05, was taken as statistically significant. The Mann-Whitney test, one-way analysis of variance (ANOVA), and Kruskal-Wallis H test and Bonferroni correction were adopted by SPSS Statistics 17.0 software, where p values smaller than 0.05 were regarded as significant.

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Author Contributions

H.T. and B.D. conceived and designed the study. H.T., B.D., H.Y., L.D., Q.Z., J.Q. and Y.Y. performed the experiments. H.T. and B.D. wrote the paper. H.Y., R.C., A.K. and P.Y. reviewed and edited the manuscript. All authors read and approved the manuscript.

Additional Information

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