Genetic polymorphisms of C-type lectin receptors in Behcet’s disease in a Chinese Han population

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C-type lectin receptors (CLRs) have been demonstrated to be involved in several autoimmune diseases. The role of CLRs in Behcet’s disease (BD) is unknown and thus was the purpose of this study. A two-stage association study was carried out and a total of 766 BD patients and 1674 healthy controls were recruited. Genotyping of 14 SNPs of 13 genes in CLRs was carried out by iPLEX Gold genotyping or polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. The expression of mannose binding lectin 2 (MBL2) and killer cell lectin like receptor C4 (KLRC4) was measured by Real-time PCR. Significantly increased frequencies of the A allele as well as AA genotype of rs1800450 in MBL2 (Pc = 2.50 × 10−6, OR = 1.494; Pc = 2.24 × 10−6, OR = 2.899; respectively) and TT genotype of rs2617170 in KLRC4 (Pc = 2.53 × 10−6, OR = 1.695) and decreased frequencies of GG genotype of rs1800450 (Pc = 1.56 × 10−3, OR = 0.689) and C allele as well as CC genotype of rs2617170 (Pc = 2.05 × 10−9, OR = 0.585; respectively) were observed in BD. Two variants, p.Gly54Asp (rs1800450) and p.Asn104Ser (rs2617170) affect MBL2 and KLRC4 protein stability and expression. Our study demonstrates that the MBL2/rs1800450 and KLRC4/rs2617170 are susceptibility factors for BD in a Chinese Han population.

Behcet’s disease (BD) is a well-known multisystem vasculitis, characterized by recurrent uveitis, oral ulcerations, genital ulcerations and typical skin lesions. It often occurs in young adulthood and causes serious disability and significant visual impairment. BD is more frequent among populations along the “silk route” from the Mediterranean Basin to East Asia. Although the pathogenesis of BD is not yet exactly known, it has been hypothesized that autoimmunity and genetic factors are responsible for this disease. Recent studies have implicated that Human Leukocyte Antigen (HLA) and non-HLA genes seem to collectively contribute to the genetic background causing this disorder among different populations. Most of the non-HLA genes such as TNFAIP3, IL23R, IL12RB2, IL10, CCR1, STAT4, KLRC4, ERAP1, FUT2, and IL12A have been reported with genome-wide significance whereas NOS3 and JAK1 were reported with study-wide significance.

C-type lectin receptors (CLRs) are a large group of extracellular Metazoan proteins expressed on immune cells that have been classified as pattern recognition receptors (PRRs) which play an important role in the binding of pathogens via their surface carbohydrate structures. CLRs not only play a pivotal role in the process of anti-inflammatory immune responses but also in the maintenance of host immune-homeostasis. Growing evidence suggests that various members of CLRs are associated with severe immune mediated diseases like juvenile idiopathic arthritis (JIA), type 1 diabetes (T1DM), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and multiple sclerosis (MS). It was recently shown that patients with BD had significantly lower median serum mannose-binding lectin (MBL) levels compared to healthy controls. Whether gene polymorphisms of CLRs are associated with the susceptibility to BD is not yet known and was therefore the purpose of our study. We identified two variants, p.Gly54Asp (rs1800450) in MBL2 and p.Asn104Ser (rs2617170) in KLRC4, to contribute to the risk of developing BD.

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**Table 1.** Clinical features, age, and sex distribution of BD patients and healthy controls.

| Clinical features | total | %   |
|-------------------|-------|-----|
| Patients with BD  | 766   |     |
| Mean age ± SD     | 35.7 ± 8.9 |    |
| Male              | 663   | 86.6|
| Female            | 103   | 13.4|
| Uveitis           | 766   | 100 |
| Oral ulcer        | 766   | 100 |
| Genital ulcer     | 454   | 59.3|
| Skin lesions      | 605   | 79  |
| Arthritis         | 122   | 15.9|
| Positive pathergy test | 123 | 16.1|
| Healthy controls  | 1674  |     |
| Mean age ± SD     | 39.5 ± 10.7 |   |
| Male              | 938   | 56  |
| Female            | 736   | 44  |

**Results**

**Clinical characteristics of BD patients.** Clinical features, age as well as gender distribution in the recruited BD patients and healthy controls are presented in Table 1. All patients had uveitis. Our BD patient group contained more males than the control group. The genotype frequencies of the 14 SNPs were tested and the results did not deviate from the Hardy-Weinberg equilibrium in the healthy controls.

**Frequency of genotypes and alleles of the examined SNPs in BD versus healthy controls in the first-phase study.** The fourteen SNPs were tested in 388 BD cases and 742 healthy controls during the first phase study. The frequencies of *MBL2/rs1800450* AA genotype (Pc = 0.02, OR = 2.556) and *KLRC4/rs2617170* TT genotype (Pc = 8.90 × 10⁻³, OR = 1.688) were significantly higher in BD. A significant lower frequency of the *KLRC4/rs2617170* C allele and CC genotype was also observed (Pc = 1.66 × 10⁻⁴, OR = 0.661; Pc = 1.02 × 10⁻², OR = 0.576; respectively) in BD (Table 2). However, we failed to find a significant association between the remaining SNPs and ocular BD (Supplementary Table 1).

**Genotype and allele frequency of the examined SNPs in BD versus healthy controls in the second phase and combined study.** To validate the results from the first stage study, a different cohort of patients (378 BD cases and 932 healthy individuals) was recruited for the second stage study. The frequencies of *MBL2/rs1800450* A allele and AA genotype in BD patients was significantly higher (Pc = 6.70 × 10⁻³, OR = 1.637; Pc = 2.32 × 10⁻¹, OR = 3.204; respectively), whereas a lower frequency of the GG genotype (Pc = 1.90 × 10⁻³, OR = 0.6) was observed in BD compared with controls (Table 2). The *KLRC4/rs2617170* C allele and CC genotype frequencies were significantly lower in BD compared to controls (Pc = 1.47 × 10⁻⁴, OR = 0.667; Pc = 1.63 × 10⁻², OR = 0.593, respectively), while a higher frequency of the TT genotype was detected in BD (Pc = 4.16 × 10⁻³, OR = 1.701) (Table 2). Combination of the data from the two stage studies confirmed that rs1800450 in *MBL2* was correlated with the risk to BD (A allele: Pc = 2.50 × 10⁻⁶, OR = 1.494; AA genotype: Pc = 2.24 × 10⁻⁶, OR = 2.899; GG genotype: Pc = 1.56 × 10⁻³, OR = 0.689;) (Table 2), and that rs2617170 in *KLRC4* also contributed to susceptibility of BD (C allele: Pc = 2.05 × 10⁻⁴, OR = 0.664; CC genotype: Pc = 1.20 × 10⁻⁵, OR = 0.585; TT genotype: Pc = 2.53 × 10⁻⁶, OR = 1.695) (Table 2).

**The Influence of *MBL2/rs1800450* and *KLRC4/rs2617170* on gene mRNA transcription and cytokine production.** In order to find a biological explanation for the association of BD with *MBL2/rs1800450* and *KLRC4/rs2617170*, the mRNA expression of *MBL2* as well as *KLRC4* of healthy genotyped individuals was measured in their PBMCs. We also evaluated whether different genotypes of *MBL2/rs1800450* and *KLRC4/rs2617170* could influence the production of cytokines important in the development of BD such as IFN-γ, IL-6, IL-8, IL-18, and TNF-α. These experiments were performed in healthy individuals to eliminate confounding effects such as the inflammatory status and immunosuppressive drug effects in our BD patient group.

Real-time PCR data demonstrated that the mRNA level of *MBL2/rs1800450* in GG carriers was remarkably higher than AG carriers (P = 0.019) (Fig. 1). We did not test AA carriers since the frequency of this genotype is very low (<3–4%). The mRNA expression of *KLRC4/rs2617170* in CC carriers showed a significant increase compared to CT/TT individuals (Fig. 2, P < 0.001).

The effect of *MBL2* and *KLRC4* genotype on cytokine production was tested in LPS treated PBMCs isolated from genotyped healthy controls. ELISA was applied to test the concentration of IFN-γ, IL-6, IL-8, IL-18, and IL-10 as well as TNF-α in the 72 hr cell culture supernatants. LPS stimulated PBMCs from GG genotype *MBL2/rs1800450* carriers secreted higher amount of INF-γ, IL-6 and IL-8 than AG carriers (P = 0.002; p = 0.009; p = 0.005; respectively) (Fig. 3a,b,c). Compared to CC (P = 0.002; P = 0.004) carriers, an elevated secretion of IL-8 and IL-10 was observed in TT *KLRC4/rs2617170* (Fig. 4a,b). No effect of the various rs1800450 and rs2617170 genotypes on the release of other cytokines could be detected. (Figs 3d,e,f and 4c,d,e).
ings also showed that CLRs are vital in immune homeostasis, which can induce both pro-inflammatory and expression of some members of CLRs are significantly different between BD cases and healthy individuals, such as compared to healthy controls. Moreover, previous reports showed that CLRs are involved in the development of extracellular proteins. CLRs can bind carbohydrate through CTLD and activate different signaling pathways, by stimulated PBMCs from CC genotype carriers of rs2617170 were increased.

**Discussion**

In this study we show that gene polymorphisms of MBL2 encoding rs1800450 and KLRC4 encoding rs2617170 are associated with BD. Furthermore, the two SNPs were found to affect their gene expression. mRNA expression of MBL2 and KLRC4 were higher in individuals with the GG (BD-protective) genotype of rs1800450 and CC (BD-protective) genotype of rs2617170 as compared to the other genotype carriers. Additionally, INF-γ, IL-6 and IL-8 production by stimulated PBMCs from GG genotype carriers of rs1800450 and IL-8, IL-10 production by stimulated PBMCs from CC genotype carriers of rs2617170 were increased.

C-type lectin receptors (CLRs), often containing the C-type lectin-like domain (CTLD), are a large family of extracellular proteins. CLRs can bind carbohydrate through CTLD and activate different signaling pathways, which induce the expression of specific cytokines ultimately affecting T cell subtype polarization. Recent findings also showed that CLRs are vital in immune homeostasis, which can induce both pro-inflammatory and anti-inflammatory immune responses. BD is a multifactorial autoimmune disease and the interactions between susceptibility genes and environmental factors may affect susceptibility. Several studies suggest that the expression of some members of CLRs are significantly different between BD cases and healthy individuals, such as the increased CD94 expression in BD patients and decreased mannose-binding lectin (MBL) concentration as compared to healthy controls. Moreover, previous reports showed that CLRs are involved in the development of certain autoimmune diseases such as JIA, T1MD, SLE, RA, MS. Based on these studies, we assumed that CLR genetic polymorphisms might also be associated with BD. To validate this hypothesis, we examined the association of polymorphisms of CLRs in BD patients and found a strong association between 2 SNPs, rs1800450 in the MBL2 gene and rs2617170 in KLRC4, with BD in a Chinese Han population. The fact that CLRs play vital roles in the innate immune response against microbial pathogens strengthens the view that BD is caused by an aberrant response against environmental stimuli.

| Gene | SNP | Allele | BD | Controls | P Value | OR(95%CI) |
|------|-----|--------|----|----------|---------|-----------|
|      |     | Genotype | N  | %      |         |           |
| MBL2 | rs1800450 | stage1 | A  | 188 | 24.2 | 284 | 19.1 | 5 × 10⁻⁴ | 0.26 | 1.351 (1.096–1.665) |
|      |     |         | G  | 588 | 75.8 | 1200 | 80.9 |         |     |                     |
|      |      |         | AA | 32  | 8.2  | 25   | 3.4  | 4.25 × 10⁻⁴ | 0.02 | 2.556 (1.492–4.308) |
|      |      |         | AG | 124 | 32   | 234  | 31.5 | 0.885 | NS   | 1.020 (0.783–1.327) |
|      |      |         | GG | 232 | 59.8 | 483  | 65.1 | 0.079 | NS   | 0.797 (0.619–1.027) |
|      | rs1800450 | stage2 | A  | 198 | 26.2 | 332  | 17.8 | 1.31 × 10⁻⁶ | 6.70 × 10⁻⁵ | 1.637 (1.339–2.002) |
|      |     |         | G  | 558 | 73.8 | 1532 | 82.2 |         |     |                     |
|      |     |         | AA | 26  | 6.9  | 21   | 2.3  | 4.54 × 10⁻⁵ | 2.32 × 10⁻³ | 3.204 (1.780–5.769) |
|      |     |         | AG | 146 | 38.6 | 290  | 31.1 | 0.009 | NS   | 1.391 (1.084–1.785) |
|      |     |         | GG | 206 | 54.5 | 621  | 66.6 | 3.72 × 10⁻³ | 1.90 × 10⁻¹ | 0.6 (0.470–0.766) |
|      | rs2617170 | combined | A  | 386 | 25.2 | 616  | 18.4 | 4.90 × 10⁻⁶ | 2.50 × 10⁻⁴ | 1.494 (1.293–1.726) |
|      |      |         | G  | 1146| 74.8 | 2732 | 81.6 |         |     |                     |
|      |      |         | AA | 58  | 7.6  | 46   | 2.7  | 4.39 × 10⁻⁸ | 2.24 × 10⁻⁴ | 2.899 (1.949–4.312) |
|      |      |         | AG | 270 | 35.2 | 524  | 31.3 | 5.40 × 10⁻² | NS   | 1.195 (0.997–1.431) |
|      |      |         | GG | 438 | 57.2 | 1104 | 65.9 | 3.06 × 10⁻³ | 1.56 × 10⁻⁴ | 0.689 (0.579–0.821) |
| KLRC4 | rs2617170 | stage1 | C  | 342 | 44.1 | 807  | 54.4 | 3.25 × 10⁻⁴ | 1.66 × 10⁻⁴ | 0.661 (0.555–0.787) |
|      |     |         | T  | 434 | 55.9 | 677  | 45.6 |         |     |                     |
|      |     |         | CC | 79  | 20.4 | 228  | 30.7 | 2.00 × 10⁻⁴ | 1.02 × 10⁻² | 0.576 (0.430–0.772) |
|      |     |         | CT | 184 | 47.4 | 351  | 47.3 | 0.97  | NS   | 1.005 (0.786–1.285) |
|      |     |         | TT | 125 | 32.2 | 163  | 22   | 1.74 × 10⁻⁴ | 8.90 × 10⁻³ | 1.688 (1.283–2.222) |
|      | combined | stage2 | C  | 333 | 44   | 1009 | 54.1 | 2.89 × 10⁻⁴ | 1.47 × 10⁻⁴ | 0.667 (0.563–0.791) |
|      |      |         | T  | 423 | 56   | 855  | 45.9 |         |     |                     |
|      |      |         | CC | 77  | 20.4 | 281  | 30.2 | 3.19 × 10⁻⁴ | 1.63 × 10⁻² | 0.593 (0.445–0.789) |
|      |      |         | CT | 179 | 47.4 | 447  | 48   | 0.84  | NS   | 0.976 (0.768–1.240) |
|      |      |         | TT | 122 | 32.2 | 204  | 21.9 | 8.16 × 10⁻⁵ | 4.16 × 10⁻³ | 1.701 (1.304–2.218) |
|      |      |         | C  | 675 | 44.1 | 1816 | 54.2 | 4.03 × 10⁻¹ | 2.05 × 10⁻⁴ | 0.664 (0.588–0.750) |
|      |      |         | T  | 857 | 55.9 | 1532 | 45.8 |         |     |                     |
|      |      |         | CC | 156 | 20.4 | 509  | 30.4 | 2.35 × 10⁻⁷ | 1.20 × 10⁻⁵ | 0.585 (0.477–0.718) |
|      |      |         | CT | 363 | 47.4 | 798  | 47.7 | 0.897 | NS   | 0.989 (0.833–1.173) |
|      |      |         | TT | 247 | 32.2 | 367  | 21.9 | 4.96 × 10⁻⁶ | 2.53 × 10⁻⁴ | 1.695 (1.401–2.051) |

Table 2. Genotype and allele frequencies of MBL2 and KLRC4 polymorphisms in BD and healthy controls. SNP, single-nucleotide polymorphism; BD, Behcet's disease; OR, odds ratio; NS, not significant; 95% CI, 95% confidence interval; Pc, Bonferroni corrected p value.
Figure 1. The influence of MBL2/rs1800450 genotypes on the expression of MBL2 by PBMCs. MBL2 mRNA level in GG individuals of SNP rs1800450 was significantly higher than in AG individuals. Data are shown as mean ± SD. *P = 0.019 (GG/AG = 12).

Figure 2. The influence of KLRC4/rs2617170 genotypes on the expression of KLRC4 in PBMCs. KLRC4 mRNA level in CC individuals of SNP rs2617170 was significantly higher than in CC/CT individuals. Data are shown as mean ± SD. **P < 0.001 (CC = 13, CT = 11, TT = 10).

Figure 3. Effect of MBL2 genotype on cytokine production by LPS stimulated PBMCs from healthy genotyped individuals. IFN-γ (a), IL-6 (b), IL-8(c), IL-1β (d), IL-10 (e) and TNF-α (f) were measured by ELISA in the cell culture supernatants. Data expressed as the mean ± SD (AG/GG = 12).
MBL2 belongs to the C-type collectin family, and plays a potential role in innate immunity. Many studies showed that a low or high serum MBL level is involved in several immune mediated diseases (e.g., RA, Crohn's disease, Sjögren disease and diabetic retinopathy\textsuperscript{22–24}). Immune defense function of MBL is associated with its serum level and oligomeric type\textsuperscript{23}. Five SNPs of the MBL2 gene, including three structure variants, codon 52 (rs5030737), 54 (rs1800450), 57 (rs1800451) and two promoter variants, −550 (rs11003125) and −221 (rs7096206) are thought to be responsible for reducing MBL2 serum levels and influencing the formation as well as the stability of oligomeric MBL2\textsuperscript{25–27}. However, others didn't find any association between genetic polymorphisms of MBL2 and BD susceptibility\textsuperscript{28}. In our study, we confirmed that MBL2 is a predisposing gene for BD in a Chinese Han population. Sample selection bias and different genetic backgrounds may explain the observed discrepancy between studies. We did not measure MBL2 levels in serum of our BD patients or controls, since the patients were often treated with immunosuppressive drugs, which may influence the serum concentration of MBL2. Further studies are needed to address this issue.

NKG2F encoded by the KLRC4 gene is a recently described member of the NKG2 family receptors, and its function has not been examined in detail\textsuperscript{29}. This receptor can activate NK cells following the binding with its ligand DAP12\textsuperscript{30}. A recent GWAS has shown that rs2617170 of KLRC4 is associated with BD in Turkish and Japanese patients\textsuperscript{31}. However, it has not yet been reported in the Chinese population. Our results indicate that only the rs2617170 association (C allele: Pc = 2.05 × 10\textsuperscript{−9}, OR = 0.664; CC genotype: Pc = 1.20 × 10\textsuperscript{−5}, OR = 0.585) exceeds the threshold for genome-wide significance (P < 5e-08). Our study confirms the results of a previous GWAS regarding the association of KLRC4/rs2617170 with BD\textsuperscript{32}. Interestingly, the C allele of rs2617170 was associated with disease risk in this GWAS study\textsuperscript{31}, whereas the C allele had a higher frequency in the controls as compared to the BD patients in our study, and would therefore seem to be associated with disease protection. On the other hand, Dixon et al\textsuperscript{32} have reported that rs2617170 is a significant eQTL for KLRC4 expression, and the C allele is associated with reduced KLRC4 gene expression. This is in contrast with our findings where we showed that the C allele is associated with higher gene expression. The reasons for these discrepancies may be due to different ancestral backgrounds of the subjects investigated and this issue clearly deserves further study. Until now, the role of KLRC4 in BD has not received much attention. It has been demonstrated that the stimulation of IL-2 and IL-15 led to an up-regulation of KLRC4 on NK cells\textsuperscript{33}. Other groups have reported that IL-15 levels were elevated in serum, cerebrospinal fluid, and aqueous humor from patients with BD\textsuperscript{33–35}. Further experiments are needed to unravel the functional role of KLRC4 variants on BD pathogenesis. It is interesting to point out that we observed a lower frequency of CC (30.4%) and a higher frequency of CT of rs2617170 in our healthy control group (47.7%) as compared to data reported in the Asian population as shown in the NCBI Resource (42.2% and 35.6%, respectively). However, our results are similar to a previous report on rs2617170 genotype frequencies\textsuperscript{36} in a Chinese Han population (27.4% and 55.8%, respectively), indicating heterogeneity between Asian populations.

Our study has a number of limitations. Firstly, since we only chose the loci with known associations between CLRs and various autoimmune or auto inflammatory diseases, it cannot be excluded that other SNPs in CLRs may have an association with BD. Detailed sequence analysis should be carried out to investigate the potential involvement of other rare variants of these factors in BD development. Secondly, our BD patients were Chinese Han patients recruited from an ophthalmic department and all had uveitis. Not all patients with BD have uveitis and depending on their complaints will see different medical departments. Further studies including BD patients...
from other medical departments (e.g., dermatology, rheumatology, stomatology) and other populations are therefore required to confirm our results and to investigate whether the observed associations are not only confined to the subpopulation of BD patients with uveitis. Due to sample size we also did not investigate whether subgrouping of our patients according to clinical features had an effect on to the CLR gene associations. Last but not least, although our study identified rs1800450 of MBL2 and rs2617170 of KLRC4 as possible risk factors contributing to the susceptibility for BD, the exact mechanism how these variants affect the disease pathogenesis are not yet exactly clarified and await further study.

In summary, our study confirmed that MBL2/rs1800450 and KLRC4/rs2617170 polymorphisms affect disease susceptibility in the Chinese Han population. Further studies are needed to reveal the crucial role of the CLRs pathways in the pathogenesis of BD.

Materials and Methods

Study population. All BD uveitis patients (n = 766) and healthy individuals (n = 1674) included in the present study (n = 2440) were ethnic Han Chinese, recruited from the First Affiliated Hospital of Chongqing Medical University from May 2008 to August 2015. The diagnostic criteria of BD strictly followed the International Study Group for BD 37. Controls were matched for age, geographic origin and ethnicity with BD patients. A case-control study including two phases was performed. In the first phase, 388 BD patients and 742 healthy individuals were included and in the second phase, 378 BD cases and 932 controls were recruited. The major clinical symptoms in the recruited BD cases are clarified in Table 1. This study was conducted under the approval of the Clinical Research Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (Permit Number: 2009-201008). All the procedures complied with the tenets of the Declaration of Helsinki. Informed written consent was provided by all patients and controls.

Single nucleotide polymorphisms (SNPs) selection. SNPs were chosen from previous studies on the correlation between CLRs and various autoimmune or auto-inflammatory diseases 31, 38–46. Minor allele frequency (MAF) and linkage disequilibrium (LD) were tested by HaploView 4.2 software based on the data of Han Chinese in the HapMap database (MAF > 0.05 as well as an r2-value of LD < 0.8). Based on this analysis, we selected a total of 14 SNPs, including 2 SNPs (rs1800450 18, rs7096206 20) in MBL2, one SNPs (911887 42) in SFTPD, one SNP (rs1323461 46) in CLEC12A, one SNP (rs2377422 46) in CLEC4A, one SNP (3764022 46) in CLEC2D, one SNP (rs2287886 26) in CD209, one SNPs (rs4763879 45) in CD69, one SNP (rs2302489 46) in KLRD1, one SNP (rs2734440 17) in KLRC1, one SNP (rs225536 46) in KLRC1, one SNP (rs2617170 22) in KLRC4, one SNP (rs4763653 46) in KLRC1 and one SNP (rs1121401 46) in KLRG1. CLEC16A 46 and selectins 46 were excluded from this study because they have been reported previously by our team.

Genomic DNA Extraction and Genotyping. Genomic DNA extraction from peripheral blood cells was performed using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, California, USA), according to instructions of the manufacturer. The concentration and quality of DNA were analyzed with a Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) and then the DNA samples were standardized and kept at −20 °C until used.

In the first stage, all SNPs in our study (except rs1800450) were genotyped by the MassARRAY platform (Sequenom, USA) and iPLEX Gold Assay. The PCR reaction was carried out by the Gene Amp PCR System 9700 instrument (ABI, Foster City, CA, USA). MassARRAY assay design software was used to design the primers (Table 3). Experimental data were analyzed through SpectroTYPEr software (version 4.0; Sequenom). Rs2617170 in the second stage was performed by the TaqManH SNP Genotyping Assay in the 7500 Real-Time PCR system (Applied Biosystems, USA). The results were examined through TaqManH Genotyper Software. Rs1800450 was genotyped by the PCR-RFLP method.

Cell isolation and culture. Ficoll-Hypaque density-gradient centrifugation was used to separate Peripheral blood mononuclear cells (PBMC) from fresh venous blood of healthy male controls. PBMCs were seeded into 24-well culture plates (1 000 μl per well) in medium RPMI 1640 (supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin). To test the production of cytokine IFN-γ, IL-6, IL-8, IL-1β, IL-10 and TNF-α, PBMCs were stimulated with 100 ng/ml lipopolysaccharide (LPS, 100 ng/ml; Sigma, Missouri, USA) for 24 h.

Real-time PCR. Total RNA extraction from PBMCs was performed using the TRIzol (Invitrogen, San Diego, California, USA) method. RNA was reverse transcribed into cDNA with a Takara transcriptase kit (Takara, Dalian, China). The assays were carried out on an ABI 7500 real-time system with the following primers (KLRC4: 5′-GGAAATGACAGACATATCGGT-3′ and 5′-GTGAGTTGAATTACTACAGACTG-3′; MBL2: GCAAGCAGAAATGGCAGTGAT and AGAGGCTTGAATCGTACA). The expression level was measured by the 2−ΔΔCt method.

Measurement of cytokines by ELISA. The concentration of IFN-γ, IL-6, IL-8, IL-1β, IL-10 and TNF-α in the supernatants of PBMCs were analyzed through the human DuoSet ELISA development kit (R&D Systems, Minneapolis, Minnesota, USA).

Statistical analysis. The differences between BD cases and healthy individuals with regard to allele and genotype frequencies were analyzed by the chi-square (χ2) test with SPSS17.0 statistical software package (version 17.0, SPSS, Chicago, IL). Hardy-Weinberg equilibrium was examined by the SHEsis website.
| SNP_ID | 2nd-PCR | 1st-PCR | UEP_SEQ |
|--------|---------|---------|---------|
| MBL2   | ACGTTGAGATCGCCTTACTCTCATTC | ACGTTGAGATCTCTTCAGACAC | TGGTCTCAGCAC |
| rs7996206 | ACGTTGAGATCGCCTTACTCTCATTC | ACGTTGAGATCTCTTCAGACAC | TGGTCTCAGCAC |
| CD69   | ACGTTGAGATCGCCTTACTCTCATTC | ACGTTGAGATCTCTTCAGACAC | TGGTCTCAGCAC |
| rs4763879 | ACGTTGAGATCGCCTTACTCTCATTC | ACGTTGAGATCTCTTCAGACAC | TGGTCTCAGCAC |
| SFTPD  | ACGTTGAGATCGCCTTACTCTCATTC | ACGTTGAGATCTCTTCAGACAC | TGGTCTCAGCAC |
| KLRD1  | ACGTTGAGATCGCCTTACTCTCATTC | ACGTTGAGATCTCTTCAGACAC | TGGTCTCAGCAC |
| KLRK1  | ACGTTGAGATCGCCTTACTCTCATTC | ACGTTGAGATCTCTTCAGACAC | TGGTCTCAGCAC |
| KLRB1  | ACGTTGAGATCGCCTTACTCTCATTC | ACGTTGAGATCTCTTCAGACAC | TGGTCTCAGCAC |
| CLEC12A | ACGTTGAGATCGCCTTACTCTCATTC | ACGTTGAGATCTCTTCAGACAC | TGGTCTCAGCAC |
| CLEC16A | ACGTTGAGATCGCCTTACTCTCATTC | ACGTTGAGATCTCTTCAGACAC | TGGTCTCAGCAC |
| CLEC2D | ACGTTGAGATCGCCTTACTCTCATTC | ACGTTGAGATCTCTTCAGACAC | TGGTCTCAGCAC |
| SFTPD  | ACGTTGAGATCGCCTTACTCTCATTC | ACGTTGAGATCTCTTCAGACAC | TGGTCTCAGCAC |

Table 3. Primers applied in the analysis of iPLEX Gold genotyping in the CLR related genes.

For multiple comparisons, the Bonferroni correction was used to adjust P values to corrected P values (Pc) according to the number of performed analyses. A p < 0.05 was viewed as significant. Expression of KLRK4, MBL2 and cytokine levels among three genotype groups was tested by the non-parametric Mann-Whitney test or student t test, with P < 0.05 (Two-tailed) taken as being statistically significant.

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Acknowledgements

The authors would like to thank all patients and healthy volunteers enrolled in the present study. This work was supported by the State Key Program for Basic Research of China (Grant No. 2016YFC0904000), National Science Foundation Major International (Regional) Joint Research Project (81320108009), Key Project of National Science Foundation (81130019), National Natural Science Foundation Project (31370893), Chongqing Key Laboratory of Ophthalmology (CSTC, 2008CSTC5003), National Key Clinical Specialties Construction Program of China, Key Project of Health Bureau of Chongqing (2012-1-003), Research fund for Traditional Chinese Medicine of Chongqing Health and Family Planning Commission (ZY201401013) and Chongqing Science & Technology Platform and Base Construction Program(cstc2014pt-sy10002).

Author Contributions

Peizeng Yang and Yi Yang conceived and designed the study. Yi Yang, Handan Tan and Bolin Deng performed the laboratory work and analyzed data. Jiayue Hu, Qingfeng Cao and Gangxiang Yuan collected blood of all patients and controls. Yi Yang wrote the paper. Hongsong Yu, Guannan Su, Aize Kjistra and Peizeng Yang reviewed and edited the manuscript. All authors read and approved the manuscript.

Additional Information

Supplementary information accompanies this paper at doi:10.1038/s41598-017-05877-x

Competing Interests: The authors declare that they have no competing interests.
