KIR and HLA Genotypes Implicated in Reduced Killer Lymphocytes Immunity Are Associated with Vogt-Koyanagi-Harada Disease

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Abstract

Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells are killer lymphocytes that provide defense against viral infections and tumor transformation. Analogous to that of CTL, interactions of killer-cell immunoglobulin-like receptors (KIR) with specific human leukocyte antigen (HLA) class I ligands calibrate NK cell education and response. Gene families encoding KIRs and HLA ligands are located on different chromosomes, and feature variation in the number and type of genes. The independent segregation of KIR and HLA genes results in variable KIR-HLA interactions in individuals, which may impact disease susceptibility. We tested whether KIR-HLA combinations are associated with Vogt-Koyanagi-Harada (VKH) disease, a bilateral granulomatous panuveitis that has strong association with HLA-DR4. We present a case control study of 196 VKH patients and 209 controls from a highly homogeneous native population of Japan. KIR and HLA class I genes were typed using oligonucleotide hybridization method and analyzed using two-tailed Fisher's exact probabilities. The incidence of Bx-KIR genotypes was decreased in VKH patients (odds ratio [OR] 0.58, P = 0.007), due primarily to a decrease in centromeric B-KIR motif and its associated KIRs 2DS2, 2DL2, 2DS3, and 2DL5B. HLA-B22, implicated in poor immune response, was increased in VKH (OR = 4.25, P = 0.0001). HLA-Bw4, the ligand for KIR3DL1, was decreased in VKH (OR = 0.59, P = 0.01). The KIR-HLA combinations 2DL2+C1/C2 and 3DL1+Bw4, which function in NK education, were also decreased in VKH (OR = 0.49, P = 0.012; OR = 0.59, P = 0.013). Genotypes missing these two inhibitory KIR-HLA combinations in addition to missing activating KIRs 2DS2 and 2DS3 were more...
common in VKH (OR = 1.90, P = 0.002). These results suggest that synergistic hyporesponsiveness of NK cells (due to poor NK education along with missing of activating KIRs) and CTL (due to HLA-B22 restriction) fail to mount an effective immune response against viral-infection that may trigger VKH pathogenesis in genetically susceptible individuals, such as HLA-DR4 carriers.

**Introduction**

Vogt-Koyanagi-Harada (VKH) disease is characterized by a bilateral granulomatous panuveitis. Extra-ocular symptoms can include aseptic meningitis, poliosis, vitiligo, and sensorineural hearing loss [1]. Clinical and experimental evidence indicates that VKH is an autoimmune disease against melanocytes [2]. Epidemiological studies, including a multistage genome-wide association study of VKH disease, have identified several genetic susceptibility factors that are associated with autoimmune disease [3,4]. Specific human leukocyte antigens (HLA), particularly HLA-DR4, is the strongest genetic risk factor associated with VKH disease [5]. However, the strength of the DR4 association varies among ethnic groups, indicating additional genetic risk factors for VKH.

HLA molecules display a remarkable degree of polymorphism and play a central role in antigen presentation to T lymphocytes that bear αβ T cell receptors [6–8]. The HLA molecules are of two classes that differ in their structure and function. HLA class II molecules (DR, DQ, DP) present antigens to CD4+ T lymphocytes, while HLA class I molecules (HLA-A, -B, -C) present antigens to cytotoxic T lymphocytes (CTL). Certain HLA class I variants also function as ligands for natural killer (NK) cells, the innate lymphocytes that are capable of lysing virally-infected cells in an antigen independent manner quickly without a “priming” period as required for T cells [9] and thus NK cells are crucial for the early control of infections [10]. NK cells discriminate infected cells from healthy cells by measuring the net input of activating and inhibitory signals perceived from infected/transformed cells through a variety of germline-encoded NK cell receptors [11].

Killer cell immunoglobulin-like receptors (KIR) are the key receptors for human NK cells that bind specific HLA class I ligands [12,13]. KIR-mediated NK cell interaction with self-HLA class I molecules begets NK cell maturation (termed licensing or education) and the subsequent ability to survey, recognize, and kill stressed target cells that have lost HLA class I molecules as a consequence of viral infection or tumor transformation [14,15]. The KIR gene family displays a high degree of diversity determined not only by the variability in KIR gene content between haplotypes, but also by allelic polymorphism [16]. Only four KIR genes (3DL3, 3DP1, 2DL4, and 3DL2) are present on all haplotypes; these are referred to as ‘framework’ genes. KIR3DL3 and 3DL2 mark the centromeric and telomeric boundaries of the KIR gene complex respectively, while 3DP1 and 2DL4 are located in the middle of the KIR gene complex. The 14 kb DNA sequence enriched with L1 repeats between 3DP1 and 2DL4 divides the KIR gene complex into two halves: 3DL3 at the 5′-end and 3DP1 at the 3′-end mark the centromeric half, while 2DL4 at the 5′-end and 3DL2 at the 3′-end mark the telomeric half [17]. KIR2DL1, 2DL2, 2DL3 and 2DS2 are only found on the centromeric half of the KIR gene complex while KIR3DL1, 3DS1, 2DS1 and 2DS4 are only found on telomeric half. Three KIR genes, 2DL5, 2DS3 and 2DS5, are found in both centromeric and telomeric locations.

On the basis of gene content, KIR haplotypes are broadly classified into two groups, A and B [18] Group A haplotypes have a fixed gene content (KIR3DL3-2DL3-2DP1-2DL1-3DP1-2DL4-3DL1-2DS4-3DL2) that encodes four inhibitory KIRs 2DL1, 2DL3, 3DL1 and 3DL2, specific
for four major HLA class I ligands, C2, C1, Bw4, and A3/A11 respectively, and an activating KIR 2DS4. In contrast, group B haplotypes vary both in number and combination of KIR genes, and comprise several genes (2DL2, 2DL5, 2DS1, 2DS2, 2DS3, 2DS5, 3DS1) that are not part of the A haplotype.

Functional studies and clinical correlations point to HLA-C as the dominant ligands for KIR. All known allotypes of HLA-C have either Asparagine (HLA-Cw1, Cw3, Cw7, Cw8, Cw12, Cw14, and Cw16—termed C1 epitope) or Lysine (HLA-Cw2, Cw4, Cw5, Cw6, Cw15, Cw17 and Cw18—termed C2 epitope) at position 80, located in the F-pocket of the peptide binding groove, and these dimorphic epitopes are recognized by different isoforms of KIR2D [19–22]. Epitopes C1 and C2 are recognized by the inhibitory KIR 2DL2/3 and 2DL1 receptors, respectively. Two unusual HLA-B allotypes (HLA-B’46:01 and HLA-B’73:01) that lack Bw4 and Bw6 epitopes but carry the C1 epitope, are recognized by the inhibitory receptor 2DL2/3 [23]. KIR3DL1 binds to the Bw4 epitope, defined by amino acid residues 77–83 in the α1 domain [24,25], which is present on approximately 40% of the HLA-B allotypes (B13, B27, B37, B38, B44, B47, B49, B51, B52, B53, B57, B58, B59, B63, B77) and 17% of HLA-A allotypes (HLA-A23, 24, 25 and 32). The HLA-A3 and HLA-A11 allotypes carry the A3/11 epitope recognized by KIR3DL2; however, the precise specificity of this receptor has not been defined [26,27]. Peptides bound by HLA-A3/11 influence its binding to KIR3DL2 [28], and HLA-A3/11-KIR3DL2 recognition does not appear to educate NK cells [29]. Very little is known about the ligands for the activating KIRs. Presumably the activating receptors recognize either ‘induced-self’ (such as MICA and MICB), ‘altered-self’ (HLA class I molecule loaded with viral peptide) or ‘non-self’ (pathogen-encoded molecules).

Given that KIR genes at chromosome-19 and HLA genes at chromosome-6 are polymorphic and display significant variations, the independent segregation of these unlinked gene families produces extraordinary diversity in the number and type of KIR-HLA pairs inherited in individuals [13,30]. KIR-HLA variation affects the KIR repertoire of NK cell clones, NK cell maturation, the capability to deliver signals, and consequently the NK cell response to human diseases [31]. Previous studies have suggested that activating KIR genes and Bx KIR haplotypes confer risk for VKH [32–34]. However, these studies are limited by small sample sizes and use of published controls. In order to better define the role of KIR and HLA variations in VKH, we analyzed a large cohort of VKH patients and healthy controls from a highly homogeneous population of Japan, where the VKH is the second most common cause of uveitis, accounting for 7.0% of cases [35].

Materials and Methods

Study subjects

Genome-wide single nucleotide polymorphism (SNP) analysis has clearly shown that most Japanese individuals fall into two main clusters: the Hondo cluster includes most of the individuals from the main islands in Japan, and the Ryukyu cluster includes most of the individuals from Okinawa [36]. The SNPs with the greatest frequency differences between the Hondo and Ryukyu clusters were found in the HLA region in chromosome 6. Moreover, HLA genotyping analysis of 2,005 individuals from 10 regions of Japan found a significant differentiation between Okinawa Island and main island Japanese [37]. Because population stratification can cause spurious associations in case-control studies, we studied the Japanese main islands other than Okinawa Island. One hundred and ninety-six patients diagnosed with VKH disease using published criteria [38] with an average age at onset of 47.9 years (59.7% female) and 209 age-, sex-, ethnically-matched healthy controls with an average age of 44.3 years (59.8% female) were recruited from the main islanders of Yokohama City University (Yokohama City, Japan) and Hokkaido University (Hokkaido, Japan). The control subjects were not related to each other.
or to the VKH patients in this study. The study was reviewed and approved by the ethics committees at Yokohama City University and Hokkaido University Hospital. All DNA samples received at UCLA were de-identified and only marked as having been obtained from patients with VKH disease or controls. Data obtained were Health Insurance Portability and Accountability Act (HIPAA) compliant, and the study adhered to the tenets of the Declaration of Helsinki.

**KIR and HLA genotyping**

DNA was isolated from peripheral blood using QIAamp blood kit (Qiagen, Valencia, CA). The quality and quantity of DNA was determined by ultraviolet spectrophotometry, and the concentration was adjusted to 100 ng/μl. The presence and absence of 15 KIR genes (2DL1, 2DL2, 2DL3, 2DL4, 2DL5A, 2DL5B, 3DL1, 3DL2, 3DL3, 3DS1, 2DS1, 2DS2, 2DS3, 2DS4, and 2DS5) and two pseudogenes (KIR2DP1 and 3DP1) was determined using the Luminex® technology based KIR sequence-specific oligonucleotide (SSO) hybridization method (One Lambda, Canoga Park, USA). Briefly, target DNA was PCR-amplified using three separate group-specific primer sets targeting Exons 3+4, 5, and 7–9. Each PCR product was biotinylated, allowing later detection using R-Phycocerythrin-conjugated Strepaavidin (SAPE). Each PCR product was denatured and allowed to hybridize to complementary DNA probes conjugated to fluorescently coded microspheres. After washing the beads, bound amplified DNA from the test sample was tagged with SAPE. A flow analyzer, the LABScan™ 100, identified the fluorescent intensity of PE (phycocerythin) on each microsphere. The assignment of genotypes was based on the reaction pattern compared to patterns associated with published KIR gene sequences in Immuno-Polymorphism Database (http://www.ebi.ac.uk/ipd/kir/). The unique and unusual KIR genotypes were further confirmed by retyping using sequence-specific priming-based-polymerase chain reaction typing system (SSP-PCR) and duplex SSP-PCR typing methods [39,40]. UCLA International KIR exchange reference DNA samples, which covers 20 common KIR genotypes, were included as controls for KIR genotyping assays. HLA-A, -B, and -C typing was performed by Luminex® technology based SSO hybridization methods (One Lambda, Canoga Park, CA). The KIR-binding HLA class I epitopes were predicted from the HLA typing results.

**Data Analysis and Statistical Methods**

Differences between controls and patients in the distribution of KIR genotypes, HLA allotypes, KIR-binding HLA motifs, and KIR-HLA gene combinations were tested by two-tailed Fisher’s exact probabilities (p), with P<0.05 considered to be statistically significant. Since each subject was tested for several HLA alleles and the same data were used to compare the frequency of all the detected alleles, it is probable that one of these alleles will by chance deviate significantly. To overcome this error, P was corrected (Pc) by the use of the Bonferroni inequality method (i.e., multiplication of the P values with the number of alleles compared). Odds ratios (OR) with 95% confidence intervals (95% CI) were calculated as the estimate of magnitude of associations of genotypes between patient and control groups. The haplotype frequencies were calculated from the genotype data by the maximum likelihood method using two different computer packages: Arlequin v3.5.2.2 [41] and LinkDos [42], and both analyses provided identical results.

**Results**

Centromeric B haplotype-specific KIR genes that are implicated in a stronger NK cell response were decreased in VKH patients

*KIR* gene content profiles were compared between 196 patients with VKH and 209 healthy controls from a homogeneous population of Yokohama and Hokkaido, Japan (Fig 1). A total
of 19 genotypes that differ by KIR gene content were identified. A significant difference between patients with VKH disease and controls was observed in the distribution of genotypes with the AA-AA (P = 0.007) or AB-AA (P = 0.05) constellations. The frequency of Bx KIR genotypes was decreased in VKH patients compared to controls (OR = 0.58 [95% CI 0.39–0.86], P = 0.007) (Fig 1 and Table 1), and consequently the frequency of AA KIR genotypes was increased in VKH patients (OR = 1.72 [95% CI 1.16–2.55], P = 0.007). The decreased frequency of Bx genotypes in patients was driven by a decrease in centromeric Bx motif (OR = 0.52 [95% CI 0.30–0.88], P = 0.018). Consequently, all variable B haplotype-specific KIR genes at the centromeric half (2DL2, 2DS2, 2DS3, 2DL5B) were reduced in patients (Table 1). There was no difference in the frequencies of telomeric AA or telomeric Bx groups between patients and controls.

Nearly half of the patients with VKH disease carry HLA-B22, which has been implicated in immunological nonresponsiveness

Distributions of HLA-A, -B, and -C allotypes in patients with VKH disease and controls are compared in Table 2. The number of observed HLA-A, B, and -C alleles and the heterozygosities for patients with VKH disease and controls were similar, and did not deviate from Hardy-Weinberg equilibrium (S1 Table). HLA-B54, B56 and Cw1 had a positive association with VKH disease, while HLA-A33, B7, B44, B46, B52 and Cw12 exhibited a negative association with the disease. Of these, the HLA-B54 antigen showed the strongest association, with an odds ratio of 5.39, P = 0.0001 (Pc < 0.005). HLA-B54, B55, and B56 are a group of related antigens originally defined using serologic methods as HLA-B22. The HLA-B22 family members differ by only 1–3 amino acids and display similar peptide binding specificities [43].
Comparison of the entire HLA-B22 group between patients and controls revealed a positive association with VKH disease; HLA-B22 family members were found in 49.5% of patients vs. 17.7% of controls (OR = 4.25 [95% CI 2.70–6.70], P = 0.0001, Pc = 0.002). The association with Cw1 is likely due to its strong linkage disequilibrium with B54 allele, which is evident from all B54 haplotypes carry Cw1 (S2 Table).

Frequencies of Bw4, a ligand for KIR3DL1, containing HLA-B allotypes were decreased in patients with VKH disease

Four of the six HLA class I molecules that displayed negative association with VKH disease function as ligands for KIR receptors–HLA-B44 and B52 carry the Bw4 ligand that binds to KIR3DL1 while HLA-B46 and Cw12 carry the C1 ligand that binds to KIR2DL2 and 2DL3. Consequently, the frequency of Bw4 ligand was decreased in patients with VKH disease (OR = 0.59 [95% CI 0.40–0.87], P = 0.010) (Table 3). Bw4 splits into two subsets that differ by the amino acid residue 80, which can be either threonine (Bw4T80) or isoleucine (Bw4I80). While the frequencies of both subsets were decreased in patients compared to controls, the decrease was statistically significant only for Bw4T80 (OR = 0.51 [95% CI 0.30–0.89], P = 0.022). No significant differences were observed in the frequencies of A3/11, Aw4 (Bw4 ligand on HLA-A23, 24, 25 and 32), C1 and C2 ligands between controls and patients (Table 3). However, genotypes with ≥3 copies of C1-ligands were reduced in patients (OR = 0.24 [95% CI 0.11–0.55], P = 0.0003).

The distributions of the 30 most frequent HLA haplotypes were compared between patients with VKH disease and controls, as well as published data in mainland Japan and Okinawa,

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**Table 1. Frequency of KIR genotypes and KIR genes in patients with VKH disease and healthy controls.**

| Location | KIR            | Control (n = 209) | VKH (n = 196) | p-value | OR (95% CI) |
|----------|----------------|------------------|--------------|---------|-------------|
|          | %F (N+)        | %F (N+)          |              |         |             |
| AA KIR genotype | 47.4 (99)       | 60.7 (119)       | 0.007        | 1.72 (1.16–2.55) |
| Bx KIR genotype  | 52.6 (110)      | 39.3 (77)        | 0.007        | 0.58 (0.39–0.86) |
| Centromeric AA   | 78.0 (163)      | 87.2 (171)       | 0.018        | 1.93 (1.13–3.29) |
| Centromeric Bx   | 22.0 (46)       | 12.8 (25)        | 0.018        | 0.52 (0.30–0.88) |
| Telomeric AA     | 59.8 (125)      | 66.3 (130)       |             |           |
| Telomeric Bx     | 40.2 (84)       | 33.7 (66)        |             |           |
| Cen-A 2DL1       | 100 (209)       | 100 (196)        |             |           |
| Cen-A 2DL3       | 99.0 (207)      | 100 (196)        |             |           |
| Cen-B 2DL2       | 22.0 (46)       | 12.2 (24)        | 0.012        | 0.49 (0.29–0.85) |
| Cen-B 2DS2       | 22.0 (46)       | 12.8 (25)        | 0.018        | 0.52 (0.30–0.88) |
| Cen-B 2DS3       | 19.1 (40)       | 7.7 (15)         | 0.0008       | 0.35 (0.19–0.66) |
| Cen-B 2DL5B      | 13.9 (29)       | 7.6 (15)         |             |           |
| Tel-B 2DL5A      | 40.2 (84)       | 33.7 (66)        |             |           |
| Tel-B 3DS1       | 36.8 (77)       | 32.1 (63)        |             |           |
| Tel-B 2DS1       | 39.7 (83)       | 33.7 (66)        |             |           |
| Tel-B 2DS5       | 26.8 (56)       | 28.1 (55)        |             |           |
| Tel-A 3DL1       | 95.2 (199)      | 96.4 (189)       |             |           |
| Tel-A 2DS4       | 95.2 (199)      | 95.9 (188)       |             |           |

Frequency (%F) of KIR gene/genotypes is expressed as percentage and defined as the number of individuals with gene/genotypes (N+) divided by the number of individuals studies in the given study group (n). KIR3DL3, 3DP1, 2DP1, 2DL4, and 3DL2 were observed in all 405 subjects. OR: odds ratio, CI: confidence interval, Cen: Centromeric, Tel: Telomeric.

**IR3DL3, 3DP1, 2DP1, 2DL4 and 3DL2 were observed in all 405 subjects.**

doi:10.1371/journal.pone.0160392.t001

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Table 2. Frequency of HLA-A, -B and -C allotypes in patients with VKH disease and healthy controls.

| KIR ligand | HLA Allotypes | Controls (n = 209) | VKH (n = 196) | P-value | OR (95% CI) |
|------------|---------------|--------------------|---------------|---------|-------------|
|            | % (N+)        | % (N+)             |               |         |             |
| A3/11      | A3            | 1.0 (2)            | 0.5 (1)       |         |             |
| A3/11      | A11           | 18.2 (38)          | 24.5 (48)     |         |             |
| Aw4        | A24           | 56.0 (117)         | 58.7 (115)    |         |             |
| Not a ligand | A1          | 1.9 (4)            | 1.5 (3)       |         |             |
| Not a ligand | A2          | 46.4 (97)          | 39.8 (78)     |         |             |
| Not a ligand | A26         | 21.5 (45)          | 25.0 (49)     |         |             |
| Not a ligand | A30         | 0.5 (1)            |               |         |             |
| Not a ligand | A31         | 13.9 (29)          | 18.4 (36)     |         |             |
| Not a ligand | A33         | 18.2 (38)          | 8.2 (16)      | 0.0033  | 0.40 (0.22–0.74) |
| Bw4-I80    | B38           | 0.5 (1)            |               |         |             |
| Bw4-I80    | B51           | 19.1 (40)          | 17.3 (34)     |         |             |
| Bw4-I80    | B52           | 21.1 (44)          | 8.7 (17)      | 0.0005  | 0.36 (0.20–0.65) |
| Bw4-I80    | B58           | 0.5 (1)            | 0.5 (1)       |         |             |
| Bw4-I80    | B59           | 4.3 (9)            | 8.2 (16)      |         |             |
| Bw4-T80    | B13           | 1.9 (4)            | 3.1 (6)       |         |             |
| Bw4-T80    | B37           | 1.4 (3)            | 1.5 (3)       |         |             |
| Bw4-T80    | B44           | 17.7 (37)          | 7.1 (14)      | 0.0015  | 0.36 (0.19–0.68) |
| Not a ligand | B7          | 12.9 (27)          | 6.1 (12)      | 0.0275  | 0.44 (0.22–0.90) |
| Not a ligand | B35         | 12.0 (25)          | 18.4 (36)     |         |             |
| Not a ligand | B39         | 8.1 (17)           | 7.7 (15)      |         |             |
| Not a ligand | B48         | 6.2 (13)           | 4.1 (8)       |         |             |
| Not a ligand | B60         | 8.6 (18)           | 13.8 (27)     |         |             |
| Not a ligand | B61         | 22.5 (47)          | 22.4 (44)     |         |             |
| Not a ligand | B54         | 8.6 (18)           | 33.7 (66)     | 0.0001  | 5.39 (3.06–9.50) |
| Not a ligand | B55         | 8.1 (17)           | 8.7 (17)      |         |             |
| Not a ligand | B56         | 1.0 (2)            | 7.1 (14)      | 0.0015  | 7.96 (1.79–35.50) |
| Not a ligand | B62         | 15.8 (33)          | 12.2 (24)     |         |             |
| Not a ligand | B67         | 1.9 (4)            | 2.6 (5)       |         |             |
| Not a ligand | B71         | 2.4 (5)            | 2.0 (4)       |         |             |
| Not a ligand | B75         | 2.9 (6)            | 2.6 (5)       |         |             |
| C1         | B46           | 14.8 (31)          | 4.6 (9)       | 0.0007  | 0.28 (0.13–0.60) |
| C1         | Cw1           | 34.0 (71)          | 48.0 (94)     | 0.0046  | 1.79 (1.20–2.67) |
| C1         | Cw7           | 26.3 (55)          | 18.9 (37)     |         |             |
| C1         | Cw8           | 18.2 (38)          | 15.8 (31)     |         |             |
| C1         | Cw9           | 22.0 (46)          | 25.5 (50)     |         |             |
| C1         | Cw10          | 19.6 (41)          | 26.5 (52)     |         |             |
| C1         | Cw12          | 21.1 (44)          | 9.2 (18)      | 0.0009  | 0.38 (0.21–0.68) |
| C1         | Cw14          | 26.8 (56)          | 20.9 (41)     |         |             |
| C2         | Cw4           | 8.6 (18)           | 13.8 (27)     |         |             |
| C2         | Cw5           | 1.0 (2)            |               |         |             |
| C2         | Cw6           | 1.9 (4)            | 2.0 (4)       |         |             |
| C2         | Cw15          | 4.8 (10)           | 6.1 (12)      |         |             |

Frequency (%F) of HLA-A, B and C allotypes is expressed as percentage and defined as the number of individuals with gene/genotypes (N+) divided by the number of individuals studies in the given study group (n). OR: odds ratio, CI: confidence interval.

doi:10.1371/journal.pone.0160392.t002
The frequencies of the HLA haplotypes in our control group are similar to published data from mainland Japan, but differ significantly from data from Okinawa, Japan. Three haplotypes (A24-B54-Cw1, A24-B60-Cw10, A11-B54-Cw1) were positively associated with VKH (OR = 5.06 [95% CI 2.52–10.14], P = 0.0001; OR = 5.28 [95% CI 1.49–18.67], P = 0.006; and OR = 7.35 [95% CI 1.64–33.02], P = 0.003, respectively) (S2 Table). Three other haplotypes (A24-B52-Cw12, A33-B44-Cw14, A2-B46-Cw1) were negatively associated with VKH (OR = 0.36 [95% CI 0.20–0.67], P = 0.002; OR = 0.34 [95% CI 0.17–0.67], P = 0.002; and OR = 0.22 [95% CI 0.09–0.52], P = 0.001, respectively). All these six haplotypes possess at least 2 KIR ligands (A11, A24, B44, B46, B52, Cw1, Cw10, Cw12, Cw14) including at least one C1 ligand.

Table 3. Frequency of KIR-binding HLA class I ligands in patients with VKH disease and healthy controls.

| Genotypes     | Controls (n = 209) | VKH (n = 196) | p-value | OR (95% CI) |
|---------------|-------------------|---------------|---------|-------------|
|               | %F    | (N+)   | %F    | (N+)   |
| HLA-A3/11     | 19.1  | (40)   | 25.0  | (49)   |
| A3            | 0.9   | (2)    | 0.5   | (1)    |
| A11           | 18.2  | (38)   | 24.5  | (48)   |
| A3+A11        | 0     | (0)    | 0     | (0)    |
| HLA-Bw4       | 55.5  | (116)  | 42.4  | (83)   | 0.0097 | 0.59 (0.40–0.87) |
| Bw4<sup>T80</sup> | 20.6  | (43)   | 11.7  | (23)   | 0.0216 | 0.51 (0.30–0.89) |
| Bw4<sup>S80</sup> | 41.6  | (87)   | 33.7  | (66)   |
| HLA-Aw4       | 56.0  | (117)  | 58.7  | (115)  |
| Bw4/Aw4       | 75.1  | (157)  | 71.4  | (140)  |
| > 2 copies (Bw4+Bw4/ Bw4+Aw4/ Aw4+Aw4) | 37.3  | (78)   | 29.6  | (58)   |
| HLA-C1        | 99.0  | (207)  | 98.5  | (193)  |
| HLA-C2        | 16.3  | (34)   | 21.4  | (42)   |
| C1+C1         | 83.7  | (175)  | 78.5  | (154)  |
| C2+C2         | 0.9   | (2)    | 1.5   | (3)    |
| C1+C2         | 15.3  | (32)   | 19.9  | (39)   |
| > 3 copies of C1 | 14.8  | (31)   | 4.1   | (8)    | 0.0003 | 0.24 (0.11–0.55) |

Frequency (%F) of HLA class I ligand is expressed as percentage and defined as the number of individuals with the ligand (N+) divided by the number of individuals studies in the given study group (n). OR: odds ratio, CI: confidence interval.

doi:10.1371/journal.pone.0160392.t003

Table 4. Frequency of KIR-HLA class I ligand combination in patients with VKH disease and healthy controls.

| KIR-HLA combinations | %F | Controls (n = 209) | VKH (n = 196) | p-value | OR (95% CI) |
|----------------------|----|-------------------|---------------|---------|-------------|
|                      |    | %F    | (N+) | %F    | (N+) |
| 2DL1+C2              | 16.3| (34)  | 21.4 | (42)  |
| 2DL2+C1/2            | 22.0| (46)  | 12.2 | (24)  | 0.012       | 0.49 (0.29–0.85) |
| 2DL3+C1              | 98.1| (205) | 98.5 | (193) |
| 2DL2/3+C1            | 99.0| (207) | 98.5 | (193) |
| 3DL1+Bw4             | 52.6| (110) | 39.8 | (78)  | 0.013       | 0.59 (0.40–0.88) |
| 3DL1+Bw4<sup>T80</sup> | 20.1| (42)  | 11.2 | (22)  | 0.02        | 0.50 (0.29–0.88) |
| 3DL1+Bw4<sup>S80</sup> | 39.2| (82)  | 31.6 | (62)  |
| 3DL1+Aw4             | 54.1| (113) | 57.7 | (113) |
| 3DL1+Aw4/Bw4         | 75.1| (157) | 73.5 | (144) |
| 3DL2+A3/11           | 19.1| (40)  | 25.0 | (49)  |

Frequency (%F) of KIR-HLA class I ligand combinations expressed as percentage and defined as the number of individuals with the KIR-HLA combination (N+) divided by the number of individuals studies in the given study group (n). OR: odds ratio, CI: confidence interval.

doi:10.1371/journal.pone.0160392.t004

Japan (S2 Table) [37,44,45]. The frequencies of the HLA haplotypes in our control group are similar to published data from mainland Japan, but differ significantly from data from Okinawa, Japan. Three haplotypes (A24-B54-Cw1, A24-B60-Cw10, A11-B54-Cw1) were positively associated with VKH (OR = 5.06 [95% CI 2.52–10.14], P = 0.0001; OR = 5.28 [95% CI 1.49–18.67], P = 0.006; and OR = 7.35 [95% CI 1.64–33.02], P = 0.003, respectively) (S2 Table). Three other haplotypes (A24-B52-Cw12, A33-B44-Cw14, A2-B46-Cw1) were negatively associated with VKH (OR = 0.36 [95% CI 0.20–0.67], P = 0.002; OR = 0.34 [95% CI 0.17–0.67], P = 0.002; and OR = 0.22 [95% CI 0.09–0.52], P = 0.001, respectively). All these six haplotypes possess at least 2 KIR ligands (A11, A24, B44, B46, B52, Cw1, Cw10, Cw12, Cw14) including at least one C1 ligand.
(Cw1, Cw10, Cw14, B46), and haplotype A2-B46-Cw1 possess 2 copies of C1 ligands (B46 and Cw1). HLA-B54 appears in two out of the three haplotypes associated with VKH disease.

Inhibitory KIR-HLA combinations that calibrate NK education and response to infection are decreased in patients with VKH disease

2DL2/3+C1, 2DL1+C2 and 3DL1+Bw4 are well-defined inhibitory KIR-HLA pairs that play a pivotal role in NK cell education and maturation. The decreases in 2DL2 and Bw4 discussed above were associated with concomitant reductions in 2DL2+C1/C2 and 3DL1+Bw4 combinations in patients with VKH disease (OR = 0.49 [95% CI 0.29–0.85], P = 0.012; OR = 0.59 [95% CI 0.40–0.88], P = 0.013 respectively) (Table 4). The difference in the frequency of 3DL1+Bw4 between groups was driven by Bw4^{720} (OR = 0.50 [95% CI 0.29–0.88], P = 0.02). The VKH population demonstrates a higher incidence of genotypes that lack these two KIR-HLA pairs, as well as two activating KIRs (2DS2 and 2DS3), compared to controls (45.4% vs. 30.6%, OR 1.90 [95% CI 1.28–2.83], P = 0.002) (Fig 2). Within the remaining 54.6% of patients with VKH disease, 20.4% carry HLA-B22, and thus 65.8% of patients either lack 4 KIR/HLA factors (3DL1+Bw4, 2DL2+C1/C2, 2DS2 and 2DS3) or carry HLA-B22, while only 41.6% of controls display such genotypes.

Discussion

Among the genetic factors that confer risk for autoimmune disease are the HLA and KIR genes, which are highly polymorphic and are very important regulators of the innate and
adaptive immune response in humans. In this study, we found that VKH is associated with a decrease in the centromeric Bx motif and its associated KIR genes—2DL2, 2DS2, 2DS3, and 2DL5B. This is in contrast to the previous findings in Mestizo [32], Saudi Arabian [34], and Japanese [33] populations, that were found to have an increased frequency of KIR B-haplotypes and certain activating KIR genes. This inconsistency is likely attributed to small sample size (<30 patients) and use of published controls in previous studies. The present study examined a much larger cohort of 196 patients with VKH disease and 209 healthy controls from a homogeneous native Japanese population of Yokohama and Hokkaido, Japan. The HLA haplotype frequency in the control population is comparable to published data from mainland Japan, indicating the cases and controls in this study were of Japanese ancestry and were well matched.

The centromeric-B KIR gene complex may decrease risk for VKH disease by bolstering the immune response against infectious and other environmental triggers for autoimmunity. The clearest demonstration of the clinical benefit of specific KIR genotypes derives from analyses in HLA-matched hematopoietic stem cell transplantation (HSCT), which revealed the beneficial effect of having a centromeric-B KIR motif in the donor [46–49]. These studies indicate that NK cells generated from centromeric-B KIR motif carriers are strongly reactive and may: 1) protect against graft versus host disease (GvHD) by depleting host antigen presenting cells; 2) facilitate engraftment by elimination of host immune barriers; 3) kill residual host tumor to protect against relapse, and; 4) decrease infectious complications. The clinical benefit conferred by the centromeric-B KIR motif could result from the presence of activating KIRs 2DS2 and 2DS3. These two activating KIRs have no detectable avidity for HLA class I [50,51], but may recognize different ligands associated with infection or tumor transformation. Many association studies have demonstrated that activating KIR genes protect against infectious disease [52]. Therefore, carrying activating KIRs 2DS2 and 2DS3 could represent a better protection against viruses, microorganisms, or other environmental precipitants of VKH disease. A similar protective role for B KIR genotypes and activating KIRs was recognized in other autoimmune diseases that were linked to viral infections, such as Multiple Sclerosis [53] and Pemphigus Foliaceus [54]. It is also important to consider the possibility of other polymorphic KIR genes that are located at the centromeric-B motif, such as 3DL3 and 2DL5B for which ligands and functions are unknown could also confer decreased risk for developing VKH disease.

HLA-B is the most polymorphic gene in humans, encoding over 3,130 distinct protein variants in human populations [55]. A striking finding of the current study is the strong association of HLA-B54 antigen (and related antigen group B22) with VKH disease. Interestingly, the association of HLA-B22 (originally called HLA-BW221) and VKH in Japanese was first reported almost 40 years ago [56,57]. However, it could not be replicated in a subsequent study with 9 VKH patients from California [58]. The discrepancy between these two studies might be due to a different ethnic background, small sample size, and/or the serological HLA typing method used that was known to have up to 25% discrepancy compared to DNA-based methods [59]. Furthermore, these first studies were soon overshadowed by the strong association of HLA-DR4 in Japanese patients [60]. It is important to note that the HLA-DR4 is in strong linkage disequilibria with HLA-B54 and HLA-Cw1, one of the common haplotypes in a Japanese population, but uncommon in other parts of the world [61].

Although we did not test the current cohorts for HLA-DR types, published association studies in Japanese VKH patients [60,62–64] and the strong linkage disequilibrium between HLA-B and HLA-DR loci prophesies a potential positive association of HLA-DR4 with the present cohort of VKH patients [61]. Ocular infiltrating CD4+ T cells from DR4+ patients with VKH disease were shown to recognize self-peptides from melanocytes (tyrosinase<sub>450-462</sub> and gp100<sub>44-59</sub>) that share high sequence homology with cytomegalovirus envelope glycoprotein H (CMV-egH<sub>290-302</sub>), suggesting that CMV infection may stimulate the production of T cells that cross-react with tyrosinase by a mechanism of molecular mimicry [65,66]. In addition...
to VKH, HLA-DR4 and the linked HLA-B54 are associated with other autoimmune diseases in Japanese patients, including Type 1 Diabetes [67] and rheumatoid arthritis [68], suggesting a common underlying mechanism for autoimmune diseases in Japanese population.

HLA-B22 confers susceptibility to several viral infectious diseases. For instance, B22 was associated with rapidly progressive disease in human immunodeficiency virus (HIV) infected individuals [69,70], immunological nonresponse to hepatitis B virus surface antigen [71], myelopathy due to human T-lymphotropic virus type I [72], and progression of liver injury [73] and unresponsiveness to interferon-α treatment in hepatitis C virus infection [74]. Nearly 50% of the VKH patients studied here carry HLA-B22, which implicates a B22-mediated immunological nonresponsiveness to viral infections, which may play a role in the pathogenesis of VKH disease. Consistent with this possibility, B lymphocyte cell lines established from patients with VKH disease contain greater amounts of Epstein-Barr virus (EBV) DNA, and express more EBV viral antigens than those established from patients with other types of uveitis, indicating EBV-specific nonresponsiveness in VKH patients [75].

The interaction of inhibitory KIR receptors with specific HLA class I molecules mediates NK tolerance to self while conferring functional competence [14,15,76–78]. NK cells generated from KIR3DL1-Bw4 carriers were shown to strongly influence progression of acquired immune deficiency syndrome (AIDS) and plasma HIV RNA levels [79]. Among the three well-defined inhibitory KIR-HLA combinations that involve in NK cell education and maturation (2DL2/3 +C1, 2DL1+C2 and 3DL1+Bw4), two (2DL2+C1/C2 and 3DL1+Bw4) were decreased in patients with VKH disease, suggesting a protective role of these inhibitory KIR-HLA combinations. Presumably the absence of these two inhibitory KIR-HLA interactions results in the generation of mostly unlicensed NK cells that fail to mount a vigorous NK response against infections implicated in VKH pathogenesis. HLA-B22 mediated unresponsiveness could act independently as well as synergistically to modify VKH disease susceptibility. These results suggest that a hyporesponsiveness of NK cells due to poor education plus missing of activating KIRs, as well as by CTLs due to nonresponsive HLA-B22, play an important role in VKH pathogenesis following viral infections in genetically susceptible individuals, such as HLA-DR4 carriers. A comprehensive model that summarizes potential mechanisms for the Immunogenetic basis of VKH pathogenesis is presented in Fig 3.

Immunotherapy, including tumor infiltrating lymphocyte therapy, interleukin-2, and the CTLA inhibitor ipilimumab, promotes T cell activation and survival in order to augment the immune response against neoplastic cells, but inadvertently tips the balance toward autoimmunity including the development of VKH [80–82]. One of the authors (RDL) has seen VKH develop in a patient whose malignant melanoma was successfully treated with BRAF and MEK inhibitors, suggesting that the release of antigens from the melanoma may induce the autoimmune response to intraocular antigens in a genetically predisposed patient (unpublished data). KIR and HLA genotyping may be useful in determining the risk of developing uveitis patients either receiving immunostimulatory therapy for cancer or MEK/BRAF treatment for malignant melanoma in particular. Patients found to be genetically at risk could then be monitored closely by an opthalmologist.

A high frequency of HLA-B22 and a low frequency of the Bx KIR genotype is the characteristic genetic composition of the Japanese population, which may play a role in pathogenesis and be part of why VKH is more common in Japanese than other populations. It is important to recognize the genetic analysis alone is unlikely to distinguish the contribution of predisposing KIR-HLA factors, and functional studies will be required to define the mechanism by which CTL and NK cells contribute to protect against VKH pathogenesis. The understanding of the mechanisms underlying the genetic basis for VKH susceptibility could have important implications for treatment in the clinic, providing new targets for therapeutic manipulation of disease process.
Supporting Information

S1 Table. Baseline characteristics of HLA genotyping for patients with VKH disease and healthy controls.

(XLS)
S2 Table. Frequency of the 30 most common HLA-A-B-C haplotypes in patients with VKH disease and healthy controls.

(Accepted Manuscript)

Acknowledgments
Dr. Levinson receives funding from the MacDonald Family Foundation and RPB. This study was partially supported by UCSF Department of Surgery seed grant to Dr. Rajalingam.

Author Contributions
Conceived and designed the experiments: RR RL.
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Contributed reagents/materials/analysis tools: AM NM SO.
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