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Host Genetic Factors in Susceptibility to Herpes Simplex Type 1 Virus Infection: Contribution of Polymorphic Genes at the Interface of Innate and Adaptive Immunity

Manuela Moraru,* Elisa Cisneros,* Natalia Gómez-Lozano,* Rosario de Pablo,* Francisca Portero,† María Cañizares,* Mercedes Vaquero,‡ Gastón Roustán,§ Isabel Millán,¶ Miguel López-Botet,* and Carlos Vilches*

HSV-1 establishes life-long latency that can result in clinical relapses or in asymptomatic virus shedding. Although virtually all adults have been exposed to HSV-1, the clinical course varies remarkably. Genetic host variability could be related to this clinical diversity. In this study, we analyzed the contribution of gene families in chromosomes 1, 6, 12, and 19, which encode key regulators of the innate and adaptive immunity, in a cohort of 302 individuals. Class I and class II alleles of the HLA system, the copy-number variation of NK cell receptor genes (KIR and NKG2C), the combinations of killer cell Ig-like receptor and their HLA ligands, and CD16A and CD32A allotypes of variable affinity for IgG subclasses were all studied. Although no major susceptibility locus for HSV-1 was identified, our results show that the risk of suffering clinical HSV-1 infection is modified by MHC class I allotypes (B*18, C*15, and the group of alleles encoding A19), the high-affinity receptor/ligand pair KIR2DL2/HLA-C1, and the CD16A-158V/F dimorphism. Conversely, HLA class II and CD32A polymorphisms and NKG2C deletion did not seem to influence the clinical course of herpetic infection. Collectively, these findings support an important role in host defense against herpetic infection for several polymorphic genes implicated in adaptive immunity and in surveillance of its subversion. They confirm the crucial role of cytotoxic cells (CTL and NK) and the contribution of genetic diversity to the clinical course of HSV-1 infection. The Journal of Immunology, 2012, 188: 4412–4420.

Most adults have suffered HSV-1 infection. The virus migrates from mucosae or skin to sensory ganglia, where it establishes life-long latent infection. Virus propagation is controlled by innate and adaptive immunity, and the course of the infection depends upon the host immune system capacity to control both the primary infection and the reactivations. HSV-1 replication in keratinocytes and mucosal cells triggers a strong cytokine response. IFN-α produced by dendritic cells (DC) and IFN-γ secreted by NK and, later, CD4+ T cells represent a first line of direct antiviral defense (1). IFNs also promote subsequent adaptive immune response by enhancing Ag presentation (2).

CD8+ CTLs eliminate infected cells presenting HSV-1 peptides on HLA class I molecules, and they are responsible for immuno-surveillance during viral latency (3, 4). In addition, DC present viral Ags bound to HLA class II molecules to CD4+ T lymphocytes, which enhance differentiation and establishment of functional memory CTLs (1) and Ab-mediated control of HSV-1.

Key components of innate cellular immunity to viruses are NK cells, which kill infected cells without presensitization, secrete antiviral cytokines, and favor DC maturation. Their behavior depends on the balance of signals transduced by activating and inhibitory receptors that survey altered molecular patterns on infected cells (5, 6). Their best-known trigger is HLA class I downregulation, monitored by several families of NK cell receptors (7). Furthermore, NK lymphocytes exert Ab-dependent cellular cytotoxicity (ADCC) against Ab-coated infected cells by means of FcγR.

HSV-1, like other herpesviruses, has developed mechanisms to circumvent the host immune response. Infected cell protein (ICP) 47 binds the transporter for antigenic peptides (TAP), thus hampering HLA class I-mediated Ag presentation by infected cells to T lymphocytes (8). Furthermore, HSV-1 glycoproteins gE and gL form an FcR on both the viral envelope and the infected cell membrane, where it can compete, directly and through Ab bipolar bridging, with host FcγR, protecting the infected cells from complement- and cell-mediated lysis (9).

Although virtually all adults have been exposed to HSV-1, the clinical course of the infection varies remarkably, ranging from asymptomatic virus shedding to frequent inflammatory herpetic lesions and potentially life-threatening episodes of herpetic encephalitis. Clinical variability is attributable to the virus itself (e.g., HSV-1 strains, amount of inoculum), the environment (e.g., exposure to UV light and other triggers), and variations in the host immune response. Herpetic encephalitis and isolated cases of fatal HSV-1 infection have been associated with rare mutations in innate
immunity-related genes (10–14). A recent linkage analysis detected in chromosome 21 a 2.5-megabase susceptibility region for herpes simplex labialis (15). However, predictive genetic markers that explain the pathogenesis of the common outcomes of HSV-1 infection are yet to be identified.

An attractive candidate to influence the course of HSV-1 infection is the enormous diversity of the HLA complex in chromosome 6, credited with a large proportion of the variability of immune responses, because it could modify the efficacy of HSV-1-antigen presentation. Several groups have analyzed the contribution of HLA allotypes to the clinical diversity of HSV-1 infection, but many results did not replicate in different populations (16–27). Killer cell Ig-like receptors (KIR), encoded by a highly polymorphic gene complex in chromosome 19, represent another series of candidates to influence the HSV-1 infection outcome because they help NK cells fight viruses that tamper with HLA expression (28). The most conspicuous aspect of KIR gene diversity is copy-number variation (CNV); most humans are natural knockouts for multiple KIR genes, with only the genes at the centromeric and the telomeric ends of the KIR complex being conserved. We have previously identified an association between symptomatic HSV-1 infection and presence in the genome of the linked genes KIR2DL2 and KIR2DS2 (29).

Also used by NK cells to sense HLA expression are CD94/NKG2 heterodimers encoded in the NK complex of chromosome 12, which comprise both activating and inhibitory receptors for HLA-E (30, 31). In sharp contrast with KIR, the CD94 and most NKG2 genes are well conserved (32), one remarkable exception being complete deletion of NKG2C (KLR2C) in some individuals (33, 34). The consequence of NKG2C deletion for immunity and health is unknown, but marked expansion of NKG2C-positive NK and T lymphocytes in response to CMV infection (35, 36) suggests a role for NKG2C in defense against herpesviruses.

Finally, the genes in chromosome 1 encoding for the FcγR CD16A (FCGR3A) and CD32A (FCGR2A) display allelic polymorphisms that modulate their affinity for IgG (37). High-affinity FcγRs could enhance ADC and compete with the decoy FcγR formed by HSV-1 γgE/gI, thus improving the efficacy of humoral response against HSV-1.

Whether and how virus evasion mechanisms may be counteracted by host defense and to which extent immunogenetic variability contributes to the clinical outcomes of herpetic infection is yet to be established. In this study, we have analyzed the contribution of the genetic diversity of the HLA system and the KIR, NKG2C, CD16A, and CD32A receptors to the clinical variability of HSV-1 infection.

Based on reported episodes of symptomatic herpes infection (most often, cold sores) after a standardized questionnaire, the total sample was subdivided into two categories: asymptomatic and symptomatic individuals. When considering the serologic findings and the frequency of HSV-1 reactivations, each of the previous groups was further separated, resulting in a total of four subcategories: 1) asymptomatic seronegatives; 2) asymptomatic seropositives; 3) donors with sporadic clinical reactivations (less than two reactivations yearly in the maximum activity period); and 4) patients with recurrent reactivations (two or more per year).

**HLA, KIR, NKG2C, FCGR2A, and FCGR3A genotyping**

HLA class I (HLA-A, -B, and -C) and class II (HLA-DRB1, -DRB3, -DRB4, and -DRB5) typing was carried out using a reverse line PCR sequence-specific oligonucleotide test (Inviron-Dynal Reli-SSO, Wirral, U.K.). KIR genotyping was performed using a PCR with a sequence-specific primer method designed by our group (38). On the basis of gene content, two categories of KIR haplotypes have been established, designated A and B. These share some KIR genes and pseudogenes (3DL2, 3DL3, and, in most cases, 2DL4 and 3DP1), whereas they diverge in the remaining KIR genes. Besides the shared framework genes, the A haplotype carries: genes coding for the inhibitory KIR 2DL1, 2DL3, and 3DL1; for KIR2DS4, as the only activator; and one pseudogene, KIR2DP1. By contrast, the hallmark of B haplotypes is the replacement of one or more of the latter six genes by variable combinations of KIR not seen in the A haplotype: 2DL2, 2DL5, and genes encoding activating KIR different from those in A. According to this, we assigned an AA genotype to individuals having all and only KIR genes corresponding to an A-haplotype; a BX genotype, when all the previous genes, as well as any of those characteristic of B-haplotypes, were present; and a BB-genotype when any of the A-haplotype was missing and one or more of those included in a B-haplotype were present. The assignment of a BX genotype was based exclusively on gene content; such designation was used instead of AB so as not to imply that the genes form an A-haplotype were inherited in cis.

Joint KIR/ligand analysis considered the interactions of KIR 2DL1 and 2DS1 with HLA-C allotypes with lysine at position 80 of the α-chain (HLA-C2 epitope); KIR 2DL2 and 2DL3 with HLA-C alleles bearing asparagine at the same position 80 (HLA-C1 epitope); KIR3DL1 with HLA-B and HLA-A allotypes having the Bw4 epitope (excluding A*25); KIR3D2L with HLA-A*0301 and A*11 alleles; the recently demonstrated interaction of full-length KIR2DS4 with HLA-A*0101 and subsets of HLA-C alleles (C*01, *02, *04, *05, *14, and *1601) (39); and KIR3DS1 with Bw4, because of its apparent genetic interaction in viral infection (40).

Homozygous and heterozygous deletion of the NKG2C gene were assessed by a PCR method based on the approach used by Miyashita et al. (34), with modifications (M. Moraru, M. Canizares, A. Muntasell, R. de Pablo, M. López-Botet, C. Vilches, submitted for publication, details available upon request).

Functional polymorphisms of FCGR2A and FCGR3A were determined using a PCR with confronting two-pair primers, as described (41).

**MHC epitope prediction**

To predict HSV-1 peptide binding to specific HLA molecules, we have used the NetCTLpan tool (http://www.cbs.dtu.dk/services/NetCTLpan/), which integrates information on proteasomal cleavage, TAP transport efficiency, and MHC binding affinity (42).

**Statistical analysis**

Gene frequencies were estimated by direct counting, and they were compared using the Fisher’s exact test. Influence of activating KIR gene number on the clinical course of HSV-1 infection was analyzed using the Mann–Whitney U test. The Mantel–Haenszel χ² test for linear trends was used to analyze cumulative influence of high-affinity KIR/ligand pairs 2DL2/C1 and 2DL1/C2, distribution of 2DL2/C1 in recurrent, sporadic, and asymptomatic patients, and allele-dose effect of CD16A valine-158. A significance level of 5% was chosen.

For forward stepwise multiple logistic regression was performed using the SPSS14 package (SPSS, Chicago, IL) to measure the independent effect of various genetic variables on the clinical course of herpetic infection and to build a predictive model of the probability of suffering symptomatic infection. A receiver operating characteristic (ROC) curve was used to plot sensitivity and specificity of the predictive model (43). For multiple logistic regression, we selected variables that showed differences with p < 0.1 in univariate analysis of their distribution in symptomatic and asymptomatic donors. To reduce the number of variables, we grouped the alleles that encode the following public HLA specificities: A9 (A*23−24), A19 (A*29−33), and A28 (A*68−69). HLA-B*35, with p slightly over 0.1.
was also included because of its previous identification as a protective factor, as was the KIR2DL1/C2 combination, for its apparently cumulative effect on the risk conferred by KIR2DL2/C1.

Results

Cohort demographic characteristics

We studied 302 adult individuals, of whom 164 had symptomatic HSV-1 infection and 138 were asymptomatic. The mean age of the whole sample was 40.3 ± 12.7 y, the symptomatic and the asymptomatic groups having similar ages (Supplemental Table I). Sixty-five percent of the donors were women, who predominated in the group developing clinical herpetic infection (p = 0.002) and, in particular, among individuals with two or more reactivations per year (p = 0.0001, when compared with the rest of the population). This finding is in line with previous observations on the gender distribution of patients with recurrent HSV-1 infection (44).

Influence of HLA diversity on the outcome of HSV-1 infection

To explore whether and how the extreme HLA polymorphism conditions the clinical efficacy of adaptive responses against HSV-1, we analyzed the HLA alleles of all individuals included in this study. HLA class II allotypes had remarkably similar distributions in symptomatic and asymptomatic donors (Fig. 1, Supplemental Table II). In contrast, several HLA class I alleles displayed less even distributions in the two subgroups (Fig. 1, Supplemental Table II), although only two of the apparent differences between symptomatics and asymptomatics reached statistical significance. In particular, HLA-B*18 was overrepresented in the asymptomatic group (p = 0.003; odds ratio [OR], 0.34), and HLA-B*35, reported to associate negatively with herpes in Italians (19), showed a trend in the same direction (one-tailed p = 0.075; OR, 0.60). On the contrary, HLA-C*15 was more frequently encountered among symptomatic individuals (p = 0.013; OR, 4.53).

The most common HLA class I allotype, A*02, was slightly less represented in asymptomatic than in symptomatic donors (Fig. 1). Stratification of asymptomatic donors for presence or absence of HSV-1–specific Abs showed that HLA-A*02 was underrepresented solely in seronegative individuals (37.3%), whereas seropositive asymptomatic and symptomatic donors all had A*02 frequencies (52.1 and 49.4%, respectively) similar to those reported in Spanish individuals (45, 46), but that difference was only close to significance (p = 0.072 for the comparison between HSV-1 seronegatives and all other donors). Comparison of the HLA allele distribution in the four subgroups of patients showed no other significant differences (Supplemental Table II).

HSV-1 peptide binding profiles of HLA class I molecules

To identify functional differences between HLA-B*18, B*35 and C*15 that could explain their divergent influence on risk of clinical HSV-1 infection, we used a tool for peptide-binding prediction to MHC molecules (42). We focused on differences in presentation of peptides derived from immediate early HSV-1 genes, because these are transcribed upon viral entry without prior viral protein synthesis, and they encode Ags expressed in latency (47). The products of all common B*18 and B*35 alleles of white populations (B*18:01, B*35:01–B*35:03, and B*35:08) were predicted to present a unique 9-mer peptide (EPAPDVWVF) from ICP0; in contrast, no ICP0-derived peptides were potential ligands for HLA-C*15 (alleles C*15:02 and C*15:05). The ICP27 gene encodes several potential nonamer ligands for HLA-B*18 and C*15, but none for B*35. Peptides from all other immediate early gene products were similarly presented by all three MHC molecules.

Influence of KIR gene diversity on the clinical course of HSV-1 infection

Previous analysis of KIR-gene CNV in 131 out of the 302 donors studied in this article showed that resistance to clinical HSV-1

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** Phenotypic frequencies of common HLA alleles in symptomatic and asymptomatic individuals. Only the alleles with a frequency >5% in at least one subgroup are represented in this figure; the exact frequencies of all studied alleles are shown in Supplemental Table II. *p < 0.05, **p < 0.01.
infection associates with the absence of KIR2DL2 and KIR2DS2 from the genome (29). In this study, we replicated that study in another group of 171 individuals of similar characteristics and origins. We observed small deviations in the same direction (e.g., 59.8% of symptomatics, versus only 55.7% of asymptomatics, had a KIR2DL2 gene) (Fig. 2, Supplemental Table III), but they were not statistically significant. Yet, in the total sample of 302 individuals, the increase of KIR2DL2 and KIR2DS2 in clinical HSV-1 infection retained statistical significance (p = 0.011, OR = 1.83; and p = 0.027, OR = 1.69, respectively). As was the case in our first study, all other KIR genes were distributed similarly in the symptomatic and asymptomatic subgroups of the replication sample (Fig. 2A), as well as in patients with recurrent and sporadic symptoms (data not shown).

On the basis of gene content, two categories of KIR haplotypes have been established, designated A and B, which combine into an array of diverse AA, BX, and BB genotypes, for which distribution was compared in donors with or without HSV-1 symptoms. Consistent with the observed differences in the frequencies of KIR 2DL2 and 2DS2, genes present only in B-haplotypes, reciprocal deviations in the frequencies of the AA and BB genotypes were seen (Fig. 2B, Supplemental Table IV), but those deviations were only close to statistical significance (p < 0.1).

The number of activating KIR encoded by different genotypes ranges from six to none (when KIR2DS4 is represented in both chromosomes of an AA genotype by null alleles, dominant in white populations), and this variation might modulate the activation threshold of NK cells. However, search for possible association between the number of genes for activating KIR and the clinical course of HSV-1 infection showed no significant differences between asymptomatic donors and patients with either sporadic or recurrent symptoms (Fig. 2C and data not shown).

**KIR/HLA ligand pairs of higher avidity increase the risk of clinical HSV-1 infection**

We refined the study of KIR genotypes by considering the simultaneous presence of a receptor and its known or presumed ligand in the same individual. The receptor/ligand pair 2DL2/HLA-C1 was more commonly encoded in symptomatic than in asymptomatic donors (p = 0.011, OR = 1.87) (Fig. 3, Supplemental Table IV). This had been originally observed in our first study (29) and was reproduced with marginal significance in the replication cohorts (58.7 versus 45.6%; OR = 1.70; single-tailed p = 0.059). Furthermore, when the HSV-1 reactivation rate was considered, a highly significant gradient in the 2DL2/C1 distribution was observed: its frequency was highest in patients with frequently recurrent HSV-1 reactivations and lowest in donors without symptoms (p = 0.005; Table II).

The proportion of individuals encoding both high-affinity receptor/ligand pairs 2DL2/HLA-C1 and 2DL1/HLA-C2 is also increased among symptomatically infected individuals (p = 0.005; OR = 2.13), in whom lack of both receptor/ligand pairs was rarer than in asymptomatic individuals (p = 0.032, OR = 0.51, Fig. 3). As a consequence, presence of both, either, or none of the two pairs caused a linear increase in the risk of having HSV-1 symptoms (Mantel–Haenszel \( \chi^2; p = 0.002 \)) (Supplemental Table IV). Coincident increase of both receptors and their respective ligands in the same group of patients is noticeable because KIR2DL2 is often inherited in haplotypes that lack a KIR2DL1 gene; furthermore, C1 and C2 are mutually exclusive allotypes of the same locus. These findings prompted analysis of C1-C2 genotype distribution in symptomatic patients, which did not fit the Hardy–Weinberg equilibrium, and this was due to predominance of heterozygosity: 29% C1C1, 58% C1C2, and 13% C2C2 (p = 0.017). This distribution differed significantly from that of asymptomatic controls (38% C1C1, 46% C1C2, 17% C2C2; p = 0.011), in whom these genotypes were in Hardy–Weinberg equilibrium. The rest of inhibitory or activating KIR/ligand pairs did not show significant differences in their distribution (Fig. 3 and data not shown).

**NKG2C gene deletion does not influence the clinical course of HSV-1 infection**

The gene encoding the HLA-E receptor NKG2C, which marks subsets of NK and T lymphocytes that expand in response to...
CMV, is deleted in some Asian and European individuals (33, 34); therefore, we searched for possible relation between NKG2C deletion and lower frequency of HSV-1 infection. Homozygous and heterozygous individuals for the NKG2C deletion were found in both the symptomatic and asymptomatic groups. The frequencies of the NKG2C wildtype, NKG2C deleted, and NKG2C double deleted genotypes were identical in the two groups (Table III) and in the range seen in Japanese and Dutch (33, 34). An apparent frequency gradient of the NKG2C wildtype genotype (highest in asymptomatic seropositive donors, lowest in patients with recurrent symptoms; Table III) was not statistically significant.

**Homzygosity for the higher avidity allele of CD16A (FCGR3A) protects from clinical HSV-1 infection**

Because FcγR mediate cellular responses (ADCC, phagocytosis) against infected cells recognized by virus-specific IgG molecules, we investigated whether dimorphisms that increase the avidity for IgG of two such receptors could improve the infection outcome. The alleles encoding valine/phenylalanine-158 of CD16A (FCGR3A) and arginine/histidine-131 of CD32A (FCGR2A) were genotyped, and their frequencies in the whole sample were in the range reported for white populations (48, 49). After excluding the individuals who lacked IgG Abs for HSV-1, comparisons showed that, as hypothesized, homozygosity for the higher affinity allele of CD16A (158V/V) was less common in patients with clinical HSV-1 infection than in seropositive individuals lacking HSV-1 symptoms (one-tailed \( p = 0.024 \), two-tailed \( p = 0.037 \); OR = 0.43; Fig. 4). Furthermore, the dose of allele CD16A-158V had a significant effect on protection from herpetic symptoms (Mantel–Haenszel test for linear trends, \( p = 0.027 \); Table IV). In contrast with CD16A, the CD32A-131H/R dimorphism had no appreciable influence on the clinical course of the infection (Fig. 4). No influence of either dimorphism was seen on the frequency of clinical HSV-1 reactivations (data not shown).

**Multiple logistic regression confirms the association of several immunogenetic factors with clinical HSV-1 infection**

Multiple logistic regression analysis was performed to determine the extent to which each of the possibly associated variables identified in the univariate tests contributes to clinical HSV-1 infection. This analysis revealed an independent and cumulative effect of the presence or absence of several variables on the probability to suffer symptomatic HSV-1 reactivations. These results confirmed that the presence of HLA-B*18 and a CD16A-158V/V genotype grant protection from HSV-1 reactivation, whereas HLA-C*15, the group of HLA-A alleles encoding the A19 allotype, and the KIR2DL2/C1 receptor/ligand pair (but neither KIR2DL2 nor KIR2DS2 alone) confer susceptibility to suffer herpetic reactivations (Table V). A predictive model on the probability of suffering herpetic lesions was built with the parameters that, in multiple regression, showed a significantly positive or negative association. Such model classified correctly 70.6% of the patients with herpetic symptoms and 58.0% of the asymptomatic donors. An ROC curve, which plots specificity and sensitivity of the model (43), enclosed 68.7% of the maximum possible area (Fig. 5).

**Discussion**

The highly variable clinical course of HSV-1 infection suggests that susceptibility or resistance host genes are involved in its control, as shown for murine CMV and experimental HSV-1 infection (50, 51). However, no major HSV-1 susceptibility locus has been identified in humans, suggesting that polygenic inheritance is responsible for variable susceptibility. In this study, we have analyzed the contribution of gene families that encode key regulators at the interface of adaptive and innate immunity: class I and II alleles and allotypes that modulate the affinity of CD16A and CD32A for IgG. Even though none of these genes arises as a major susceptibility locus, uni- and multivariate analyses indicate that MHC class I alleles, combinations of KIR and their HLA-encoded ligands, and allotypes that modulate the affinity of CD16A and CD32A for IgG. Even though none of these genes arises as a major susceptibility locus, other immune mediators are also involved in the clinical outcome of HSV-1 infection. In contrast, polymorphisms of HLA class II, NKG2C, and CD32A appear to play no role in susceptibility to HSV-1.

Among HLA class I alleles, B*18 was significantly less common among herpetic patients, an association not observed in other populations. Previous studies indicated that B35 might confer protection against HSV-1 (19, 23, 25, 27); we observed a coincident deviation, but it was only close to significance in uni- and multivariate analysis. In contrast with those HLA-B alleles, C*15 emerged as a susceptibility factor. This allele had never been studied in HSV-1 infection, because it remained undiscovered until DNA typing methods became available in the mid-1990s (52, 53). Strong association of C*15 with B51 (46) might be related to previous reports on B51 (the common split of the public Ag HLA-B5) behaving as a predisposing factor for symptomatic herpes (17, 18, 20, 26, 27). Finally, multiple regression analysis identified the group of alleles encoding an A19 Ag as possible risk factors; similar results were obtained previously by some (16, 25, 27), but not all (19, 21), groups.

The picture that emerges from comparison of different studies is one in which MHC class I polymorphism appears consistently associated with HSV-1 infection, the predisposing or protective alleles varying in each study. Low reproducibility of results in different populations might bear relation with geographical differences in distribution and haplotypic combinations of HLA.
alleles. Confounding effects can also contribute to conflicting results; among them, small cohorts of <50 patients in many previous studies, control groups composed of general population instead of herpes-resistant donors, and imprecision of serological HLA typing. We have addressed these problems by studying >150 symptomatically infected individuals, and a similarly sized control group comprising only asymptomatic individuals. Furthermore, this is the first study, to our knowledge, performed entirely with DNA-based genotyping methods, of greater precision and reproducibility, and the only techniques currently accepted for HLA-C and class II alleles.

With the exception of C*15 and A*32 (part of the A19 allotype), the HLA class I alleles that appear to modify the outcome of HSV-1 infection do not encode known KIR ligands. Therefore, their divergent roles in the infection should derive from differential functions as Ag-presenting molecules to CD8+ CTLs. A peptide prediction tool that takes into account MHC binding, proteasomal C-terminal cleavage, and TAP transport efficacy showed that the peptide RQEsIKPR, encoded by HLA-Cw*08, is able to bind the cognate receptor HLA-C1 pairs in the same individual. This is the first study, to our knowledge, performed entirely with DNA-based genotyping methods, of greater precision and reproducibility, and the only techniques currently accepted for HLA-C and class II alleles.

Another aspect in which MHC molecules diverge functionally is their capacity to overcome the effect of viral immunoevasins. Differences in KIR/ligand affinity are possibly relevant because IFN-γ decreases in HLA-C expression levels induced by HSV-1 and/or herpes, because it might render NK cells less sensitive to modest decreases in HLA-C expression levels induced by HSV-1 and/or hamper surveillance of viral reactivations by memory CD8 T cells expressing NK receptors. Differences in KIR/ligand affinity are possibly relevant because IFN-γ could attenuate HSV-1 blockade by protein ICP47 during HSV-1 infection (58). Tapasin-independence of HLA class I expression on infected epithelial cells (65). Consequently, tapasin-independence cannot be currently predicted reliably from the primary structure of an HLA allotype, but B18 could share that feature with B35 because it carries a combination of two motifs (aspartate-114 and serine-116) that associate with tapasin-independence (59–62).

Subversion of HLA class I expression in infected cells could facilitate HSV-1 evasion from CTLs, but it is also a trigger for cytotoxicity and cytokine secretion by NK cells, which sense it by means of KIR and other MHC receptors. We have previously reported that KIR genes 2DL2 and 2DS2 contribute to the susceptibility to suffer frequent, clinically relevant HSV-1 reactivations (29). Current results show that these genes by themselves increase only modestly the risk of symptomatic herpes. It is mainly the combination of KIR2DL2 with HLA-C alleles encoding its epitope ligand [KIR2DS2 lacks any detectable affinity for HLA (63)] that increases noticeably susceptibility to this disease.

KIR2DL2 is currently considered a 2DL3 allotype of higher affinity (64). Stronger inhibition by KIR2DL2 in the presence of its cognate ligands could explain its association with symptomatic herpes, because it might render NK cells less sensitive to modest decreases in HLA-C expression levels induced by HSV-1 and/or hamper surveillance of viral reactivations by memory CD8 T cells expressing NK receptors. Differences in KIR/ligand affinity are possibly relevant because IFN-γ could attenuate HSV-1 blockade of HLA class I expression on infected epithelial cells (65). Consequently, tapasin-independent HLA molecules could therefore be advantageous by presenting HSV-1 Ags early postinfection/reactivation. Unfortunately, tapasin-independence cannot be currently predicted reliably from the primary structure of an HLA allotype, but B18 could share that feature with B35 because it carries a combination of two motifs (aspartate-114 and serine-116) that associate with tapasin-independence (59–62).

Materials and Methods

Table IV. CD16A-158 V allele dose and protection from symptomatic HSV-1 infection

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Asymptomatic Seropositives (n = 70)</th>
<th>Symptomatics (n = 163)</th>
<th>OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD16A-158 F/F</td>
<td>20 (28.6)</td>
<td>63 (38.6)</td>
<td>1.0</td>
</tr>
<tr>
<td>CD16A-158 V/V</td>
<td>35 (50.0)</td>
<td>83 (51.2)</td>
<td>0.75</td>
</tr>
<tr>
<td>CD16A-158 V/V</td>
<td>15 (21.4)</td>
<td>17 (10.4)</td>
<td>0.36</td>
</tr>
</tbody>
</table>

*p of linear trend = 0.027.

*In the Mantel–Haenszel test for linear trends, the reference genotype is assigned an arbitrary OR value of 1, to which the OR of the other genotypes are referred. The test measures the significance of a linear relationship among an ordinal variable, the dose of a risk or protective factor (the CD16A-158 V allele), and probability of an outcome (being symptomatic).

Table V. Variables that contribute independent and significantly to the risk of symptomatic HSV-1 infection, according to multiple logistic regression analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>p Value</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-B*18</td>
<td>0.003</td>
<td>0.31</td>
<td>0.14–0.66</td>
</tr>
<tr>
<td>HLA-C*15</td>
<td>0.007</td>
<td>5.93</td>
<td>1.63–21.64</td>
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<td>HLA-A19</td>
<td>0.035</td>
<td>1.71</td>
<td>1.04–2.82</td>
</tr>
<tr>
<td>KIR2DL2/HLA-C1</td>
<td>0.002</td>
<td>2.15</td>
<td>1.32–3.51</td>
</tr>
<tr>
<td>CD16A-158V/V</td>
<td>0.017</td>
<td>0.42</td>
<td>0.21–0.85</td>
</tr>
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</table>

The following variables were also submitted to stepwise multiple logistic regression, according to the criteria defined in Materials and Methods, but they were found not to associate independent and significantly with symptomatic HSV-1 infection: HLA-A alleles encoding the public specificities A9 and A28; HLA-B*35; and the KIR2DL1/HLA-C2 pair. Coincidence of the KIR2DL1/HLA-C2 and the KIR2DL2/HLA-C1 pairs in the same individual neither modified noticeably the results shown above, nor did they improve the derived predictive model and its associated ROC curve, when included as an additional variable in multiple regression analysis (results not shown).

FIGURE 4. CD16A-158 (A) and CD32A-131 (B) genotype frequencies in asymptomatic and symptomatic individuals. *p < 0.05.

FIGURE 5. ROC curve (43) plotting the specificity and sensitivity of a predictive model on the probability of suffering herpetic lesions, built with the parameters found to contribute independent and significantly in multiple regression analysis: HLA-C*15, HLA-A19-associated alleles, and the KIR2DL2/C1 pair as risk factors and HLA-B*18 and CD16-158V/V as protective factors (Table V). The diagonal line (y = x) corresponds to the random speculation of a class.
sistent with this model, another high-affinity receptor/ligand pair, 2DL1/C2, further increased the risk conferred by 2DL2/C1. According to this reasoning, association of symptomatic herpes with KIR2DS2 would only reflect its strong linkage disequilibrium with 2DL2. As an anecdotal evidence, KIR genes 2DL2 and 2DS2 segregated separately in three donors of this study: two 2DL2+ 2DS2− donors had HSV-1 symptoms, whereas the single 2DL2− 2DS2+ individual was asymptomatic.

Our study on KIR diversity focused on CNV, but did not take into account possible effects of allelic polymorphism. KIR2DL2 is represented in white populations mostly by two alleles, KIR2DL2*001 and *003 (66). Their products share the three substitutions that make KIR2DL2 a stronger receptor than KIR2DL3 and differ from each other by two amino acid replacements: one in the second Ig-like domain (isoleucine/threonine-200, respectively) and one in the cytoplasmic tail (alnine/threonine-313). The former variation, located far from the ligand-binding region, seems not to modify the receptor affinity (64), but more subtle effects of those polymorphisms on KIR2DL2 function and, hence, on its genetic association with HSV-1 infection, cannot be excluded. Similarly, we have not considered quantitative differences in the affinity of KIR2DL2 for various HLA-C1, and even -C2, ligands (64). Of note in the latter regard, C*15:02, the predominant C*15 allele in Spain (46, 52), encodes a C2 epitope for which KIR2DL2 lacks affinity, and it is recognized with average affinity by KIR2DL1 (64); functional effects of this interaction (e.g., inhibition, education) are yet to be defined. Current data thus provide no clues to understand a possible NK cell-mediated mechanism underlying the observed association of C*15 with HSV-1 infection.

The KIR repertoire of different humans is enormously variable and variegated, not simply because of gene CNV and allelic structural polymorphism; both the receptor density on the cell surface and the proportion of NK cells expressing each KIR vary in each individual. Regulation of the KIR repertoire is incompletely understood, and it is genetically controlled in part by KIR genes and by the HLA context (67). It is possible that certain HLA-C ligands modulate differently the contribution of KIR2DL2 to the KIR repertoire or license to a different extent the effector capacity of NK cells. Further investigation is warranted to determine whether variable usage of KIR2DL2 modulates the influence exerted on defense against HSV-1 by mere presence in the genome of the genes/alleles encoding the receptor and its ligands.

Unidentified ligands for orphan KIR could have escaped our analysis, but no other KIR and their known ligands associated with the clinical course of HSV-1 infection. And neither did CNV of NKG2C, including its homozygous deletion. Confirmation of this result would imply that this lectin-like NK receptor is redundant or plays no major role in defense against HSV-1.

Finally, the higher affinity allotype of CD16A (158V) was identified as a protective factor for symptomatic herpes. Valine-158 possibly enhances efficacy of the humoral response against HSV-1 by inducing a stronger interaction of CD16A with the lower hinge of IgG (37). Importance of this interaction is illustrated by the decay FcγR evolved by several human viruses, including HSV-1, which, reciprocally, are possibly better counteracted by competing host FcγR allotypes of higher affinity for IgG. Also supporting the importance of CD16A for defense against HSV-1 would be the association of mutations at aa 48 of CD16A with severe infections by herpesviruses, but that association is controversial (68–71). Contrasting with the protective effect of CD16A-158V is the lack of association between clinical herpess and CD32A-131 allotypes of variable affinity. Lack of association might reflect greater importance of effective NK cell-mediated cytotoxicity over phago-

cytosis in the elimination of HSV-1–infected cells and/or predominance of the IgG subclasses bound preferentially by CD16A in the humoral response to HSV-1.

This is, to our knowledge, the widest, hypothesis-based, immunogenetic study so far performed on susceptibility to HSV-1 infection, and it includes several genes never tested before. We have examined as many as 107 immunogenetic markers as potential risk factors for symptomatic herpes (74 HLA allotypes, 16 KIR genes and genotypes, 8 KIR/ligand pairs, 3 NKG2C, and 6 FCGR genotypes). Paradoxically, this is also a pitfall of our approach, because the high number of comparisons performed increases the probability of finding spurious associations. Following Bonferroni’s correction for multiple tests would require setting a statistical significance threshold at ∼0.0005 (0.05 divided by 107 primary comparisons). For ORs and allele frequencies in the range seen in this study, such a confidence level could only have been achieved by studying ∼500–1000 donors. Consequently, the newly identified associations (in particular those not suspected a priori, such as those of C*15 and B*18) should be considered preliminary until validated in other populations.

In summary, we have shown that susceptibility to clinical HSV-1 infection is modulated by polymorphism in genes encoding molecules that control the effector functions of cytotoxic T and NK lymphocytes (HLA class I, combinations of KIR and their ligands, and CD16A), whereas the course of the infection is not modified by genetic polymorphism in HLA class II and CD32A, molecules more implicated in cooperation between CD4 T cells, B lymphocytes, and phagocytic cells. Collectively, our results are consistent with a crucial role of CTLs and NK cells in host defense against herpetic infection, and they show that polymorphisms in genes controlling adaptive immune responses, and surveillance of its sabotage, explain part of the variable susceptibility to HSV-1 infection. Further research is warranted to determine the extent to which diversity of innate immunity-related genes contributes to this disease.

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Disclosures

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