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MAJOR ARTICLE

Association of Inhibitory Killer Cell Immunoglobulin-like Receptor Ligands With Higher *Plasmodium falciparum* Parasite Prevalence

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Killer cell immunoglobulin-like receptors (KIRs) and their HLA ligands influence the outcome of many infectious diseases. We analyzed the relationship of compound KIR-HLA genotypes with risk of *Plasmodium falciparum* infection in a longitudinal cohort of 890 Ugandan individuals. We found that presence of HLA-C2 and HLA-Bw4, ligands for inhibitory KIR2DL1 and KIR3DL1, respectively, increased the likelihood of *P. falciparum* parasitemia in an additive manner. Individuals homozygous for HLA-C2, which mediates strong inhibition via KIR2DL1, had the highest odds of parasitemia, HLA-C1/C2 heterozygotes had intermediate odds, and individuals homozygous for HLA-C1, which mediates weaker inhibition through KIR2DL2/3, had the lowest odds of parasitemia. In addition, higher surface expression of HLA-C, the ligand for inhibitory KIR2DL1/2/3, was associated with a higher likelihood of parasitemia. Together these data indicate that stronger KIR-mediated inhibition confers a higher risk of *P. falciparum* parasitemia and suggest that KIR-expressing effector cells play a role in mediating antiparasite immunity.

Keywords. KIR; HLA; malaria; *Plasmodium falciparum*; NK cells.

Plasmodium falciparum malaria has caused hundreds of millions of deaths throughout history and continues to kill nearly half of a million people annually [1]. As a result, *P. falciparum* has exerted strong selective pressure on human evolution [2, 3]. Most of the human genetic mutations known to alter malaria susceptibility lead to erythrocyte defects or cytoadhesion variants [2, 3]. However, a 2019 genome-wide association study of 17 000 individuals estimated that known variants account for only 11% of total host genetic influence on malaria susceptibility [4]. Surprisingly, only a few gene variants implicated in the host immune response have been robustly associated with malaria susceptibility. Such associations could be particularly valuable in illuminating causal mechanisms of immune protection. Notably, most studies of host genetic susceptibility to malaria, including the study noted above, have compared severe malaria cases to controls with nonsevere (but symptomatic) malaria [5, 6]. This analytic framework could overlook genes that influence protection at the earlier pre-erythrocytic stages of the *P. falciparum* life cycle, before blood-stage infection is established.

Human killer cell immunoglobulin-like receptors (KIRs) are a family of inhibitory and activating transmembrane receptors whose known ligands are primarily HLA class I molecules. The KIR locus is the second most polymorphic region in the human genome after HLA, and it is evolving even more rapidly, likely in part owing to selective pressure from pathogens [7]. Human haplotypes vary both in the number of KIR genes and in the proportion that are inhibitory or activating. While some KIR genes are universal, others are expressed by only a subset of individuals [8]. Moreover, within an individual, only a fraction of cells express a given KIR molecule [9]. KIRs are expressed on natural killer (NK) cells, some γδ T cells, and some CD8 T cells, all of which play a role in the antimalarial immune response [9–12]. Interactions of KIRs with their HLA ligands modulate the activation threshold of the cell and have been shown to influence the outcome of several, predominantly viral, infections [13–15]. Because the KIR and HLA loci are on different chromosomes and segregate independently, associations of KIR-HLA compound genotypes with susceptibility to particular

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pathogens can shed light on mechanisms critical for immunity and immunopathogenesis.

To date, the association of KIRs and their HLA ligands with susceptibility to malaria has been examined in very few studies and almost entirely in the context of case-control comparisons of severe versus nonsevere malaria [5, 6]. It has been hypothesized that KIR expression may influence activation of NK cells during malaria, and one study noted an association between KIR genotype and the in vitro NK cell response to *P. falciparum*–infected red blood cells (iRBCs) [16]. Here, we examined the influence of well-defined KIR-HLA ligand pairs on the prevalence of parasitemia and malaria in a longitudinal cohort of 890 individuals in Uganda to determine the influence of KIRs on susceptibility to *P. falciparum* infection. This analysis revealed that individuals with more inhibitory KIR-HLA pairs have higher odds of parasitemia, strongly supporting a role for cellular immunity in host restriction of the parasite.

METHODS

Study Approval

Ethical approval was granted by the Makerere University School of Medicine Research and Ethics Committee, the London School of Hygiene and Tropical Medicine Ethics Committee, the University of California, San Francisco, Committee on Human Research, and the Uganda National Council for Science and Technology. All participants and/or their parents/guardians gave written informed consent.

Clinical and Parasitological Outcomes

Subjects were enrolled into East Africa International Center of Excellence for Malaria Research longitudinal cohorts at 3 sites in Uganda with differing malaria transmission intensity [17]. Households were randomly selected; all children aged 6 months to 10 years and 1 caregiver were offered enrollment. Further details are provided in the Supplementary Material.

Participants visited the clinic quarterly for thick blood smears. Caregivers were instructed to bring children to study clinics for free care for any illness. Thick blood smears were performed in children presenting with fever (>38.0°C) or with reported fever in the previous 24 hours. Parasitemia was determined by microscopy and parasite density was calculated, as described elsewhere [18]. Loop-mediated isothermal amplification (LAMP) was performed on samples negative by microscopy at routine visits throughout follow-up for children and for part of the follow-up for adults. At each household, mosquitoes were collected monthly using Centers for Disease Control and Prevention light traps and tested for sporozoites with an enzyme-linked immunosorbent assay [19], and an annual entomological inoculation rate was calculated [18].

KIR-HLA Genotyping

HLA genotyping was performed using next-generation sequencing of polymorphic exons of HLA-A, HLA-B, and HLA-C genes. KIR genotyping for the presence/absence of each KIR was conducted by polymerase chain reaction with sequence-specific priming, as described elsewhere [20], with some modifications detailed in the Supplementary Material.

HLA-C Expression Levels

HLA-C expression levels were imputed using estimates based on previously published measurements of HLA-C allotypes [21].

Statistical Analysis

We analyzed the association of KIR-HLA ligands with 5 outcomes related to malaria immunity, as follows: (1) parasite prevalence, defined as ≥1 symptomatic or asymptomatic parasite positive visit per quarter (by microscopy); (2) annual malaria incidence rate, defined as the number of symptomatic malaria episodes per person-year; (3) parasite density, defined as the log parasite density measured during a visit; (4) the probability of symptoms if infected, or whether a participant was febrile if parasitemic; and (5) the objective temperature at parasitemic visits, conditional on parasite density.

We used multilevel models with random effects at the individual and household levels, adjusted for site, household log entomological inoculation rate, and sex. Models included a continuous linear term for age, a binary indicator of age group (children vs adults), and an interaction between the 2 because the study did not enroll participants aged 11–17 years. Temperature models also controlled for log parasite density at that visit. We used Monte Carlo permutation tests to calculate exact *P* values and controlled for multiple testing separately for each outcome, using the false discovery rate (FDR) approach. All analysis used Stata 15.1 software (StataCorp). Sensitivity analyses are described in the Supplementary Materials.

RESULTS

Clinical Cohort and KIR-HLA Genotyping

We analyzed data from 657 children and 233 adults recruited from 292 households at 3 study sites in Uganda (Table 1). KIR and HLA class I loci genotyping was performed (Table 2), and individuals were categorized based on the presence of well-characterized KIR ligand groups (ie, HLA-C2 for KIR2DL1, HLA-C1 for KIR2DL2/3, and HLA-Bw4 for KIR3DL1; Supplementary Table 1). The prevalence of inhibitory KIR genes KIR2DL1 and KIR3DL1 was very high (both >98.5%), consistent with previous reports in East African populations [22]. To control for KIR-HLA differences in ethnicity, participants were categorized as Bantu or non-Bantu based on the

Table 1. Characteristics of Study Population

Characteristic	Jinja	Kanungu	Tororo
Households	n = 97	n = 100	n = 95
Household EIR, median (range)	2.1 (1.5–22.0)	6.3 (3.0–44.5)	67.6 (11.5–588.4)
Adults	n = 89	n = 78	n = 66
Symptomatic malaria incidence, median (range), episodes per person-year	0.0 (0.0–2.0)	0.2 (0.0–3.7)	0.0 (0.0–3.0)
Parasite prevalence, % of quarters parasite positive	4.9	12.8	12.0
Children	n = 187	n = 267	n = 203
Age at enrollment, median (range), y	4.4 (0.5–10.0)	4.7 (0.5–9.9)	5.1 (0.6–10.0)
Symptomatic malaria incidence, median (range), episodes per person-year	0.2 (0.0–5.1)	1.2 (0.0–8.1)	2.1 (0.0–8.8)
Parasite prevalence, % of quarters parasite positive	12.8	36.4	64.2
All participants	n = 276	n = 345	n = 269
Follow-up time, median (range), mo[a]	55.1 (2.3–58.6)	56.7 (0.0–58.3)	39.0 (1.8–40.3)
Sickle cell result, no. (%)			
AA	201 (72.8)	323 (93.6)	195 (72.5)
AS	72 (26.1)	22 (6.4)	70 (26.0)
SS	0 (0.0)	0 (0.0)	2 (0.7)
No result	3 (1.1)	0 (0.0)	2 (0.7)
Bantu ethnicity, no. (%)	180 (65.2)	341 (98.8)	2 (0.7)

Abbreviation: EIR, entomological inoculation rate.

[a]Three individuals in the sample had only 1 visit. Tororo visits after 31 December 2014 were excluded, because transmission changed dramatically at this site after the start of intensive indoor residual spraying campaigns .

language of their consent form (Table 1). However, because ethnicity was largely collinear with site, including it in models did not improve model fit beyond site fixed effects; it was therefore omitted from final analyses.

Subjects were followed up from August 2011 to June 2016, with blood smears performed every 3 months to assess *P. falciparum* parasitemia. Reflecting the varying transmission intensity between sites, the prevalence of parasitemia was highest among children in Tororo (64%), followed by Kanungu (36%) and Jinja (13%). The incidence of malaria, defined as parasitemia accompanied by fever, also differed among the sites and was inversely associated with age [18].

To assess whether KIRs and their HLA ligands influence the risk of *P. falciparum* infection or its clinical manifestations, we tested associations of individual KIR genes, HLA ligand groups, and KIR-HLA compound genotypes with 5 malaria outcome measures: prevalence of parasitemia, clinical malaria incidence, parasite density (stratified by routine quarterly visits and malaria visits), conditional probability of clinical malaria given parasitemia, and temperature at parasitemic visits (conditional on parasite density). For each association, we report the *P* value from a multilevel model, an empirical *P* value after permutation tests (P^*), and a *q* value, calculated by applying the false discovery rate correction for multiple comparisons to the empirical *P* values. There was no association between the presence of any individual KIR gene and any of these outcomes (Supplementary Tables 2*A*–2*F*).

Inhibitory KIR2DL1 Ligand HLA-C2 Increases Risk of *P. falciparum* parasitemia

Among the best characterized KIR-HLA ligand interactions are inhibitory KIR2DL1 with the HLA-C2 subgroup of HLA-C alleles (defined by lysine at amino acid position 80) and KIR2DL2/L3 with the HLA-C1 subgroup (asparagine at position 80) [23]. Because KIR2DL1 (98.6%) and KIR2DL2/3 (99.3%) were both present in virtually all subjects,

Table 2. Killer Cell Immunoglobulin-like Receptors and Ligand Prevalence

Ligand	Participants With Ligand, % (N = 890)[a]
KIR ligands	
KIR2DL1 (n = 888)	98.6
KIR2DL2	54.7
KIR2DL3 (n = 883)	84.8
KIR2DL4	99.7
KIR2DL5	57.9
KIR2DP1	98.1
KIR2DS1	23.5
KIR2DS2	49.0
KIR2DS3	23.0
KIR2DS4	68.3
KIR2DS5	42.6
KIR3DL1	98.7
KIR3DL2	99.6
KIR3DS1	13.0
HLA ligands	
C1/C2 ligand group zygosity (n = 889)	
C1/C1	19.5
C1/C2	53.1
C2/C2	27.4
Bw4 ligand group (≥1 copy)	74.8

Abbreviation: KIR, Killer cell immunoglobulin-like receptor.

[a]N = 890 except where otherwise noted; numbers were reduced for some ligands and KIR owing to failed amplification or lack of DNA.

Table 3. Effect of HLA-C Zygosity on Parasite Prevalence

C1/C2 Zygosity	OR	*P* Value	Empirical *P* Value	Empirical FDR *q* Value
C1/C1 (n = 173)	Reference	…	…	…
C1/C2 (n = 472)	1.26	.04	.04	0.11
C2/C2 (n = 244)	1.54	.001	.001	0.008

Abbreviations: FDR, false discovery rate; OR, odds ratio.

the presence of their HLA ligands determines which inhibitory KIR/HLA-C pair(s) an individual has. Furthermore, within these inhibitory KIR/HLA-C ligand interactions, there is a hierarchy of inhibition. KIR2DL1/HLA-C2 provides the strongest inhibition, followed by KIR2DL2/HLA-C1, while KIR2DL3/HLA-C1 provides the weakest inhibition [24–26]. We found that individuals with ≥1 copy of the KIR2DL1 ligand HLA-C2 had 1.31-fold higher odds of being parasitemic (P = .02; P^*=.01; q = 0.05). Conversely, having ≥1 HLA-C1 allele decreased the odds of being parasitemic (odds ratio, 0.79; P = .02; P^* = .01; q = 0.05).

Next, we assessed the zygosity of HLA-C ligand groups to determine whether the copy number of HLA-C1 or HLA-C2 influenced parasite prevalence. HLA-C1/C1 individuals are able to receive inhibitory signals only via KIR2DL2/3, whereas HLA-C2/C2 individuals receive inhibitory signals predominantly via KIR2DL1; heterozygous individuals have the ligand for all 3 [23]. Comparison of these groups revealed a stepwise increase in parasite prevalence, with HLA-C2/C2 individuals having 1.54-fold higher odds of parasitemia compared with HLA-C1/C1 individuals (Table 3). Heterozygous (HLA-C1/C2) individuals had intermediate odds. These results suggest that engagement of KIR2DL1 with its ligand HLA-C2, the most inhibitory of the KIR/HLA-C ligand pairs, increases parasite prevalence relative to engagement of KIR2DL2/3 with their ligand HLA-C1, which provide weaker inhibitory signals. KIR2DL1-expressing cells from HLA-C2/C2 homozygous individuals will receive stronger inhibitory signals, potentially dampening the effector cell response to *P. falciparum* infection.

KIR2DL2 and KIR2DL3 segregate as alleles of the same locus, but KIR2DL2 provides stronger inhibition when bound to HLA-C1 [24]. Overall, no difference in parasitemia risk was observed between individuals in the KIR2DL3/C1 and KIR2DL2/C1 subgroups (Table 4). However, KIR2DL2 is in linkage disequilibrium with the homologous activating receptor KIR2DS2 (Wn* = 0.8 [27]; P < .001 [Fisher exact test]), which may be capable of binding HLA-C1 in a peptide-dependent manner [28, 29]. To test whether KIR2DS2 has an opposing effect that obscures the stronger inhibitory impact of KIR2DL2, we examined the impact of KIR2DL2 and KIR2DL3 among individuals lacking KIR2DS2. This revealed that presence of ≥1 copy of KIR2DL2 increased the odds of being parasitemic 1.6-fold compared with those with KIR2DL3 only (Table 4). While KIR2DL3/HLA-C1, the least inhibitory KIR-HLA combination in the hierarchy, was associated with lower parasite prevalence, previous data indicate that it markedly increases the risk of cerebral malaria, an inflammatory complication of malaria [5]. Together these data further support a hierarchy of inhibition in which KIR-HLA combinations with stronger inhibitory potential increase parasite prevalence.

Inhibitory KIR3DL1 Ligand HLA-Bw4 and Risk of *P. falciparum* Parasitemia

Another inhibitory KIR molecule, KIR3DL1, recognizes a subset of HLA-A and HLA-B alleles that contain the HLA-Bw4 motif, defined by amino acid residues 77–83 [30]. Like KIR2DL1, KIR3DL1 was nearly universal in our cohort (98.7%); hence, its effect on parasite prevalence could not be estimated directly. However, only 74.8% of individuals had an HLA-Bw4 ligand, enabling us to estimate the effect of the KIR-HLA pair. Among individuals with HLA-Bw4, the odds of parasitemia were 1.31-fold higher than in individuals lacking HLA-Bw4 (P = .01; P^* = .006; q = 0.04). This further suggests that increased KIR inhibition increases the likelihood of parasitemia, perhaps owing to dampened cellular immunity.

Because both HLA-C2 and HLA-Bw4 were found to increase parasite prevalence and have previously been shown to be in linkage disequilibrium, we looked for an additive effect of the 2 ligand groups by analyzing the combined genotype. Having

Table 4. Effect of KIR2DL2/3 on Parasite Prevalence

KIR-HLA Ligand	OR	*P* Value	Empirical *P* Value	Empirical FDR *q* Value
2DL2/3				
No C1 (n = 244)	1.24	.06	.04	0.12
C1 + 2DL2 only (n = 87)	1.03	.84	.84	0.84
C1 + 2DL2/2DL3 (n = 273)	0.93	.54	.51	0.64
C1 + 2DL3 only (n = 278)	Reference	…	…	…
2DL2/3 among those without 2DS2				
No C1 (n = 136)	1.19	.20	.17	0.35
C1 + 2DL2 only or 2DL2/2DL3 (n = 45)	1.60	.02	.02	0.06
C1 + 2DL3 only (n = 272)	Reference	…	…	…

Abbreviations: FDR, false discovery rate; OR, odds ratio.

both HLA-C2 and HLA-Bw4 increased the odds of parasitemia by 1.46-fold compared with HLA-C2 alone (P = .001; P^* < .001; q = 0.008) and by 1.51-fold compared with HLA-Bw4 alone (P = .002, P^* = .001, q = 0.01), suggesting an additive inhibitory effect of the 2 KIR-HLA ligand pairs. Together, this supports a model wherein the more inhibitory signals that KIR-expressing effector cells can receive, the greater the risk of parasitemia.

Association Between Surface Expression of Inhibitory KIR Ligand HLA-C and Odds of Parasitemia

To further elucidate how HLA-C influences *P. falciparum* infection, we examined the relationship of HLA-C surface expression levels to parasite prevalence. HLA-C molecules have been ascribed 2 distinct roles in cellular immunity—as ligands for inhibitory KIRs, and as antigen-presenting molecules for CD8 T cells—both of which can influence infectious disease outcomes [13, 14, 31]. In contrast to HLA-A and -B molecules, surface expression of HLA-C is 10–15-fold lower and varies substantially among individual allotypes [32]. During viral infections such as human immunodeficiency virus (HIV) infection, HLA-C alleles that are more highly expressed at the cell surface have been associated with a more potent CD8 T-cell response that correlates with enhanced viral control [33, 34].

We hypothesized that if the protection associated with certain HLA-C ligand groups is attributable to differences in peptide presentation, then higher HLA-C expression would similarly correlate with a reduced likelihood of parasitemia. We assigned each HLA-C allele a value based on previously determined expression levels [21] and calculated a total HLA-C expression level for each individual. We found that higher HLA-C expression was associated with an *increase* in the odds of *P. falciparum* parasitemia (1.12-fold increase per 1 standard deviation of expression; P = .01; P^* = .006; q = 0.04). This contrasts with HIV infection, where highly expressed HLA-C allotypes are protective. These data suggest that the mechanism by which HLA-C molecules influence restriction of *P. falciparum* parasitemia is not antigen presentation to T cells, but rather a role in KIR-mediated inhibition of effector cells.

Relationship of KIR-HLA Genotype to Other Parasitological and Malaria Outcomes

Finally, because immune mechanisms responsible for restricting parasitemia may differ from those that protect against symptomatic malaria, we also examined the association of KIR-HLA ligand pairs with the other parasitological and clinical outcomes noted above (Supplementary Tables 2*B*–2*F*). We did not observe an association of KIR-HLA ligand pairings with incidence of clinical malaria, probability of clinical malaria if parasitemic, body temperature at parasitemic visits, or parasite density. Together these results are most consistent with an influence of KIR-expressing effector cells on restriction of parasite replication, rather than on the downstream inflammatory consequences that eventuate in fever and other clinical symptoms.

In addition, we performed multiple sensitivity analyses to assess the robustness of our results. A more sensitive measure of parasitemia (LAMP) was performed on samples that were negative by microscopy at routine visits. For adults, these data were available for only a portion of follow-up time. When the definition of parasitemia was expanded to include *P. falciparum* infections detectable only by LAMP among children for the entire follow-up period (Supplementary Table 3) and among children and adults for the period LAMP data were available for both (Supplementary Table 4), similar associations were observed for all results stated above. In addition, we analyzed models (1) stratified by site (Supplementary Table 5*A*–5*C*) to determine whether our results were robust across transmission intensities and ethnicities; (2) restricted to children, because they experience the largest burden of parasitemia (Supplementary Table 6); and (3) excluding individuals with hemoglobin S (sickle cell) mutations to ensure that our results were not confounded by hemoglobin variants (Supplementary Table 7). These secondary analyses yielded similar results, further strengthening our findings.

DISCUSSION

Using longitudinal data from individuals with varying malaria exposure, we found that the inhibitory KIR ligands HLA-C2 and HLA-Bw4 were both associated with a higher prevalence of *P. falciparum* parasitemia. Our data indicate that both the number of inhibitory KIR-HLA ligand pairs and the strength of their cellular inhibitory signaling influence the risk of *P. falciparum* parasitemia but, notably, do not affect clinical malaria. Our findings suggest that the cellular immune response plays a critical role either in the prevention or clearance of blood-stage *P. falciparum* infection. These associations represent a novel genetic determinant of *P. falciparum* parasitemia, and they support an expanded role for KIRs in modulating host immunity to parasitic infections.

This novel association of KIR-HLA compound genotypes with parasite prevalence is somewhat paradoxical, given that *P. falciparum* spends most of its life cycle outside of HLA-expressing cells. After intradermal inoculation by mosquitoes, *P. falciparum* sporozoites migrate via the dermis and lymphatics to infect hepatocytes, the only stage in the parasite life cycle within an HLA-expressing host cell [35]. Within the hepatocyte, parasites expand 2000–40 000-fold, then emerge synchronously as merozoites, which establish a self-propagating cycle of erythrocytic infection [36, 37]. Because erythrocyte membranes contain little to no HLA [38], we hypothesize that the influence of KIRs on cell-mediated antiparasite immunity may occur primarily during the liver stage when the multiplicity of infection

is also lowest. It has previously been shown that increasing KIR-mediated NK cell inhibition is associated with poorer clearance of HCV and HBV in the liver [13, 14].

Alternatively, the observed impacts of KIR-HLA pairs on parasite prevalence may be due to enhanced spontaneous clearance of parasitemia rather than cessation of infection at the liver stage. Here, it is notable that no association of any individual KIR gene with clinical outcomes was observed, suggesting that the influence of KIRs is dependent on interactions with an HLA-bearing cell. Previous evidence suggests that NK cell activation by iRBCs is dependent on contact with monocytes and myeloid dendritic cells, creating a point at which KIR-mediated inhibition of NK cells may occur, despite the absence of HLA on RBCs themselves [39]. While NK-iRBC conjugates have been observed, direct activation of NK cells by iRBCs has not been conclusively demonstrated [16]. While conventional αβ T cells express KIRs only infrequently, KIRs are often expressed on γδ T cells. Furthermore, we have shown KIRs to be highly expressed on the CD16 + Vγ9Vδ2 T-cell subset, which is increased with frequent malaria exposure and can be activated by opsonized iRBCs in a T-cell receptor–independent manner [40]. Thus, KIRs may modulate the antibody-dependent cellular cytotoxicity not only of NK cells but also of γδ T cells [41, 42].

It is also notable that we did not observe an influence of KIRs or their HLA ligands on symptomatic malaria. In endemic regions, "clinical immunity"—defined as protection from symptomatic malaria—develops gradually with increasing age and exposure to *P. falciparum*, but sterilizing immunity (ie, protection against parasitemia) does not [43]. Hence, it is likely that the immune mechanisms underlying sterile protection and restriction of parasite replication differ from those that mediate clinical immunity, with the latter involving immunoregulatory processes that dampen the inflammation responsible for fever and other symptoms, while fostering successful long-term parasitism.

By definition, sterile immunity is directed at the pre-erythrocytic stages of parasite development. The pre-erythrocytic immune response can prevent or greatly limit the establishment of blood-stage parasitemia and is believed to be mediated by CD8 T cells [12, 44], although recent data suggest that NK cells [45–47] and γδ T cells [48–50], which both frequently express KIRs, may also play important roles. It remains possible that KIR-HLA interactions exert an impact on the symptomatic or inflammatory manifestations of malaria that we were underpowered to detect, given that all study subjects had access to free clinical care that afforded prompt diagnosis and treatment of malaria. Indeed, Hirayasu et al [5] reported that the risk of cerebral malaria, an inflammatory complication, is markedly elevated among individuals with KIR2DL3/HLA-C1, which is the least inhibitory pairing among KIR2DL1/2/3 ligands.

Immunopathology contributes substantially to the neurological damage caused by cerebral malaria [51], and NK cells have been found to accumulate in the brain microvasculature [52]. Thus, lower KIR inhibition could allow unrestrained NK cell activation resulting in immune-mediated pathology. This would be consistent with the hypothesis that mechanisms preventing initial *P. falciparum* infection and those preventing severe disease are distinct and may have a balancing effect on the maintenance of different KIRs and HLA ligands in malaria-endemic populations.

Our results also underscore that case-control studies of severe versus nonsevere malaria have important limitations as a framework to identify immune susceptibility genes. Because such comparisons are restricted to individuals in whom parasitemia is already established, they cannot identify genetic factors influencing host restriction at the pre-erythrocytic stages of infection. Moreover, the development of severe malaria is likely to be influenced by genetic factors governing inflammation, as well as nongenetic factors such as socioeconomic status and access to care. We believe that additional genetic determinants of malaria may be uncovered through analytic methods that incorporate rigorous longitudinal assessments of both parasite infection and symptoms, enabling evaluation of immune protection at varying stages of malaria, from initial infection and establishment of parasitemia, to development of symptomatic or even severe malaria.

While it is well established that KIRs influence the immune response to and ultimate outcome of numerous viral infections, their impact on the control of nonviral infections has been less certain. Our finding that inhibitory KIR ligands increase the risk of *P. falciparum* parasitemia indicates that the influence of KIRs on host immunity extends to parasitic infections. Moreover, our findings suggest an important role for the cellular immune response in restricting *P. falciparum* infection, perhaps during the earliest stages of infection, before blood-stage parasitemia is established. These findings represent a novel genetic determinant of malaria susceptibility and further our understanding of antimalarial immunity with implications for vaccine design.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases online*. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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References

1. World Health Organization. World malaria report 2017. Geneva, Switzerland: World Health Organization, **2018**.
2. Kariuki SN, Williams TN. Human genetics and malaria resistance. Hum Genet **2020**; 139:801–11.
3. Kwiatkowski DP. How malaria has affected the human genome and what human genetics can teach us about malaria. Am J Hum Genet **2005**; 77:171–92.
4. Band G, Le QS, Clarke GM, et al. Insights into malaria susceptibility using genome-wide data on 17 000 individuals from Africa, Asia and Oceania. Nat Commun **2019**; 10:1–19.
5. Hirayasu K, Ohashi J, Kashiwase K, et al. Significant association of KIR2DL3-HLA-C1 combination with cerebral malaria and implications for co-evolution of KIR and HLA. PLOS Pathogens **2012**; 8:e1002565.
6. Lourembam SD, Sawian CE, Baruah S. Differential association of KIR gene loci to risk of malaria in ethnic groups of Assam, Northeast India. Infect Genet Evol **2011**; 11:1921–8.
7. Kulkarni S, Martin MP, Carrington M. The yin and yang of HLA and KIR in human disease. Semin Immunol **2008**; 20:343–52.
8. Middleton D, Gonzelez F. The extensive polymorphism of KIR genes. Immunology **2010**; 129:8–19.
9. Andersson S, Fauriat C, Malmberg JA, Ljunggren HG, Malmberg KJ. KIR acquisition probabilities are independent of self-HLA class I ligands and increase with cellular KIR expression. Blood **2009**; 114:95–104.
10. Burrack KS, Hart GT, Hamilton SE. Contributions of natural killer cells to the immune response against *Plasmodium*. Malaria J **2019**; 18:321.
11. Dantzler KW, Jagannathan P. γδ T cells in antimalarial immunity: new insights into their diverse functions in protection and tolerance. Front Immunol **2018**; 9:2445.
12. Lefebvre MN, Harty JT. You shall not pass: memory CD8 T cells in liver-stage malaria. Trends in parasitology. **2020**; 36:147–57.
13. Di Bona D, Aiello A, Colomba C, et al; KIRIIND (KIR Infectious and Inflammatory Diseases) Collaborative Group. KIR2DL3 and the KIR ligand groups HLA-A-Bw4 and HLA-C2 predict the outcome of hepatitis B virus infection. J Viral Hepat **2017**; 24:768–75.
14. Khakoo SI, Thio CL, Martin MP, et al. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. Science **2004**; 305:872–4.
15. Mori M, Leitman E, Walker B, Ndung'u T, Carrington M, Goulder P. Impact of HLA allele-KIR pairs on HIV clinical outcome in South Africa. J Infect Dis **2019**; 219: 1456–63.
16. Artavanis-Tsakonas K, Eleme K, McQueen KL, et al. Activation of a subset of human NK cells upon contact with *Plasmodium falciparum*-infected erythrocytes. J Immunol **2003**; 171:5396–405.
17. Kamya MR, Arinaitwe E, Wanzira H, et al. Malaria transmission, infection, and disease at three sites with varied transmission intensity in Uganda: implications for malaria control. Am J Trop Med Hyg **2015**; 92:903–12.
18. Rodriguez-Barraquer I, Arinaitwe E, Jagannathan P, et al. Quantification of anti-parasite and anti-disease immunity to malaria as a function of age and exposure. eLife **2018**; 7:e35832.
19. Kilama M, Smith DL, Hutchinson R, et al. Estimating the annual entomological inoculation rate for *Plasmodium falciparum* transmitted by *Anopheles gambiae* s.l. using three sampling methods in three sites in Uganda. Malar J **2014**; 13:111.
20. Martin MP, Carrington M. KIR locus polymorphisms: genotyping and disease association analysis. Methods Mol Biol **2008**; 415:49–64.
21. Apps R, Qi Y, Carlson JM, et al. Influence of HLA-C expression level on HIV control. Science **2013**; 340:87–91.
22. Nakimuli A, Chazara O, Farrell L, et al. Killer cell immunoglobulin-like receptor (KIR) genes and their

HLA-C ligands in a Ugandan population. Immunogenetics **2013**; 65:765–75.
23. Biassoni R, Falco M, Cambiaggi A, et al. Amino acid substitutions can influence the natural killer (NK)-mediated recognition of HLA-C molecules: role of serine-77 and lysine-80 in the target cell protection from lysis mediated by "group 2" or "group 1" NK clones. J Exp Med **1995**; 182:605–9.
24. Moesta AK, Norman PJ, Yawata M, Yawata N, Gleimer M, Parham P. Synergistic polymorphism at two positions distal to the ligand-binding site makes KIR2DL2 a stronger receptor for HLA-C than KIR2DL3. J Immunol **2008**; 180:3969–79.
25. Winter CC, Gumperz JE, Parham P, Long EO, Wagtmann N. Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-C allotype recognition. J Immunol **1998**; 161:571–7.
26. Carrington M, Wang S, Martin MP, et al. Hierarchy of resistance to cervical neoplasia mediated by combinations of killer immunoglobulin-like receptor and human leukocyte antigen loci. J Exp Med **2005**; 201:1069–75.
27. Single RM, Martin MP, Meyer D, Gao X, Carrington M. Methods for assessing gene content diversity of KIR with examples from a global set of populations. Immunogenetics **2008**; 60:711–25.
28. Moesta AK, Parham P. Diverse functionality among human NK cell receptors for the C1 epitope of HLA-C: KIR2DS2, KIR2DL2, and KIR2DL3. Front Immunol **2012**; 3:336.
29. Naiyer MM, Cassidy SA, Magri A, et al. KIR2DS2 recognizes conserved peptides derived from viral helicases in the context of HLA-C. Sci Immunol **2017**; 2:eaal5296.
30. Gumperz JE, Litwin V, Phillips JH, Lanier LL, Parham P. The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. J Exp Med **1995**; 181: 1133–44.
31. Marco MD, Schuster H, Backert L, Ghosh M, Rammensee HG, Stevanović S. Unveiling the peptide motifs of HLA-C and HLA-G from naturally presented peptides and generation of binding prediction matrices. J Immunol **2017**; 199:2639–51.
32. Apps R, Meng Z, Del Prete GQ, Lifson JD, Zhou M, Carrington M. Relative expression levels of the HLA class-I proteins in normal and HIV-infected cells. J Immunol **2015**; 194:3594–600.
33. Thomas R, Apps R, Qi Y, et al. HLA-C cell surface expression and control of HIV/AIDS correlate with a variant upstream of HLA-C. Nat Genet **2009**; 41:1290–4.
34. Corrah TW, Goonetilleke N, Kopycinski J, et al. Reappraisal of the relationship between the HIV-1-protective single-nucleotide polymorphism 35 kilobases upstream of the HLA-C gene and surface HLA-C expression. J Virol **2011**; 85:3367–74.
35. Mota MM, Pradel G, Vanderberg JP, et al. Migration of *Plasmodium* sporozoites through cells before infection. Science **2001**; 291:141–4.
36. Sturm A, Amino R, van de SC, et al. Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. Science **2006**; 313:1287–90.
37. Crutcher JM, Hoffman SL. Malaria. In: Baron S, ed. Medical microbiology. 4th ed. Galveston, TX: University of Texas Medical Branch at Galveston; **1996**.
38. Moras M, Lefevre SD, Ostuni MA. From erythroblasts to mature red blood cells: organelle clearance in mammals. Front Physiol **2017**; 8:1076.
39. Newman KC, Korbel DS, Hafalla JC, Riley EM. Cross-talk with myeloid accessory cells regulates human natural killer cell interferon-gamma responses to malaria. PLoS Pathog **2006**; 2:e118.
40. Farrington LA, Callaway PC, Vance HM, et al. Opsonized antigen activates Vδ2+ T cells via CD16/FCγRIIIa in individuals with chronic malaria exposure. PLoS Pathog **2020**; 16:e1008997.
41. Lisovsky I, Kant S, Tremblay-McLean A, et al. Differential contribution of education through KIR2DL1, KIR2DL3, and KIR3DL1 to antibody-dependent (AD) NK cell activation and ADCC. J Leukoc Biol **2019**; 105:551–63.
42. Parsons MS, Zipperlen K, Gallant M, Grant M. Killer cell immunoglobulin-like receptor 3DL1 licenses CD16-mediated effector functions of natural killer cells. J Leukoc Biol **2010**; 88:905–12.
43. Tran TM, Li S, Doumbo S, et al. An intensive longitudinal cohort study of Malian children and adults reveals no evidence of acquired immunity to *Plasmodium falciparum* infection. Clin Infect Dis **2013**; 57:40–7.
44. Hafalla JC, Cockburn IA, Zavala F. Protective and pathogenic roles of CD8+ T cells during malaria infection. Parasite Immunol **2006**; 28:15–24.
45. Hart GT, Tran TM, Theorell J, et al. Adaptive NK cells in people exposed to *Plasmodium falciparum* correlate with protection from malaria. J Exp Med **2019**; 216:1280–90.
46. Arora G, Hart GT, Manzella-Lapeira J, et al. NK cells inhibit *Plasmodium falciparum* growth in red blood cells via antibody-dependent cellular cytotoxicity. eLife Sci **2018**; 7:e36806.
47. Suscovich TJ, Fallon JK, Das J, et al. Mapping functional humoral correlates of protection against malaria challenge following RTS,S/AS01 vaccination. Sci Transl Med **2020**; 12:eabb4757.
48. Mamedov MR, Scholzen A, Nair RV, et al. A macrophage colony-stimulating-factor-producing γδ T cell subset prevents malarial parasitemic recurrence. Immunity **2018**; 48:350–363.e7.
49. Zaidi I, Diallo H, Conteh S, et al. γδ T cells are required for the induction of sterile immunity during irradiated sporozoite vaccinations. J Immunol **2017**; 199:3781–8.

50. Ribot JC, Neres R, Zuzarte-Luís V, et al. γδ-T cells promote IFN-γ–dependent *Plasmodium* pathogenesis upon liver-stage infection. Proc Natl Acad Sci U S A **2019**; 116:9979–88.
51. Grau GE, Heremans H, Piguet PF, et al. Monoclonal antibody against interferon gamma can prevent experimental cerebral malaria and its associated overproduction of tumor necrosis factor. Proc Natl Acad Sci U S A **1989**; 86:5572–4.
52. Hansen DS, Bernard NJ, Nie CQ, Schofield L. NK cells stimulate recruitment of $CXCR3^+$ T cells to the brain during *Plasmodium berghei*-mediated cerebral malaria. J Immunol **2007**; 178:5779–88.