Hierarchy of resistance to cervical neoplasia mediated by combinations of killer immunoglobulin-like receptor and human leukocyte antigen loci

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Killer immunoglobulin-like receptor (KIR) recognition of specific human histocompatibility leukocyte antigen (HLA) class I allotypes contributes to the array of receptor–ligand interactions that determine natural killer (NK) cell response to its target. Contrasting genetic effects of *KIR*/*HLA* combinations have been observed in infectious and autoimmune diseases, where genotypes associated with NK cell activation seem to be protective or to confer susceptibility, respectively. We show here that combinations of *KIR* and *HLA* loci also affect the risk of developing cervical neoplasia. Specific inhibitory *KIR*/*HLA* ligand pairs decrease the risk of developing neoplasia, whereas the presence of the activating receptor *KIR3DS1* results in increased risk of disease, particularly when the protective inhibitory combinations are missing. These data suggest a continuum of resistance conferred by NK cell inhibition to susceptibility involving NK cell activation in the development of cervical neoplasia and underscore the pervasive influence of *KIR*/*HLA* genetic variation in human disease pathogenesis.

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NK cells are an important component of the innate immune system, providing the first line of defense in the early stages of the immune response against viral infections and tumors by production of cytokines or direct cytotoxicity (1, 2). NK cell activity is controlled partially through interactions between killer immunoglobulin-like receptor (KIR) expressed on NK cells and their respective HLA class I ligands expressed on target cells (3–5). *HLA* class I loci are characterized by extreme allelic polymorphism, whereas *KIR* variation stems both from the polygenic nature of *KIR* haplotypes (i.e., they vary in the number and types of genes present) and from allelic polymorphism at some of the *KIR* genes (6, 7). Independent segregation of *HLA* (6p21) and *KIR* (19q13.4) genes along with KIR specificity for particular HLA allotypes raises the possibility that any given individual may express KIR molecules for which no ligand is present or vice versa, resulting in the lack of NK cell signaling through that specific KIR. Thus, the requirement for specific ligand–receptor interactions at these two polymorphic loci implies an epistatic relationship (i.e., a synergistic relationship involving variation at two or more loci, specific combinations of which are essential for the phenotype to occur) between KIR and HLA in NK cell–mediated immunity.

KIR genes encode molecules that convey either inhibitory or activating signals to NK

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cells and to a subset of $CD8^+$ T cells. Binding of inhibitory KIR (designated 2DL and 3DL) to specific HLA allotypes has been clearly demonstrated (3, 8), correlating with their ability to inhibit NK cytolysis of target cells bearing those HLA molecules. The inhibitory KIR3DL1 binds HLA-B allotypes that have the Bw4 epitope (determined by amino acid positions 79–83 of the molecule) (9). The ligand for KIR3DS1, an activating KIR that shows 99% amino acid sequence similarity to KIR3DL1 in its extracellular domains, has not been determined, and, indeed, its cell surface expression has not been definitively shown. However, the presence of this gene along with *HLA-B* alleles encoding Bw4 have an epistatic protective effect on AIDS progression (10), suggesting that, like KIR3DL1, KIR3DS1 might recognize some Bw4 allotypes in HIV^+ individuals. Specificity of inhibitory KIR for HLA-Cw allotypes is dictated to a large extent by the presence of asparagine or lysine at position 80 of the HLA-Cw molecule (11, 12). KIR2DL1 recognizes group 2 HLA-Cw molecules that are characterized by Lys80, whereas KIR2DL2 and KIR2DL3 (referred to as KIR2DL2/3 throughout the remainder of the paper) prefer group 1 HLA-Cw molecules containing Asn80. *KIR2DL1*, *KIR2DL2/3*, and *KIR3DL1* are present in nearly all individuals of European descent, so the presence or absence of their HLA ligands determines the functional significance of these inhibitory KIR in any given individual.

The effect of genetic variation at the *KIR* locus in combination with genes encoding their HLA ligands on cancer has not been studied previously, even though variation at these genes may be important in the development of cancers that are linked to viruses because *KIR* genes have been shown to be associated with outcome to viral infections (10, 13). To test the possibility that the *KIR* genes are involved in risk of developing cervical neoplasia, a cancer caused by human papilloma virus (HPV; reference 14), we examined *KIR* and *HLA* in three large cervix studies.

RESULTS AND DISCUSSION

To test the possibility that the *KIR* genes are involved in risk of developing cervical neoplasia, a cancer caused by HPV (14), we grouped *HLA-B* (*HLA-B Bw4*) and *HLA-Cw* (groups 1 and 2) class I alleles according to KIR ligand specificity using *HLA* genotypes determined previously from participants in three large cervical studies and compared the *HLA* group frequencies in cytologically normal versus cervical intraepithelial neoplasia 3 (CIN3)/cancer patients. Using a dominant model, both *HLA-Cw* group 2 and *HLA-B Bw4* allelic groups were significantly associated with a decreased risk of disease when data from the three studies were merged (odds ratio [OR] = 0.71, P = 0.02, and OR = 0.70, P = 0.015, respectively; Fig. 1 and Table S1, available at http://www.jem.org/cgi/content/full/jem.20042158/DC1), a trend that was reflected in each individual study (although not significantly in most comparisons) and either consistent or more pronounced when restricted to HPV^+ controls (Cw group 2, OR = 0.77, 95% confidence interval = 0.53–1.12; Bw4, OR = 0.56, 95% confidence interval = 0.38–0.81). Although we cannot entirely rule out the possible association between these alleles and HPV infection, the observation of similar associations in HPV-restricted controls supports a role for these alleles in cervical cancer pathogenesis. The presence of both *Bw4* and *Cw* group 2 showed a somewhat stronger effect than the presence of an allele or alleles from only one of these groups (OR = 0.56, P = 0.002 for both, vs. OR = 0.65, P = 0.024 for only one; Table I). Alternatively, the presence of at least one copy of *Cw* group 1 was significantly associated with increased risk of disease in the Eastern U.S. study (OR = 2.14, P = 0.02) but not in the Costa Rica or Portland studies (Table S1). However, *Cw* group 1 in the absence of both *Cw* group 2 and *Bw4* did associate with susceptibility in each of the three studies (OR = 1.47–2.09), although, again, significance was reached

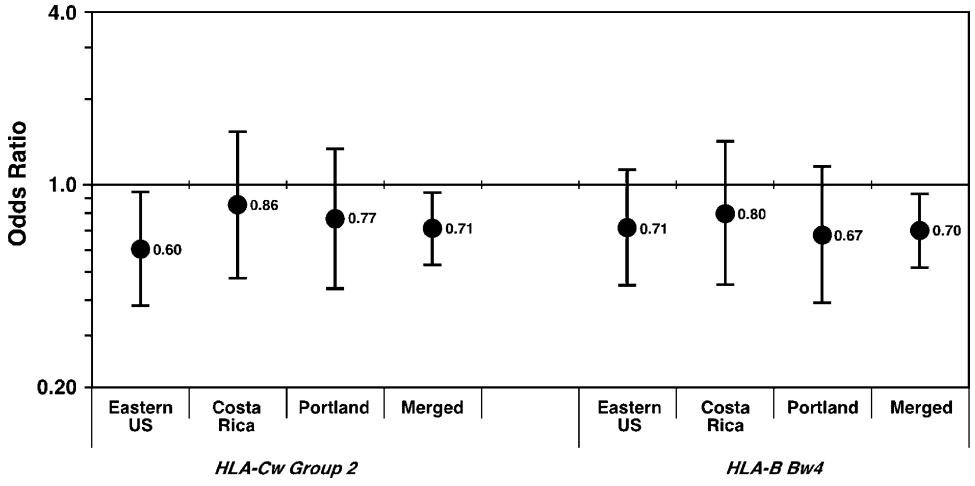

Figure 1. Independent associations between HLA ligands for KIR and CIN3/cancer outcome in three independent studies. Odds ratios (circle) and 95% confidence intervals (dashes) are shown separately for *HLA-Cw* group 2 and *HLA-B Bw4*, for the Eastern U.S., Costa Rica, and Portland studies, followed by estimates for data from all three studies merged.

Table I. Combined effect of *HLA-B Bw4* and *HLA-Cw* group 2 on CIN3 cancer

	Merged			
KIR/HLA	Cases (n = 257)	Controls (n = 675)	OR (95% CI)	p-value
	n (%)	*n* (%)		
Bw4$^-$/*Grp2*$^-$	75 (29)	134 (20)	1.00 (ref)	–
Bw4$^+$/*Grp2*$^-$ or *Bw4*$^-$/*Grp2*$^+$	87 (34)	239 (35)	0.65 (0.45–0.95)	0.0243
Bw4$^+$/*Grp2*$^+$	95 (37)	302 (45)	.56 (0.30–0.81)	0.0020

CI, confidence interval; OR, odds ratio.

only in the Eastern U.S. study (for merged data, OR = 1.6, P = 0.0066). Overall, these data suggest that one mode of influence conferred by HLA class I on cervical neoplasia may stem from their capacity as ligands for inhibitory KIR molecules (inhibitory *KIR* genes that encode products recognizing Cw group 1, Cw group 2, and Bw4 are present in >95% of individuals studied here). Specifically, effector cell inhibition mediated by KIR2DL1 and KIR3DL1 (the receptors for Cw group 2 and Bw4, respectively) may participate in decreasing the risk of cervical neoplasia.

Unlike several of the inhibitory *KIR*, the activating *KIR* are present on only a fraction of *KIR* haplotypes. Given the protective influence of *Bw4* and *Cw* group 2 alleles, which encode the high-affinity ligands for two inhibitory KIR, we hypothesized that the presence of activating *KIR* may confer a susceptibility effect. Two of the three cervix studies from which DNA was available (Eastern U.S. and Costa Rica) were typed for the presence or absence of six activating and five inhibitory *KIR* genes, which, along with three anchor *KIR* loci that are always present, constitute the known assortment of *KIR* haplotypes (Table S2, available at http://www.jem.org/cgi/content/full/jem.20042158/DC1). *KIR3DS1*, an activating receptor that shows high sequence similarity to *KIR3DL1*, was observed significantly more frequently in CIN3/cancer cases than in controls (OR = 1.64, P = 0.009). The activating *KIR* genes, *KIR2DS1* and *KIR2DS5*, showed a weak association with increased risk of CIN3/cancer that bordered on significance (Table S2), possibly because of their strong linkage disequilibrium with *KIR3DS1* (15, 16). Thus, activating and inhibitory *KIR* seem to have opposing effects on the risk of developing cervical neoplasia.

When effects of *KIR3DS1* were evaluated jointly with *Cw* group 2 (Fig. 2, left) or *Bw4* (Fig. 2, right), a significant gradient of effects from susceptibility to protection was observed in the following order (Fig. 2 and Table S3, available at http://www.jem.org/cgi/content/full/jem.20042158/DC1): (a) presence of *KIR3DS1* in the absence of ligand for the inhibitory *KIR2DL1* or *KIR3DL1* (OR = 1; baseline), (b) absence of both activating *KIR3DS1* and inhibitory *KIR* ligand (OR = 0.69–0.81), (c) presence of both *KIR3DS1* and inhibitory *KIR* ligand (OR = 0.60–0.67), and (d) absence of *KIR3DS1* in the presence of inhibitory *KIR* ligand (OR = 0.44–0.47). These trends were evident for *KIR3DS1* in combination with *Cw* group 2 (ligand for *KIR2DL1*; P trend = 0.0026) as well as *Bw4* (ligand for *KIR3DL1*; P trend = 0.0025), supporting a scenario in which the risk of developing cervical neoplasia is, to some extent, a function of the level of NK cell activation contributed by KIR.

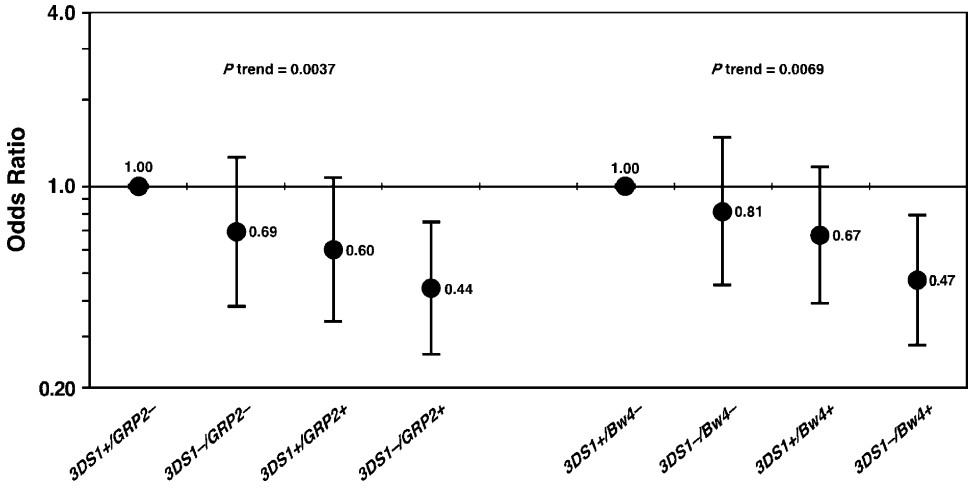

Figure 2. Effect of compound genotypes *KIR 3DS1/ HLA-Cw* group 2 and *KIR3DS1/HLA-B Bw4* on CIN3/cancer. Odds ratios (circle) and 95% confidence intervals (dashes) are shown separately for *KIR 3DS1* and *HLA-Cw group 2*, and for *KIR3DS1* and *HLA-B Bw4*. The referent groups, *KIR3DS1*$^+$*/Cw group 2*$^-$ (left) and *KIR3DS1*$^+$*/HLA-B Bw4*$^-$ (right), are those with which all other genotypically defined groups are compared, and the odds ratio for each referent group is set at 1. Middle genotypes in both analyses were grouped together to generate the P trend values because we have no functional criteria to suggest that one genetic factor (*HLA-Cw* group 2, *HLA-B Bw4*, or *KIR3DS1*) is dominant over the others. We list the various genotypes separately and ordered by decreasing odds ratios as a means to compare odds ratios of individual genotypes.

The *HLA-B* and *-C* loci are in strong linkage disequilibrium, being separated by only 81.5 kb. Indeed, *HLA-B Bw4* and *HLA-Cw* group 2 are in significant but not complete linkage disequilibrium (D′[Q2] = 0.413, P < 0.0001). Thus, it can be difficult to distinguish between disease associations involving one or both of these loci in the context of their function as inhibitory KIR ligands, particularly because the receptors that recognize these ligands, KIR3DL1 and KIR2DL1, are present in 95–98% of all individuals (Table S2). To address this issue with regard to the *KIR3DS1/HLA-Cw* group 2 and *KIR3DS1/HLA-B Bw4* associations in cervical disease, we tested for independent and additive effects of these compound genotypes, which were suggested in the analysis of the *HLA* groups where *KIR* genotype was not considered (Table I). A significant test for trend was observed for odds ratios determined in a progressive manner from genotypes expected to result in the most activation to those resulting in the most inhibition as follows (P trend = 0.0012; Fig. 3 and Table S4, available at http://www.jem.org/cgi/content/full/jem.20042158/DC1): (a) absence of both *Bw4* and *Cw* group 2, presence of *KIR3DS1*; (b) presence of either *Bw4* or *Cw* group, presence of *KIR3DS1*; (c) absence of *Bw4* and *Cw* group 2, absence of *KIR3DS1*; (d) presence of *Bw4* and *Cw* group 2, presence of *KIR3DS1*; (e) presence of *Bw4* and/or *Cw* group 2, absence of *KIR3DS1*. Although numbers are limited, the data suggest that having both *Bw4* and *Cw* group 2 may be more protective than having only one of these inhibitory ligands when *KIR3DS1* is present (compare the second and fourth sets in Fig. 3). However, in the absence of *KIR3DS1*, there did not seem to be an additive effect of having both *Bw4* and *Cw* group 2 relative to having only one of these ligands (*Bw4* alone, OR = 0.48, P = 0.06; *Cw* group 2 alone, OR = 0.43, P = 0.04; *Bw4* and *Cw* group 2, OR = 0.44, P = 0.02). These statistics also indicate independent protective effects of both *Bw4* and *Cw* group 2, as opposed to a single protective locus that is simply marked by a second locus through linkage disequilibrium.

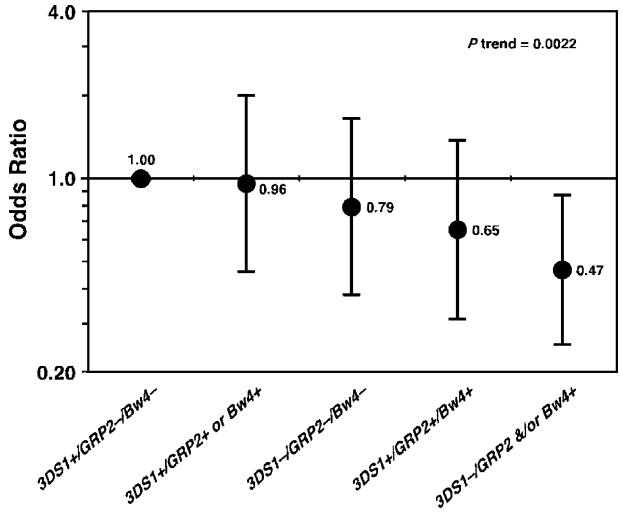

Figure 3. Combined effect of activating *KIR3DS1* and HLA ligands (*HLA-Cw* group 2 and *HLA-B Bw4*). Odds ratios (circle) and 95% confidence intervals (dashes) are shown for each combination. In this analysis, the genotype of the referent group (for which the odds ratio is set at 1) was *KIR3DS1*$^+$*/HLA-Cw* group 2-*/HLA-B Bw4*$^-$. As in Fig. 2, middle genotypes were grouped together to generate the P trend values (Fig. 2).

Previous experimental data are consistent with the hypothesis that KIR2DL1 provides a stronger inhibitory signal than do KIR2DL2 and KIR2DL3 (17), and previously reported genetic epidemiological data support this model (13, 18). We suggest that the data reported herein present another illustration of the distinction among KIR2DL receptors in effector cell signaling, in which KIR2DL1 binding to Cw group 2 ligands may send a stronger inhibitory signal to the effector cells than does KIR2DL2 or KIR2DL3 upon binding to Cw group 1 ligands on target cells. The potentially strong inhibition mediated by KIR2DL1–Cw group 2, along with that conferred by KIR3DL1–Bw4 interactions, may avert to some extent the immune activation, which we propose is linked to cervical pathogenesis. A model for the gradation of responses from most activating (susceptible) to most inhibitory (protective) is shown in Fig. 4.

Cervical neoplasia is the third disease with which *KIR3DS1* has been associated (10), underscoring the need to determine whether this receptor is expressed, and, if so, to identify its ligand. Rather than being directly involved in disease processes, *KIR3DS1* may simply mark a neighboring gene that is directly involved in pathogenesis of cervical neoplasia. Indeed, all individuals in this study with *KIR3DS1* also had *KIR2DS1*. However, four individuals who had *KIR2DS1* were missing *KIR3DS1*, and all four were disease-free, accounting for the weaker association between *KIR2DS1* and disease (Table S2) and suggesting that *KIR2DS1* may not independently increase risk of this disease. Given the strong linkage disequilibrium among the *KIR* genes, it is difficult to rule out the possibility that both *KIR2DS1* and *KIR3DS1* mark another locus that directly influences the risk of developing cervical neoplasia.

The existence of functionally opposing KIR molecules suggests that the health benefits derived from any given activating or inhibitory KIR may be coupled to certain risks, so that a *KIR* gene (or haplotype) conferring protection against one disease may actually predispose to another. This model is corroborated by genetic epidemiological data, which show that specific *KIR/HLA* compound genotypes expected to result in an activating phenotype are associated with protection against some infectious diseases (10, 13) but are also associated with susceptibility to autoimmune pathogenesis (19–21). In this regard, cervical neoplasia may more closely resemble autoimmune disease, in spite of its infectious etiology.

HPV infection is necessary, but not sufficient, for development of cervical cancer (22). Nevertheless, HPV infections rarely progress to cervical cancer; most HPV infections

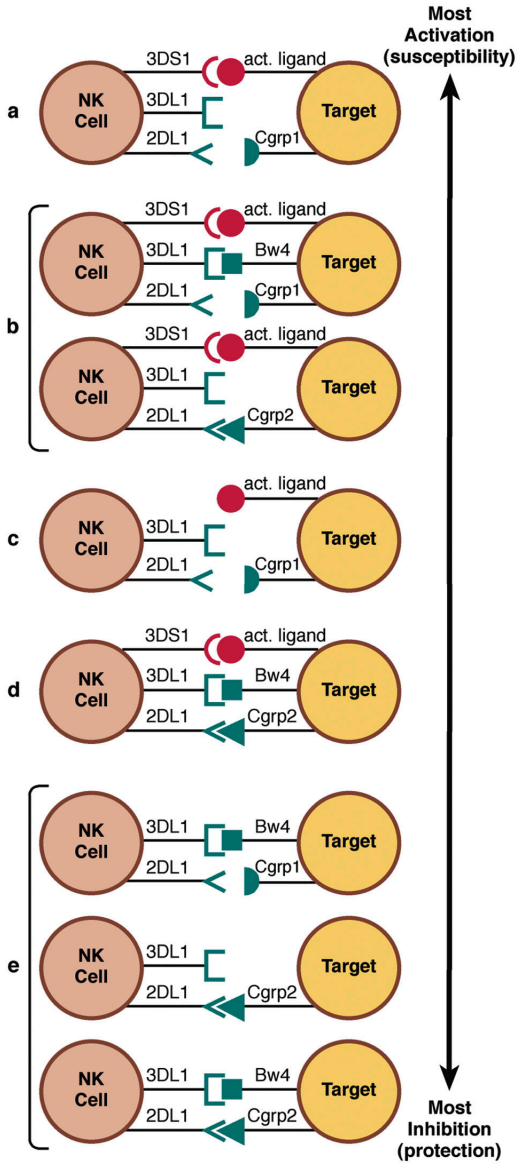

Figure 4. Model of the spectrum of KIR-mediated NK phenotypes associated with risk of developing cervical neoplasia. Resistance to cervical neoplasia increases when genotypes are ordered by their ability to confer the most activation (susceptibility) to the most inhibition (protection). (a) Presence of KIR3DS1 and absence of ligand for inhibitory KIR results in strong activation (most susceptibility). (b) Presence of KIR3DS1 and presence of one or the other ligand for the inhibitory KIR results in weak activation. (c) Absence of the activating KIR3DS1, and absence of ligands for inhibitory KIR result in relative neutrality. (d) Presence of KIR3DS1 along with ligands for one or both inhibitory KIR confers weak inhibition. (e) Presence of ligands for inhibitory KIR in the absence of KIR3DS1 results in strong inhibition (most protection). Note that expression of KIR3DS1 and a potential ligand for this receptor have not been defined, and that its hypothetical expression and binding to a ligand are proposed in this model based solely on the data presented herein. Red shapes, activating receptor and ligand; Green shapes, inhibitory receptors and ligands.

cause no or only mild cytologic abnormalities that subsequently regress to normalcy. Cervical inflammation does not seem to correlate significantly with HPV infection but has been associated with high-grade lesions in oncogenic HPV-infected women (23). Thus, although acquired immune responses to HPV may deter development of high-grade lesions and cancer, inappropriate localized hyperresponsiveness, mediated in part by KIR–ligand interactions, may actually increase the risk of disease progression. The findings described here indicate that further studies of the inflammatory process in the progression to cervical neoplasia and of the role of NK cells (and CTLs) in exacerbating this process are justified.

MATERIALS AND METHODS

Study population. Informed consent was obtained from all participants in accordance with U.S. Department of Health and Human Services guidelines. The study protocol was approved by the Institutional Review Board of the National Institutes of Health and local review boards. Participants were selected from three cervix studies sponsored by the National Cancer Institute and for whom specimens were available for the current investigation: a 24,000-woman cohort in Portland, Oregon (24); a 10,077-woman population-based cohort in Guanacaste, Costa Rica (25); and a 570 woman multicenter study of histologic subtypes of cervical neoplasia in the Eastern U.S. (26). In brief, all cervical cancer and CIN3 cases were included from the three studies; all controls from the U.S.-based case control study were included, and a selected group of population-based controls were included from the two cohort studies as previously described (24, 27, 28) for a nested-case control design. Although *HLA* class I typing was completed in a subset of all three studies, samples were available for *KIR* typing only in the latter two studies. Our final analytic group for *HLA* analysis included 257 women with cancer (squamous cell carcinoma) or CIN3 and 675 normal controls. Because *KIR* typing was not conducted in the Portland cohort (because of lack of DNA), analysis of combinatorial *KIR/HLA* effects included 196 CIN3/cancer cases and 330 controls.

HPV testing. Cervicovaginal samples were tested by PCR for HPV DNA, as described elsewhere (24–26). In the Portland cohort, HPV was typed with MY09/11 consensus primers using dot blot. In the Eastern U.S. case-control study, HPV was also typed with MY09/11 consensus primers, but by strip technology. In the Costa Rica cohort, HPV typing was done by both PCR (with MY09/11) and the hybrid-capture tube test.

HLA genotyping. HLA class I genotypes had been determined previously for all three cohorts (28). Briefly, protocols involving sequence-specific oligonucleotide probe hybridization developed by the 13th International Histocompatibility Workshop (www.ihwg.org/protocols/protocol.htm) were employed.

***KIR* genotyping.** DNA was available for *KIR* typing in participants of the Costa Rica cohort and the study of cervical neoplasia in the Eastern U.S. (see Study populations). Genomic DNA was genotyped for presence or absence of the following genes: *2DL1*, *2DL2*, *2DL3*, *2DL4*, *2DL5*, *2DS1*, *2DS2*, *2DS3*, *2DS4*, *2DS5*, *3DL1*, *3DL2*, *3DL3*, *3DS1*, and *2DP1*. Genotyping was performed using PCR amplification with two pairs of locus-specific primers (PCR-SSP) as previously described (19). Internal control primers that amplify a 796-bp fragment of the third intron of *DRB1* were also included in each PCR to confirm robust PCR amplifications. Amplification was performed in a volume of 5 μl containing 200 μM dNTP, 500 nM primer, 1.5 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, and 0.5 U of Platinum *Taq* DNA polymerase (Invitrogen). Cycling was performed as follows: 2 min at 94°C; 5 cycles of 94°C for 5 s, 65°C for 15 s, 72°C for 30 s; 21 cycles of 94°C for 5 s, 60°C for 15 s, 72°C for 30 s; 4

cycles of 94°C for 5 s, 55°C for 1 min, 72°C for 2 min; and a final extension step of 10 min at 72°C. PCR products were electrophoresed in 2% agarose gels containing ethidium bromide, and predicted size products were visualized under ultraviolet light.

Statistical methods. A case-control analysis was conducted in which patients with cancer or CIN3 were compared with population control subjects. *HLA-B Bw4*, *HLA-Cw* group 2, and *KIR* genotype frequencies were initially determined for cases and controls; ORs and 95% CIs were calculated to determine the magnitude and statistical significance of associations (29). In merged analyses, the analyses were adjusted by study to account for potential differences among the populations. Additional adjustment by HPV cofactors for CIN3/cancer endpoints (smoking, parity, oral contraceptive use) did not affect our risk estimates. Because these HPV cofactors were not associated with *KIR/HLA* genotypes, we present results from the more robust, parsimonious study-adjusted model. Statistical analyses were performed using SAS software (version 8.12; SAS Institute). All tests of statistical significance are 2-sided. Linkage disequilibrium between the group of *HLA-B Bw4* and the *Cw* group 2 alleles was tested by computing D′ (30).

Online supplemental material. Table S1 shows the raw data for Fig. 1. Table S2 shows independent associations between all KIR genes tested and CIN3/cancer. Table S3 shows the raw data for Fig. 2 with the middle genotypes considered separately (Table S3, A) and grouped together (Table S3, B). Table S4 shows the raw data for Fig. 3 with the middle genotypes considered separately (Table S4, A) and grouped together (Table S4, B). Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20042158/DC1.

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