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Activating KIR receptors 3DS1 and 2DS1 protect against developing the severe form of recurrent respiratory papillomatosis

Vincent R. Bonagura^{1,2,3}, Zeying Du⁵, Elham Ashouri⁵, Lihui Luo⁵, Lynda J. Hatam¹, James A. DeVoti¹, David W. Rosenthal^{1,2,3}, Bettie M. Steinberg^{1,3,4}, Allan L. Abramson^{1,4}, David W. Gjertson⁵, Elaine F. Reed⁵, and Raja Rajalingam⁵

¹Feinstein Institute for Medical Research, North Shore-Long Island Jewish Health System, Manhasset, NY 11030.

²Department of Pediatrics, Division of Allergy and Immunology, Schneider Children's Hospital, New Hyde Park, NY 11040.

³Elmezzi Graduate School of Molecular Medicine, North Shore-Long Island Jewish Health System, Manhasset, NY 11030.

⁴The Department of Otolaryngology and Communicative Disorders, Long Island Jewish Medical Center, New Hyde Park, NY 11040.

⁵UCLA Immunogenetics Center, Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, University of California at Los Angeles, Los Angeles, CA 90095.

Abstract

The polymorphic killer-cell immunoglobulin-like receptors (KIR) control natural killer (NK) cell response against viral infection and tumor transformation. Here we investigated if select *KIR* genes are associated with recurrent respiratory papillomatosis (RRP), a rare disease of the larynx and upper airway caused by human papilloma viruses (HPV)-6/11. DNA from 66 RRP patients and 195 healthy controls were characterized for *KIR* and *HLA* gene polymorphism. Patients lacking activating *KIR* genes *3DS1* and *2DS1* were more common in severe RRP compared to mild-moderate RRP (78.8% vs. 48.5%, $p=0.019$). Further, patients carrying any of the known susceptible *HLA-DRB1/DQB1* alleles were more frequently negative for *KIR3DS1* ($p=0.006$), *KIR2DS1* ($p=0.003$) or *KIR2DS5* ($p=0.004$) compared to controls carrying any of these HLA allotypes. Nearly 80% of the severe patients were missing the protective *HLA-DQB1*0602* allele as well as both *KIR3DS1* and *KIR2DS1* genes. Phenotyping of papilloma-infiltrating mononuclear-cells revealed an elevated numbers of NK cells and CD57⁺CD4⁺ T-cells in *KIR3DS1*⁻*KIR2DS1*⁻ patients compared to patients carrying either one or both of these *KIRs*. Our data suggest that NK cells expressing activating receptors *KIR3DS1* and *KIR2DS1* may be necessary to trigger an effective early immune response against HPV-infected targets to establish resistance to RRP development.

Address for correspondence and reprint requests to: Raja Rajalingam, Ph.D., UCLA Immunogenetics Center, Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, University of California at Los Angeles, 1000 Veteran Avenue, Room# 1-536, Los Angeles, CA 90095-1652, USA. Phone: 310-825-1467; Fax: 310-206-3216; rrajalingam@mednet.ucla.edu or Vincent R. Bonagura, M.D., Division of Allergy/Immunology, 865 Northern Boulevard, Suite 101, Great Neck, NY 11021. Phone: 516-622-5070; Fax: 516-622-5060; bonagura@lij.edu.

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Keywords

Killer cell immunoglobulin-like receptors; natural killer (NK) cells; recurrent respiratory papillomatosis; human papilloma viruses (HPV)-6 and 11; HLA association

Introduction

Recurrent respiratory papillomatosis (RRP) is a rare disease characterized by recurrent benign tumor formation within the larynx and upper airway, with an incidence in the United States of $4.3/10^5$ children and $1.8/10^5$ adults [1]. Although a benign disease, the outcome of RRP can be life threatening because of airway obstruction, distal airway spread, and malignant transformation. Patients with RRP often require repeated surgeries and close clinical surveillance. Almost all RRP lesions are caused by small non-enveloped, double-stranded DNA human papilloma viruses (HPV) types 6 and 11 that replicate within the nuclei of keratinocytes [2].

Impaired cell-mediated immunity is largely responsible for the development of RRP [3]. Overrepresentation of $CD8^+CD28^-$ T-cells [3], increased expression of T_H2 -like cytokines in papillomas and by peripheral blood mononuclear cells responding to autologous papilloma tissue [4], and aberrant $CD4^+$ T-cell responses to HPV-6 and -11 peptides [5], have been shown to contribute to the compromised cell-mediated immune responses made against HPV proteins in RRP patients. Select alleles of polymorphic human leukocyte antigen (HLA) class II genes (*DRB1*0102*, *DRB1*0301*, *DQB1*0201* or *DQB1*0202*) were also shown to be the critical host genetic factors responsible for HPV-specific hyporesponsiveness [5,6]. However, not everyone carrying these disease-susceptibility HLA alleles develops RRP following HPV infection, indicating that additional host susceptibility factors are important in the RRP development.

Discovery of HLA class I-specific killer-cell immunoglobulin-like receptors (KIR) led to a series of epidemiological studies that implicated *KIR* genes in the susceptibility or resistance to various human diseases [7]. KIRs are the key receptors of human natural killer (NK) cells [8], a subset of lymphocytes that trigger early innate immune responses against infection and tumors [9]. In addition to NK cells, $CD8^+$ cytotoxic T-cells with a memory phenotype also express KIRs, and therefore KIRs can further modulate antigen-specific adaptive immune responses [10].

Fourteen KIR genes encoding receptors with either inhibitory (3DL1-3, 2DL1-3, 2DL5) or activating functions (3DS1, 2DS1-5), or both inhibitory and activating functions (2DL4), and two non-functional pseudogenes (2DP1, 3DP1) have been identified [8,11,12]. Inhibitory KIR receptors recognize distinct HLA class I molecules and trigger signals that stop NK cell function. KIR2DL2 and 2DL3 bind HLA-C allotypes containing an Asparagine⁸⁰ (group C1 alleles), KIR2DL1 binds HLA-C allotypes with Lysine⁸⁰ (group C2 alleles), KIR3DL1 binds HLA-B allotypes containing the Bw4 epitope, and KIR3DL2 binds HLA-A3/11. The ligands for activating KIRs have yet to be discovered. Studies in mice predict that activating KIRs may recognize cellular targets expressed on the surface of infected-cells that trigger NK cytotoxicity [13,14]. Based on genetic epidemiological studies [15], *in vitro* activation [16], and weak binding of KIR-Fc proteins [17] or tetramers [18], three activating KIR receptors are thought to bind the same HLA class I ligands, as do their homologous inhibitory counterparts at low affinity. KIR3DS1 shares 97% sequence similarity with KIR3DL1 in the extracellular Ig-domains and is thought to bind the HLA-Bw4 ligand [15]. Similarly, KIR2DS1 (homologue of 2DL1) and 2DS2 (homologue of 2DL2) are thought to bind weakly to HLA-C2 and HLA-C1 respectively [17,18].

KIR genes are tightly clustered at chromosome 19q13.4 [19]. The number and type of *KIR* genes show considerable variability between haplotypes, resulting in substantial differences in *KIR* gene content between individuals [20]. Since *KIR* genes at chromosome 19q13.4 and *HLA* genes at chromosome 6p21.3 are polymorphic, and independent segregation of these unlinked gene families produce a wide diversity in the number and type of *KIR-HLA* gene combinations inherited in individuals [21]. To test the possibility that certain *KIR-HLA* gene combinations confer risk for developing RRP and disease severity, we characterized both *KIR* and *HLA* genes in 66 RRP patients and, compared with those we previously characterized in 195 healthy controls [21].

Materials and Methods

Study subjects and index of RRP disease severity

Sixty six randomly selected RRP patients of varying disease severity were studied from a large RRP population that receive care at the Long Island Jewish Medical Center which is a major national referral center for this disease. The study population is not of local origin and includes most patients from throughout the United States. All patients enrolled signed informed consent approved by the Institutional Review Board of North Shore-Long Island Jewish Health System. Fifty-two males and 14 females, a male:females ratio characteristic of RRP [22] were studied. Twenty-six patients had juvenile onset RRP and 40 had adult onset RRP. Fifty patients were Caucasians (75.7%), 11 were African Americans (16.7%) and 5 were Hispanics (7.6%). All patients had either HPV type 6 or 11 infection, documented by HPV typing [23]. The demographics and the disease severity scores of the RRP are listed in table 1.

Clinical severity was determined by extent of disease at time of surgery and frequency of recurrence. At each surgery, the number of disease sites, the anatomic surface area of disease, and extent of luminal obstruction was documented to yield a composite score as described [24]. This composite score is divided by the number of days elapsed since the previous surgery, yielding a growth rate that is a measure of disease severity. The mean growth rate from multiple surgeries defined the overall severity score for an individual patient. An overall disease severity score of ≥ 0.06 , or tracheal extension, was defined as severe. An overall severity score of < 0.06 , and no tracheal extension was defined as mild-moderate disease [24–26]. This scoring system was used throughout all of our clinical studies and was developed using a much larger number of patients ($n > 150$) [24]. Minimal variation in patients' scores was observed over time, and using the mean score further improved the reliability of this variable [27]. Thirty three patients had severe disease and 33 others had mild-moderate disease.

One hundred and ninety-five unrelated Caucasian blood donors from throughout the United States were included in this study. All these samples were typed for both *KIR* and *HLA* class I genes [21], 133 were typed for *HLA-DRB1* alleles [6], and 112 were typed for *HLA-DQB1* alleles [6].

Determination of *KIR-HLA* gene combinations

Genomic DNA samples from all RRP patients were PCR-typed for presence and absence of 16 *KIR* genes using the multiplex *KIR*-SSO typing kit (Tepnel Lifecodes, Stamford, CT). This kit consists of (i) two PCR reaction mixtures for the amplification of *KIR*-exons 4, 5, 7, 8 and 9; (ii) a mixture of color coded microspheres (Luminex Corporation, Austin, TX) each conjugated with locus-specific oligonucleotide probes. Each DNA sample was PCR amplified and the PCR product hybridized with the probe-labeled microspheres according to the manufacturer. The hybridized plate was read in a Luminex instrument and analysis was performed according to the manufacturer. A set of 10 DNA standards comprising most commonly occurring *KIR* genotypes from the UCLA *KIR* exchange reference program were

processed as standards along with patient samples, and the results were identical to the known typing results.

HLA-A, -B, and -C typing was performed by either sequence-specific primer-directed PCR amplification (PCR-SSP) or sequence-specific oligonucleotide (SSO) hybridization methods using commercial kits (One Lambda, Canoga Park, CA) as previously described [6,21]. The KIR-binding HLA class I epitopes were predicted from the *HLA* typing results. If the *HLA* typing results were ambiguous, we PCR-SSP typed the KIR-binding HLA motifs as described [21]. The allele-level typing of *HLA-DRB1* and *DQB1* loci for all patients and most control subjects were performed using sequencing-based typing method as described [6]. All *KIR3DS1*-positive patients and controls were further typed for the unexpressed null allele 3DS1*049N as described [28]. Only those carrying a functional allele were considered as positive for the *KIR3DS1* gene. The presence of *KIR-HLA* gene combination, encoding the following receptor-ligand pairs were analyzed in each individual: 2DL1+HLA-C2, 2DL2/3+HLA-C1, 3DL1+HLA-Bw4, 3DL2+HLA-A3/11, 2DS1+HLA-C2, 2DS2+HLA-C1, and 3DS1+Bw4.

Phenotyping of papilloma-infiltrating mononuclear cells

Papilloma biopsies were minced, filtered, and plated in tissue culture medium 1640, supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine (Gibco, Gand Island, NY), 10% bovine serum (Hyclone Laboratories, Inc., Logan UT), and 25 units/mL IL-2 (Boehringer Mannheim, Germany). This medium was replaced every 3–4 days and cultures were harvested at day 14 and immunophenotyped as previously described [3]. The yield from each papilloma ranged from 2×10^4 to 8.6×10^8 ($\bar{x} = 10.7 \times 10^6 \pm 2.06 \times 10^6$) mononuclear cells. The success rate in obtaining sufficient mononuclear cells for immunophenotyping from a given papilloma was 72%. At day 14, cell surface marker analysis was performed on mononuclear cells using combinations of FITC-, PE-, or PerCP-conjugated antibodies specific for CD4, CD8, CD14, CD19, CD28, CD45, CD56, CD57, TCR γ , TCR δ , TCR α , or TCR β (Becton-Dickinson Immunocytometry Systems, San Jose, CA). Lymphocyte subsets were enumerated by flow cytometry by FACS analysis as described [29].

Data analysis and statistical methods

Statistics were performed using Stata Statistical Software: Release 10.1 (Stata Corporation, College Station, TX). The percentage of each *KIR* gene was determined by direct counting (number of individuals positive for the gene divided by the number of individuals tested per group $\times 100\%$). Differences between study groups in the distribution of each *KIR* gene and genotype profile were assessed using Fisher's exact test (two-tailed), and $p < 0.05$ was considered to be statistically significant. Lymphocyte subsets (expressed as percentages) were compared by separate variance t-tests using a Welch correction (GraphPad Software: San Diego, CA). When phenotyping was performed on multiple samples per patient over time, one sample was randomly selected by algorithm (an atmospheric noise randomness program at www.random.org) for analysis.

Results

Patients lacking the activating *KIR* genes *3DS1* and *2DS1* developed severe RRP

To determine if select *KIR* genes were associated with the risk of developing RRP, we PCR typed 16 known *KIR* genes in 66 RRP patients (Figure 1). No difference was observed in the frequency of any *KIR* gene between RRP patients and a randomly collected population of 195 healthy Caucasian controls. However, when the patients were grouped on the basis of their disease severity, one of the activating receptors, *KIR2DS1* was significantly underrepresented in patients with the severe disease compared to patients with mild-moderate disease (21.2%

vs. 48.5%, $p=0.038$). *KIR3DS1* and *KIR2DS5*, the genes that are in strong linkage disequilibrium with *KIR2DS1* were also underrepresented in patients with the severe disease compared to patients with mild-moderate disease. However, these differences were not statistically significant.

The *KIR* gene content of a given individual is called the “*KIR* genotype”. Within the group of RRP patients, there were 26 distinct *KIR* genotypes observed that differed in gene content (Figure 1), of which 11 were found to be unique in patients. The majority of the *KIR* genotypes identified in healthy controls (21 of 36, 58.3%) were not detected in RRP patients. The overall difference in the distribution of *KIR* genotypes between controls and patients was not statistically significant. However, genotypes lacking activating *KIR* genes *3DS1* and *2DS1* (#1–16) were significantly overrepresented in RRP patients with severe disease (78.8% in severe RRP vs. 48.5% in mild-moderate RRP, $p=0.019$). Of these 16 genotypes, six (#4, 7–11) were observed only in severe patients and five (#12–16) were observed only in controls. Eleven of these 16 genotypes (#1–6, 8, 10, 12, 15 and 16) further lacked an additional activating gene, *KIR2DS5*. Consequently, 78.8% of patients with severe RRP disease were negative for both *KIR3DS1* and *KIR2DS1* genes while only 48.5% ($p=0.019$) of the patients with mild-moderate disease lacked both of these *KIR* loci (Figure 2). *KIR3DS1**049N, the null allele-specific typing revealed that all patients and controls typed here that were positive for *KIR3DS1* gene carried a functional *KIR3DS1* allele. To avoid a possible ethnic-specific deviation caused by the increased frequency of *KIR3DS1*[−]*KIR2DS1*[−] carriage in African Americans [21], we also performed a selective analysis within the Caucasian patients only, representing 74.2% of the total patient cohort. Within the Caucasian RRP patients ($n=50$), 75% of those with severe disease lacked both *KIR3DS1* and *KIR2DS1* genes, while only 44.8% ($p=0.045$) of the patients with mild-moderate disease lacked both of *KIR* genes (Figure 2). Of the 11 African American patients with RRP, all seven of the African American patients with severe RRP disease (100%) lacked both *KIR3DS1* and *KIR2DS1* genes, as did 2 of 3 patients with mild moderate RRP compared to 77.6% of the reported frequency in African American controls [21]. The one remaining African American patient with mild-moderate RRP carried only one of these activating *KIR* genes, *KIR2DS1*.

RRP patients expressing “disease-susceptible” *HLA-DRB1/DQB1* alleles lack activating *KIR* genes *3DS1*, *2DS1* and/or *2DS5*

To determine the status of *KIR* genes in patients carrying disease-susceptibility or disease-protective *HLA-DRB1/DQB1* alleles [5,6], we assessed the cognate combinations of *KIR* genes, *DRB1* alleles, and *DQB1* alleles, by a three way association test using a best log-linear model (Figure 3 and Figure 4). Patients carrying at least one of the four disease-susceptible *HLA* class II allele (*DRB1**0102, *DRB1**0301, *DQB1**0201 and *DQB1**0202) more frequently lacked either *KIR3DS1* ($p=0.006$), *KIR2DS1* ($p=0.003$) or *KIR2DS5* ($p=0.004$), compared to controls expressing any of these disease-susceptibility *HLA* alleles (Figure 3). Although differences were not statistically significant for Caucasians only, a similar trend was observed (data not shown). Of note, there was also a trend in patients who lacked expression of the disease-protective *HLA-DQB1**0602 allele and the absence of *KIR3DS1* and *KIR2DS1* genes in RRP patients who had severe disease, $p=0.06$ (Figure 4).

***KIR*-*HLA* class I gene combinations were comparable between patients and controls**

Since *KIRs* on chromosome 19q13.4 and *HLA* on chromosome 6p21.3 are substantially polymorphic, their independent segregation produces diverse *KIR*+*HLA* class I gene combinations among individuals [21]. To investigate whether certain combinations of *KIR*-*HLA* combinations confer risk for RRP, we identified the well-defined inhibitory *KIR*+*HLA* class I ligand gene combinations in each subject (Table 2). No difference was observed in the frequencies of *KIR2DL1*+*HLA*-C2, *KIR2DL2/3*+*HLA*-C1 and *KIR3DL1*+*HLA*-Bw4.

However, the *KIR3DL2+HLA-A3/11* combination was significantly reduced in RRP patients compared to the controls (21.2% vs. 35.4%, $p=0.023$). In contrast, all three predicted activating *KIR+HLA* class I ligand combinations (*KIR3DS1+HLA-Bw4*, *KIR2DS1+HLA-C2* and *KIR2DS2+HLA-C1*) were distributed equally between patients and controls (Table 2).

Papillomas from *KIR3DS1*⁻ *KIR2DS1*⁻ RRP patients contained more NK cells and CD57⁺CD4⁺ T cells

To determine if the activating *KIR* genes *3DS1* and *2DS1* affect the lymphocyte subpopulations in papillomas from patients with RRP, we retrospectively reviewed our immunophenotyping data obtained from patients we previously described [6]. Of interest, the number of CD3⁺CD56⁺ NK cells and the CD57⁺CD4⁺ T cell subsets were significantly ($p<0.05$) elevated in papillomas of RRP patients who lacked both *KIR3DS1* and *KIR2DS1* genes compared to patients who expressed one or both of these *KIR* genes (Figure 5). No other subpopulation of mononuclear cells in papillomas was significantly different in patients who did or did not carry these activating *KIR* genes (data not shown).

Discussion

Natural killer cells are an integral component of innate immunity and provide a crucial initial defense against pathological organisms during the time (0–5 days) when the adaptive immune system is processing antigen [30]. NK cell responses to virally-infected target cells are mediated by the interplay of polymorphic inhibitory receptors, activating receptors and their corresponding ligands [31]. Activating NK cell receptor LY49H in mice recognizes mouse cytomegalovirus (MCMV)-encoded determinants expressed on the surface of infected cells and trigger the NK activation cascade leading to infected cell cytolysis [13,14]. Although such direct receptor-ligand interactions have not been determined in humans, epidemiological studies revealed that the presence of activating receptor *KIR3DS1* slowed down the AIDS progression in HIV-1 infected patients, compared to patients lacking *KIR3DS1*, thus indicating a potential anti-HIV effect of *KIR3DS1* [15]. The *KIR3DS1* receptor was also shown to be weakly protective to hepatitis-C virus (HCV) infection [32]. Analogous to these findings, we report here that patients with HPV-6/11 infection who lacked *KIR3DS1* and *KIR2DS1* genes were prone to develop severe RRP, compared to the patients carrying one or both of these *KIR* genes, thus indicating the anti-HPV effect of these activating *KIR* receptors. Taken together, the *KIR* receptors *3DS1/2DS1* on NK cells presumably recognize select viral-encoded/-induced determinants expressed on the surface of infected target cells, and enhance NK cell response against viral infection (Figure 6). The activating *KIR* genes *3DS1*, *2DS1* and *2DS5* are linked together as a cluster in the telomeric half of the *KIR* gene complex [19]. Underrepresentation of this cluster in RRP patients also suggests the possibility that other unknown but linked factors may contribute resistance of developing RRP.

Of interest to RRP, the presence of the activating receptor *KIR3DS1* was shown to associate with cervical neoplasia progression to cervical cancer, especially in the absence of protective inhibitory *KIR3DL1* and *HLA-Bw4* ligand combination [7,33]. The difference in clinical disease expression induced by different HPVs in *KIR3DS1*⁺ patients could be due to other host and viral genetic factors, such as (i) differences in the HPV strains that causes cervical cancer (HPV-16 and 18) vs. RRP (HPV-6 and 11), and thus the interaction of *KIR3DS1* with putative HPV strain-specific ligands leading to either killing of HPV-6/11-infected cells keeping the host from developing RRP, or leading to inappropriate tissue-specific hyperresponsiveness promoting growth of cervical cancer, vs. benign respiratory papillomas, (ii) differences in the *HLA* alleles associated with cervical cancer (protection: *DQB1**03, *DRB1**1501/*DQB1**0602, *DRB1**13 and *DQB1**0603) [34] vs. RRP disease (susceptibility: *DRB1**0102, *DRB1**0301, *DQB1**0201 and *DQB1**0202, protection: *DQB1**0602) [5,6] with different *HLA* class II

restrictions diversifying the CD4⁺ T-cell immune response to HPV strain-specific antigens favoring malignant vs. benign HPV-induced disease development, and (iii) differences in other immunoregulatory, and malignancy-related genes in papillomas [35] that are important in directing the outcome of HPV infection that may alter CD4⁺ T-cell immune responses made to HPV strain-specific antigens.

In addition to KIR3DS1-mediated activation, decreased inhibition triggered by HLA-C and HLA-B binding inhibitory KIR receptors has been implicated in the development of cervical neoplasia [33] and HCV resolution [32]. Although no difference was observed in the distribution of the HLA-C and -B binding inhibitory KIR-ligand combinations, a decrease in HLA-A binding inhibitory receptor-ligand pair (KIR3DL2+HLA-A3/11) in RRP patients suggests a possible protective effect by this interaction against RRP development. Furthermore, the majority of the RRP patients with severe disease carried at least one of the known disease-susceptibility HLA class II alleles (*DRB1**0102, *DRB1**0301, *DQB1**0201 and/or *DQB1**0202) but lacked both *KIR3DS1* and *KIR2DS1* genes. These observations suggest that certain combination of *KIR* and *HLA* genes could provide a permissive immune microenvironment that fosters chronic HPV-6/11 infection and ultimately tumor development.

In addition to the gene content variation, each *KIR* gene exhibits considerable sequence diversity [36]. Eleven alleles of *KIR3DS1* and five alleles of *KIR2DS1* have been described to date (<http://www.ebi.ac.uk/ipd/kir/index.html>). The sequence polymorphisms that distinguish the alleles of *KIR3DS1* and *KIR2DS1* may influence expression, ligand binding, cytolytic, and cytokine secretion as described for other KIR receptors [37–39]. Furthermore, as the KIR receptors are clonally expressed on NK cells in a stochastic manner such that each NK cell clone expresses only a portion of the genes within the genotype [40], a substantial fraction of NK cells in RRP patients may not express KIR3DS1 and KIR2DS1 receptors even if patients carry the corresponding genes; thus NK cells from these patients may still ignore HPV-infected targets leading to RRP. Future studies of KIR gene polymorphism and NK clonal expression to investigate the possible dominance of unexpressed and underexpressed variants of *KIR3DS1/2DS1* in RRP patients who carry these activating KIR genes are needed.

It was surprising that there was an increased frequency of NK cells and CD57⁺CD4⁺ T-cells in papillomas from RRP patients who lacked both activating *KIR3DS1* and *KIR2DS1*, compared to patients carrying one or both of these genes. It is possible that the larger fraction of NK cell infiltration in *KIR2DS1*[−] *KIR2DS3*[−] papillomas represents a compensatory response to counteract the absence of these activating KIR genes by generating an effective NK response against HPV-infected keratinocytes using receptors other than KIR3DS1/2DS1.

CD57⁺CD4⁺ T cells have been reported to represent: 1) a chronically activated CD45⁺ memory subset of immunoregulatory cells that express IL-4 as described in *Mycobacterium tuberculosis* [41], 2) senescent T-cells undergoing apoptosis in HIV infection [42], or 3) a subset of cells marking advanced chronic lymphocytic leukemia [43]. It is possible that the predominance of this T cell subset in *KIR2DS1*[−] *KIR2DS3*[−] papillomas represents immunoregulatory T-cells that express IL-4. This would be consistent with our previous studies that showed increased IL-4, but not IFN- γ expression in papillomas [3], and that severe disease was associated with a greater IL-4/IFN- γ expression ratio compared to mild-moderate disease [4]. Thus, it is possible that this CD57⁺CD4⁺ T-cell subpopulation is in part responsible for enhanced IL-4 expression in RRP, generated by chronic activation induced by HPV-6/11 infection, supporting the development of a T_H2-like, regulatory microenvironment that is characteristic of RRP [3,4,6]. Alternatively, recent studies suggest that these cells can express granzyme and perforin, and thus, may function as cytolytic T-cells [44]. If this is the case, then they may also represent a compensatory immune mechanism to counteract defective, HPV-specific, NK cytotoxicity in RRP patients who lack *KIR3DS1* and *KIR2DS1* genes. Further

investigation of the functional of these interesting subpopulations of T- and NK cells is ongoing in our laboratory.

Of interest was the finding that 10 of the 11 African American patients with RRP lacked both *KIR3DS1* and *KIR2DS1*, and the 11th lacked the *KIR3DS1* allele, but had *KIR2DS1*. Of note, the 11th patient also had mild-moderate disease. While the absence of these activating *KIR* genes is more common in African Americans [21,45–47], the fact that all of our RRP patients showed complete or partial absence of these *KIR* genes is intriguing. It is known that RRP is more common in African Americans and in juvenile onset disease [48]. It is thought that the increased frequency of RRP in African Americans is associated lower socioeconomic conditions with increased birth canal HPV colonization [49,50]. However, while the number of African American patients in our study is small, it is possible that another risk factor of developing RRP in these individuals may be related to their higher incidence of the absence of activating *KIR* gene carriage that further predisposes HPV exposed African American infants to develop RRP.

In summary, NK cells expressing activating *KIR* receptors 3DS1 and 2DS1 are likely to be critical in removing HPV-infected keratinocytes. Thus individuals carrying and expressing these genes may better mount an effective early NK cell response against HPV-6/11, thereby clearing HPV early after infection (Figure 6), or suppressing latent virus activation. In contrast, NK cells that do not express *KIR3DS1/2DS1* may not recognize HPV-infected keratinocytes efficiently, leading to a failure to contain HPVs. If these *KIR3DS1*[−]*KIR2DS1*[−] individuals carry one of the susceptible *HLA-DRB1/DQB1* alleles, the skewed T_H2 response may further prevent recognition of HPV-infected keratinocytes, thus enhancing the development of clinical disease. The presence of the protective *HLA-DQB1**0602 gene likely further protects individuals who fail to carry or express *KIR3DS1* and *KIR2DS1*. Finally, our findings may provide a rationale to develop new and better treatments that target cells involved in innate as well as in adaptive immune responses in patients with severe RRP.

Abbreviations

RRP	Recurrent respiratory papillomatosis,
HPV	Human papilloma viruses,
HLA	Human leukocyte antigen,
KIR	Killer-cell immunoglobulin-like receptors,
NK cell	Natural killer cell,
MCMV	Mouse cytomegalovirus,
HCV	Hepatitis-C virus,
SSP	Sequence-specific primer-directed amplification,
SSO	Sequence-specific oligonucleotide hybridization.

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Genotype #	KIR genes																Genotype Frequency									
																	Controls (n=195)		RRP Mild-Moderate (n=33)		RRP Severe (n=33)					
	2DL1	2DL2	2DL3	2DL4	2DL5	3DL1	3DL2	3DL3	3DS1	2DS1	2DS2	2DS3	2DS4	2DS5	2DP1	3DP1	%F (N)	%F (N)	%F (N)							
1																	30.8 (60)	27.3 (9)	30.3 (10)							
2																	5.1 (10)	6.1 (2)	12.1 (4)							
3																	11.8 (23)	15.2 (5)	9.1 (3)							
4																	0 (0)	0 (0)	6.1 (2)							
5																	3.1 (6)	0 (0)	3.0 (1)							
6																	1.0 (2)	0 (0)	3.0 (1)							
7																	0 (0)	0 (0)	3.0 (1)							
8																	0 (0)	0 (0)	3.0 (1)							
9																	0 (0)	0 (0)	3.0 (1)							
10																	0 (0)	0 (0)	3.0 (1)							
11																	0 (0)	0 (0)	3.0 (1)							
12																	1.0 (2)	0 (0)	0 (0)							
13																	1.0 (2)	0 (0)	0 (0)							
14																	1.0 (2)	0 (0)	0 (0)							
15																	0.5 (1)	0 (0)	0 (0)							
16																	0.5 (1)	0 (0)	0 (0)							
17																	2.6 (5)	3.0 (1)	0 (0)							
18																	0.5 (1)	0 (0)	0 (0)							
19																	0.5 (1)	0 (0)	0 (0)							
20																	0.5 (1)	0 (0)	0 (0)							
21																	0.5 (1)	0 (0)	0 (0)							
22																	0 (0)	3.0 (1)	0 (0)							
23																	2.1 (4)	0 (0)	0 (0)							
24																	1.5 (3)	0 (0)	0 (0)							
25																	1.0 (2)	0 (0)	0 (0)							
26																	1.0 (2)	3.0 (1)	0 (0)							
27																	1.0 (2)	0 (0)	0 (0)							
28																	9.7 (19)	6.1 (2)	6.1 (2)							
29																	4.6 (9)	9.1 (3)	3.0 (1)							
30																	4.1 (8)	12.1 (4)	0 (0)							
31																	3.1 (6)	0 (0)	0 (0)							
32																	2.6 (5)	0 (0)	0 (0)							
33																	1.5 (3)	0 (0)	0 (0)							
34																	1.0 (2)	0 (0)	0 (0)							
35																	1.0 (2)	3.0 (1)	0 (0)							
36																	1.0 (2)	3.0 (1)	0 (0)							
37																	1.0 (2)	0 (0)	0 (0)							
38																	0.5 (1)	3.0 (1)	0 (0)							
39																	0.5 (1)	0 (0)	0 (0)							
40																	0.5 (1)	0 (0)	0 (0)							
41																	0.5 (1)	0 (0)	0 (0)							
42																	0.5 (1)	3.0 (1)	0 (0)							
43																	0.5 (1)	0 (0)	3.0 (1)							
44																	0 (0)	3.0 (1)	0 (0)							
45																	0 (0)	0 (0)	3.0 (1)							
46																	0 (0)	0 (0)	3.0 (1)							
47																	0 (0)	0 (0)	3.0 (1)							
																	RRP Severe (n=33)			Gene Frequency (%F)						
																	RRP Mild-Moderate (n=33)									
																	Controls (n=195)									
																	97.0	54.6	84.9	100	48.5	97.0	100			
																	97.0	57.6	97.0	100	54.6	90.9	100	45.5	48.5	57.6
																	96.9	49.2	88.7	100	52.8	94.9	100	100	39.5	37.4
																									49.7	28.2
																									95.4	35.9
																									98.0	
																									100	

(p=0.038)

Figure 1. Genotypes missing both *KIR3DS1* and *KIR2DS1* are prevalent in RRP patients with severe disease

Twenty-six distinct genotypes differed from each other by the presence (shaded box) and absence (white box) of 16 *KIR* genes in RRP patients compared to those found in healthy controls. Percent population of each genotype was defined as the number of individuals (N) having the genotype divided by the number of individuals studied (n) in the given study group. The genotype of most patients with severe RRP lacked both *KIR3DS1* and *KIR2DS1* genes (genotype#1–16). *KIR2DS1* occurred at a significantly lower frequency in severe RRP patients compared to those with mild-moderate disease ($p=0.038$).

Figure 2. Patients with severe RRP lack both *KIR3DS1* and *KIR2DS1* genes

Individuals carrying either *KIR3DS1*, *KIR2DS1*, or both genes, and individuals lacking both *KIR3DS1* and *KIR2DS1*, were equally distributed in healthy controls and in mild-moderate patients. In contrast, the majority of the patients with severe RRP lacked both *KIR3DS1* and *KIR2DS1* genes compared to patients who carried one or both of these genes. NS = Not significant.

■ Controls (n=133); ▨ Mild-Moderate RRP (n=33); ■ Severe RRP (n=33)

Figure 3. RRP patients expressing disease-susceptible HLA-DRB1/DQB1 alleles frequently lack activating KIR genes 3DS1, 2DS1 and/or 2DS5

Severe RRP patients carrying at least one of the four disease-susceptible HLA class II allele (DRB1*0102, DRB1*0301, DQB1*0201 and DQB1*0202) more frequently lacked either KIR3DS1, KIR2DS1 or KIR2DS5, compared to controls expressing any of these disease-susceptibility HLA alleles.

Figure 4. Patients with severe RRP lack protective *HLA* and *KIR* genes

Most patients with severe RRP were lacked the protective genes of *HLA* (*DQB1*0602*) and *KIR* (*3DS1* and *2DS1*) loci. NS = Not significant.

Figure 5. NK cells and CD57⁺CD4⁺ T-cells are enriched in *KIR3DS1*⁻/*KIR2DS1*⁻ papillomas
 The percent of CD3⁺CD56⁺ NK cells (panel A) and CD57⁺CD4⁺ T-cells (panel B) in infiltrating mononuclear cells found in papillomas from patients with RRP who carry (n=12), or lack (n=24) *KIR3DS1-KIR2DS1* genes. Data are depicted by box plots showing the median and 75th and 25th percentiles. Whiskers represent the 90th and 10th percentiles, and outliers outside the 95th and 5th percentiles are shown (where applicable). Means are represented by the dotted lines and medians by solid lines.

Figure 6. A model depicting how the compound *KIR-HLA* genotypes may confer the risk of developing RRP

NK cells expressing activating KIR receptors 3DS1 and 2DS1 are presumably well equipped to recognize and kill HPV-infected keratinocytes, and mount an effective early NK cell response. In contrast, NK cells that do not express KIR3DS1/2DS1 receptors may not have the ability of sensing HPV-infected targets cells, and thus, the infection may spread to neighboring cells. If *KIR3DS1*[−]*KIR2DS1*[−] individuals carry one of the susceptible *HLA-DRB1/DQB1* alleles, the skewed T_H2 response may further block eradication of infection and support the development of clinical disease. The expression of the protective *HLA-DQB1**0602 type may help protect in individuals who lack *KIR3DS1/2DS1* genes. HPV-p – Human papilloma viral protein, APC – Antigen presenting cell.

Table 1

Demographics and the disease severity scores of patients with RRP.

Patients ¹		Number	Juvenile Onset/ Adult Onset
Caucasian [#]	All	55	18/36
	Severe	16	4/12
	Mild Moderate	30	8/21
	Tracheal Involvement [*]	9	6/3
Black	All	11	8/3
	Severe	6	4/2
	Mild Moderate	3	2/1
	Tracheal Involvement [*]	2	2/0

* Patients with tracheal involvement are by definition, a subset of the severe group.

[#] Five Hispanics are included in the Caucasian group.

Table 2

Frequency of KIR-HLA combinations in RRP patients and controls.

KIR-HLA combination	RRP patients (n=66)		Controls (n=195)		p-value
	%	N+	%	N+	
<u>Inhibitory KIR-HLA class I ligand</u>					
KIR2DL1+HLA-C2	66.7	(44)	55.9	(109)	0.321
KIR2DL2/3+HLA-C1	81.8	(54)	80.0	(156)	0.858
KIR3DL1+HLA-Bw4	60.6	(40)	55.4	(108)	0.778
KIR3DL2+HLA-A3/11	21.2	(14)	35.4	(69)	0.023
<u>Activating KIR-HLA class I ligand</u>					
KIR3DS1+HLA-Bw4	24.2	(16)	21.5	(42)	0.866
KIR2DS1+HLA-C2	28.8	(19)	22.5	(44)	0.403
KIR2DS2+HLA-C1	57.6	(38)	49.7	(97)	0.579