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Evidence for Natural Killer Cell–Mediated Protection from Metastasis Formation in Uveal Melanoma Patients


PURPOSE. In uveal melanoma, low human leukocyte antigen (HLA) class I expression on primary tumors is associated with a decreased risk of metastasis. Consequently, it has been suggested that natural killer (NK) cells, which detect decreased expression of HLA class I, are involved in the immune control of metastases. In this study, three novel lines of evidence were identified that support a role for NK cells.

METHODS. Uveal melanoma cell lines were used to determine the expression of NK cell receptor ligands (MICA, MICB, ULBP1–3, CD112, CD155, and HLA class I) and to examine sensitivity to lysis by human NK cell lines. Because interactions between polymorphic killer immunoglobulin receptors (KIRs) and HLA regulate NK cell function, KIR and HLA genotyping was performed on 154 patients with uveal melanoma and 222 healthy control subjects.

RESULTS. First, all 11 uveal melanoma cell lines tested expressed ligands for activating as well as inhibitory NK cell receptors. Second, such cell lines were lysed efficiently by human NK cells in vitro. Finally, the HLA-C genotype was related to the risk of metastasis-related death in patients with uveal melanoma: The patients carrying HLA-C alleles encoding ligands for KIR2DL1 and KIR2DL2/3 (HLA-C group 1/group 2 heterozygous patients), both inhibitory NK receptors, had a longer metastasis-free survival than did those carrying HLA-C ligands for either KIR2DL1 (HLA-C group 2 homozygotes) or KIR2DL2/3 (HLA-C group 1 homozygotes).

CONCLUSIONS. Together, the data support a role for NK cells in the prevention of uveal melanoma metastases. (Invest Ophthalmol Vis Sci. 2009;50:2888–2895) DOI:10.1167/iovs.08-2733

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Uveal melanoma is a rare (incidence, 5–7 cases per million per year in Caucasoids) tumor originating from melanocytes in the eye and usually strikes people who are in their sixties. Metastases develop in approximately one in every two patients, mostly through hematogenic spread to the liver. Since there is hardly any effective treatment for such metastases, they constitute the primary cause of the high mortality rate associated with this malignancy.

Several studies indicate a role for HLA class I in the occurrence of uveal melanoma. Genetic association studies have indicated that several HLA-A and -B alleles confer either susceptibility to or protection from uveal melanoma or its metastases. Loss of heterozygosity at the HLA class I locus is a frequent event in uveal melanoma and is found in approximately 50% of primary tumors. Lack of HLA-A or -B expression on the cell surface of uveal melanoma cells is associated with longer patient survival. In line with this observation, the responses of mouse NK cells to human uveal melanoma cell lines in vivo and in vitro are inversely correlated with the HLA class I expression levels on the tumor cells. 11,12 But differences between human and mouse NK cell receptor systems make it difficult to extrapolate these findings to humans. Since natural killer (NK) cells are able to detect the loss of HLA class I from the surface of a target cell, these findings have led to the hypothesis that NK cells can remove from the circulation micrometastases that have downregulated HLA class I.

NK cells are lymphocytes of the innate immune system that are able to lyse target cells without the need for prior sensitization. NK cell activation is controlled by the integration of signals emanating from multiple inhibitory and activating receptors. To recognize cells that have lost expression of one or more HLA class I loci, NK cells carry inhibitory receptors specific for HLA class I allotypes, termed inhibitory killer immunoglobulin receptors (KIRs, reviewed in Ref. 15). Such inhibitory receptors specifically recognize subsets of HLA-A, -B, and -C allotypes. The inhibitory KIRs with 3 IgSF (KIR3DL) domains interact with HLA-A or -B alleles. KIR3DL2 binds HLA-A3 and -A11 in a peptide-specific manner, 16 and KIR3DL1 binds HLA-B molecules carrying the Bw4 motif. The inhibitory KIRs with 2 extracellular immunoglobulin superfamly (IgSF) domains (KIR2DL) interact with HLA-C allotypes. KIR2DL1 interacts with HLA-C molecules characterized by a lysine at position 80 of the α chain (group 2 HLA-C: C2), whereas KIR2DL2 and KIR2DL3 interact with HLA-C molecules characterized by an asparagine at this position (group 1 HLA-C: C1). Because all individuals possess HLA-C ligands for inhibitory KIR, whereas individuals with HLA-A or -B ligands are less frequent, HLA-C is considered the main ligand for inhibitory KIRs.

NK cell cytotoxicity and IFNγ production are triggered by activation of NK receptors. These tend to recognize ligands induced by cellular stress, expressed on many tumor cell types. For example, all NK cells express the activating receptor NKG2D, which binds the stress-induced non-classical HLA molecules MICA, MICB, ULBP1 to -4, and RAET1G. Expression of
MICA, MICB, and the ULBPs has been detected in skin melanoma and other tumor cell lines but also in normal tissues. Other activating receptors include DNAM-1, which binds CD112 and -155, also expressed on tumor cell lines. However, for many activating NK receptors, the ligands are not known. Such orphan activating receptors include the natural cytotoxicity receptors and also most activating KIR.

The KIRs are the most polymorphic human NK receptors by far. The KIR locus is polymorphic with respect to both gene content and sequence. KIR haplotypes contain between 5 and 12 highly homologous KIR genes, and up to 24 alleles for individual KIR genes have been described (see http://www.ebi.ac.uk/ipd/kir/ provided in the public domain by the European Bioinformatics Institute, Cambridge, UK). In fact, the degree of polymorphism of the KIR locus is paralleled only by the HLA locus and, similarly, many studies have shown that particular KIR genotypes are associated with susceptibility to infection and autoimmune disease. Since KIRs are expressed predominantly by NK cells, these studies are indicative of NK cell involvement in infection and autoimmunity.

Little information is available on associations between KIR/HLA compound genotype and cancer. In cervical carcinoma, which is strongly associated with HPV infections, inhibitory KIRs protect in the presence of their HLA-encoded ligand, whereas the presence of the activating KIR3DS1 increases disease risk. It is unclear whether these effects are due primarily to antiviral or antitumor immune responses. In melanoma of the skin, which is not associated with viral infections, there does not appear to be a direct association between the KIR genotype and the presence of malignant melanoma, or melanoma progression.

With regard to uveal melanoma, the association between the presence of HLA class I on the primary tumor and increased metastasis risk, as well as the hematogenous spread of the tumor both suggest a role for NK cells in tumor surveillance. In this study, we determined for the first time the presence of ligands for NK receptors on uveal melanoma cell lines and their susceptibility to lysis by human NK cells. Also for the first time, we compared the KIR and HLA genotypes of patients with uveal melanoma in whom metastases developed with the genotypes of those who did not have metastases, in a large cohort of patients with uveal melanoma with extensive follow-up. Together, the data support a role for NK cells in the prevention of uveal melanoma metastases.

**Materials and Methods**

**Cell Lines**

Uveal melanoma cell lines 92.1; MEL-202, -270, -285, and -290; OMM-2, -23, -2, and -1; and OCM-1, -5 and -were analyzed. Cell lines MEL-270, OMM-2, and OMM-2.5 were derived from a primary melanoma and two of its liver metastases, respectively, from a single individual. The cell lines were cultured in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 8% fetal calf serum (FCS). NK cells were enriched from peripheral blood mononuclear cell (PBMC) preparations from blood bank donors by using negative selection (NK cell isolation kit II; Miltenyi Biotec, Utrecht, The Netherlands) and subsequently expanded in AIM-V medium (Gibco/Invitrogen, Breda, The Netherlands) supplemented with 10% human serum and 72°C for 30 seconds, annealing during the first five cycles, consisting of denaturation at 94°C for 1 minute, annealing at 60°C 30 seconds, and extension

**Cell Cytometry Staining**

Melanoma cells were harvested by trypsinization and resuspended in cell cytometry buffer containing PBS, 0.5% FCS, and 0.02% azide. The cells were incubated with unconjugated mouse IgG antibodies to ULBP-1, -2, and -3; MICA and -B, (all from R&D Systems, Abingdon, UK); CD155 (Coulter Immunotech, Mijdrecht, The Netherlands), HLA-B/C (B1.25.2), and -A/B/C/E (W6/32); unconjugated human IgM antibodies specific for HLA-C alleles (WK4C11, TRA2G9, Ref. 34–36), and PE-conjugated mouse IgG antibodies to CD54, CD58, MICA/B (BD Biosciences, San Jose, CA) and CD112 (Beckman Coulter Immunotech, Fullerton, CA); or the appropriate isotype control antibodies. WK4C11 binds Cw*01, *03, *08, *12, and *14 (present in 92-1; MEL-202; EOM-3; OMM-1; OCM-1, -3, and -8; and MEL-270 and -290), and TRA2G9 binds HLA-Cw*01, *03, and *14 (present on 92-1; OMM-1; and OCM-1, -3, and -8).

After two washing steps in buffer, mouse IgG antibodies were detected with PE-conjugated goat anti-mouse Fab(b)2 (BD Biosciences) and human IgM with PE-conjugated rabbit anti-human IgM (Dako, Glostrup Denmark). After another two washing steps, the cells were fixed in 0.5% paraformaldehyde and kept at 4°C until acquisition on a flow cytometry system (FACScalibur and CellQuest Pro software; BD Biosciences).

**NK Cell Activation Assays**

Cytotoxicity was determined in standard chromium release assays. Briefly, effector cells were incubated with 2500 51Cr-labeled target cells at multiple effector-target ratios for 4 hours. Spontaneous and maximum release of 51Cr was determined by incubating targets with medium or hydrochloric acid, respectively. Specific 51Cr release was calculated as follows: (measured release − spontaneous release)/(max release − spontaneous release) × 100%.

**Patients**

Between 1991 and 2006 and after informed consent, DNA was obtained from 154 Dutch patients with a diagnosis of primary uveal melanoma first seen at the Department of Ophthalmology at the Leiden University Medical Center in the period 1995 to 2005. Eighty-four patients were women and 70 were men. The mean age at diagnosis was 61 years (range: 27–85, SD 12). Of these patients 66 underwent enucleation, the other 88 were treated by ruthenium brachytherapy, often in combination with transcutaneous thermotherapy. The control group consisted of 222 unrelated and randomly selected Dutch individuals. The research adhered to the tenets of the Declaration of Helsinki and was approved by the LUMC Medical Ethics Committee.

**KIR and HLA-C Genotyping**

KIR genotyping was performed as described. The KIR2DS4-specific PCR was performed in a volume of 10 μL, containing 10 ng of genomic DNA, 3 picomoles of each specific primer, 200 μM dNTPs (Promega Benelux, Leiden, The Netherlands), 5% glycerol (Invitrogen-Gibco, Carlsbad, CA) containing cresol red, and 0.5 unit of Taq DNA polymerase (Promega). 1× amplification buffer (Promega), 1× bovine serum albumin (New England Biolabs, Ipswich, MA), and 1.5 mM MgCl2 (Promega). An internal control, specific for a human growth hormone gene fragment (485 bp), was included at 1 picomole of each primer to check for genomic DNA quality. PCR was performed (Peltier Thermal Cycler; PTC-200; MJ Research, Waltham, MA). After an initial denaturation step at 95°C for 2 minutes, touchdown PCR was used to increase specificity of primer annealing during the first five cycles, consisting of denaturation at 94°C for 30 seconds, annealing at 65°C–66°C for 30 seconds, and extension at 72°C for 2 seconds, followed by 30 cycles with an annealing step at 60°C 30 seconds. Finally, a 2-minute extension step was performed at 72°C. For visualization, all the amplification products were run on a 1.5% agarose gel (Boehringer Mannheim, Mannheim, Germany) prestained with ethidium bromide.

NK Cell Protection against Uveal Melanoma Metastasis

**NK Cell Protection against Uveal Melanoma Metastasis**

NK cells were enriched from peripheral blood mononuclear cell (PBMC) preparations from blood bank donors by using negative selection (NK cell isolation kit II; Miltenyi Biotec, Utrecht, The Netherlands) and subsequently expanded in AIM-V medium (Gibco/Invitrogen, Breda, The Netherlands) supplemented with 10% human serum and 10 ng/mL II-15 (Bender Medical Systems, Vienna, Austria). After at least 2 weeks, these cultures contained >98% NK cells.

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Statistical Analyses

The χ² and Fisher exact tests were used to compare frequencies. Regression analysis was used to estimate genotypic odds ratios (ORs) and the corresponding 95% confidence intervals (95% CIs). The frequencies of KIR genes were derived from counting the genotypes in the patients and control subjects, and each individual was coded as being a carrier or not a carrier for the KIR gene in question. The effect

![Figure 1](image-url). Uveal melanoma cell lines express multiple ligands for activating and inhibitory NK receptors. MEL-270 and cell lines derived from two of its liver metastases (OMM2.3 and -2.5) were analyzed by flow cytometry for surface expression of ligands (left) and for activating receptors and adhesion molecules expressed by NK cells (right). Histograms compare staining with specific antibody (thick line) with the appropriate isotype control antibody (thin line). The data for these and eight additional uveal melanoma cell lines are summarized in Table 1.
of each KIR was assessed by comparing the gene frequencies in the patients with those in the control subjects. In the patients, mortality data were analyzed as prospective cohort and the person-years follow-up was calculated for each individual and then for each of the genetic variants. Mortality analysis was performed by calculating incidence rates of death per 100 person-years of follow-up for the KIR, HLA-B, or HLA-C genotypes. The exact two-sided P value was calculated for each KIR gene and for HLA-B and HLA-C genotypes. Point estimates and 95% CI for the incidence rate ratio of mortality were calculated for each KIR gene.

RESULTS

Multiple NK Receptor Ligands in Uveal Melanoma Cell Lines

As a first step toward elucidating the susceptibility of uveal melanoma cells to human NK cells, the expression levels of known ligands for activating NK receptors on eleven uveal melanoma cell lines were determined by flow cytometry (Fig. 1, Table 1). One cell line (MEL-270) did not express detectable levels of ULBP1 to 3, MICA, or MICB, but the other ten cell lines expressed at least one ligand for the activating receptor NKG2D. All cell lines also expressed DNAM-1 ligands, with CD155 generally being more abundant than CD112. In addition, all cell lines expressed the adhesion molecules CD54 and/or -58. Thus, these uveal melanoma cell lines expressed ligands for activating receptors and for adhesion molecules expressed by NK cells.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Uveal melanoma cell lines are sensitive to NK cell lysis in vitro. 51Cr labeled uveal melanoma cells were incubated 4 hours with purified allogeneic NK cells that had been expanded with IL-15. The data are representative of at least three experiments on NK cells from six randomly selected donors.

### Table 1. Surface Expression of Ligands for NK Receptors on Uveal Melanoma Cell Lines

<table>
<thead>
<tr>
<th>HLA class</th>
<th>MoAb</th>
<th>MEL-270</th>
<th>OMM2.3</th>
<th>OMM2.5</th>
<th>MEL-290</th>
<th>92.1</th>
<th>OMM-1</th>
<th>MEL-202</th>
<th>OCM-1</th>
<th>OCM-8</th>
<th>OCM-3</th>
<th>MEL-285</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-C</td>
<td>WK4C11</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HLA-C</td>
<td>TRA2G9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HLA-A/B/C</td>
<td>B1.23.2</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>HLA-A/B/C</td>
<td>W6/32</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
</tbody>
</table>

### Table 2. KIR and HLA Typing of Patients and Control Subjects

<table>
<thead>
<tr>
<th>KIR</th>
<th>Patients (n = 154)</th>
<th>Control (n = 222)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitory KIR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2DL1</td>
<td>98</td>
<td>98</td>
<td>0.900</td>
</tr>
<tr>
<td>2DL2</td>
<td>50</td>
<td>49</td>
<td>0.860</td>
</tr>
<tr>
<td>2DL3</td>
<td>88</td>
<td>93</td>
<td>0.130</td>
</tr>
<tr>
<td>2DL5</td>
<td>43</td>
<td>48</td>
<td>0.510</td>
</tr>
<tr>
<td>3DL1</td>
<td>95</td>
<td>96</td>
<td>0.600</td>
</tr>
<tr>
<td>Activating KIR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2DS1</td>
<td>33</td>
<td>36</td>
<td>0.560</td>
</tr>
<tr>
<td>2DS2</td>
<td>50</td>
<td>49</td>
<td>0.860</td>
</tr>
<tr>
<td>2DS3</td>
<td>40</td>
<td>44</td>
<td>0.450</td>
</tr>
<tr>
<td>2DS4*001</td>
<td>40</td>
<td>44</td>
<td>0.450</td>
</tr>
<tr>
<td>2DS5</td>
<td>24</td>
<td>28</td>
<td>0.390</td>
</tr>
<tr>
<td>3DS1</td>
<td>32</td>
<td>34</td>
<td>0.690</td>
</tr>
<tr>
<td>HLA-C</td>
<td></td>
<td></td>
<td>0.760</td>
</tr>
<tr>
<td>C1/C1</td>
<td>39</td>
<td>43</td>
<td>0.760</td>
</tr>
<tr>
<td>C1/C2</td>
<td>44</td>
<td>42</td>
<td>0.760</td>
</tr>
<tr>
<td>C2/C2</td>
<td>16</td>
<td>15</td>
<td>0.760</td>
</tr>
<tr>
<td>HLA-B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bw4/Bw4</td>
<td>16</td>
<td>14</td>
<td>0.800</td>
</tr>
<tr>
<td>Bw4/Bw6</td>
<td>41</td>
<td>41</td>
<td>0.800</td>
</tr>
<tr>
<td>Bw6/Bw6</td>
<td>44</td>
<td>45</td>
<td>0.800</td>
</tr>
</tbody>
</table>

Data are expressed as percentage of the total group that were identified as carriers.
Loss of specific HLA class I alleles can be detected by inhibitory KIRs. Absence of expression of one or more HLA-A and -B alleles has been demonstrated in several uveal melanoma cell lines, but little is known about HLA-C. It has been suggested that HLA-C, which provides specific ligands for the inhibitory KIR2DL1, -2, and -3 receptors, is not expressed on uveal melanoma cells in situ. We made use of two HLA-C allotype–specific human monoclonal antibodies, both of which specifically stained the cell lines with the appropriate HLA genotype. For example, the WK4C11 antibody (specific for HLA-Cw*01, *03, *08, *12, *14) stained all cell lines except MEL-285, the only cell line whose genotype (HLA-Cw*06 homozygous) did not include an epitope for this antibody. Taken together, these experiments revealed that most, if not all, uveal melanoma cell lines express HLA-C and therefore provide ligands for inhibitory KIRs.

**Table 3. The Incidence of Mortality Due to Metastasis in Relation to the KIR and HLA-B/C Genotypes**

<table>
<thead>
<tr>
<th>Inhibitory KIR</th>
<th>Patients (n)</th>
<th>Incidence of Death Due to Metastasis (%)</th>
<th>Mean Follow-up (mo)</th>
<th>Incidence Rate of Death per 100 Person Months</th>
<th>Incidence Rate Ratio or Incidence Rate Difference † Estimate (95% CI) Exact Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>2DL1 Negative</td>
<td>3</td>
<td>0.0</td>
<td>40</td>
<td>0.00</td>
<td>Ref</td>
</tr>
<tr>
<td>2DL1 Positive</td>
<td>138</td>
<td>29.0</td>
<td>79</td>
<td>0.370</td>
<td>0.370 (0.007 to 0.014) 0.60</td>
</tr>
<tr>
<td>2DL1 Negative</td>
<td>70</td>
<td>28.6</td>
<td>75</td>
<td>0.380</td>
<td>Ref</td>
</tr>
<tr>
<td>2DL1 Positive</td>
<td>70</td>
<td>28.6</td>
<td>80</td>
<td>0.360</td>
<td>0.94 (0.51 to 1.75) 0.80</td>
</tr>
<tr>
<td>2DL3 Negative</td>
<td>17</td>
<td>23.5</td>
<td>76</td>
<td>0.310</td>
<td>Ref</td>
</tr>
<tr>
<td>2DL3 Positive</td>
<td>124</td>
<td>29.0</td>
<td>78</td>
<td>0.370</td>
<td>1.20 (0.43 to 3.37) 0.70</td>
</tr>
<tr>
<td>2DL5 Negative</td>
<td>79</td>
<td>32.9</td>
<td>73</td>
<td>0.450</td>
<td>Ref</td>
</tr>
<tr>
<td>2DL5 Positive</td>
<td>61</td>
<td>21.3</td>
<td>85</td>
<td>0.240</td>
<td>0.56 (0.29 to 1.07) 0.08</td>
</tr>
<tr>
<td>3DL1 Negative</td>
<td>8</td>
<td>0.0</td>
<td>105</td>
<td>0.00</td>
<td>Ref</td>
</tr>
<tr>
<td>3DL1 Positive</td>
<td>135</td>
<td>30.1</td>
<td>76</td>
<td>0.390</td>
<td>Ref</td>
</tr>
<tr>
<td>Activating KIR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2DS1 Negative</td>
<td>94</td>
<td>29.8</td>
<td>73</td>
<td>0.410</td>
<td>Ref</td>
</tr>
<tr>
<td>2DS1 Positive</td>
<td>47</td>
<td>25.5</td>
<td>89</td>
<td>0.290</td>
<td>0.70 (0.36 to 1.37) 0.31</td>
</tr>
<tr>
<td>2DS2 Negative</td>
<td>70</td>
<td>28.6</td>
<td>75</td>
<td>0.380</td>
<td>Ref</td>
</tr>
<tr>
<td>2DS2 Positive</td>
<td>71</td>
<td>28.2</td>
<td>81</td>
<td>0.340</td>
<td>0.92 (0.49 to 1.71) 0.79</td>
</tr>
<tr>
<td>2DS3 Negative</td>
<td>107</td>
<td>29.0</td>
<td>77</td>
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<td>2DS3 Positive</td>
<td>55</td>
<td>24.2</td>
<td>83</td>
<td>0.290</td>
<td>0.78 (0.36 to 1.70) 0.50</td>
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<td>0.72 (0.37 to 1.42) 0.35</td>
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<td>HLA-C C1/C1</td>
<td>55</td>
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<td>69</td>
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<tr>
<td>HLA-C C1/C2</td>
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<tr>
<td>HLA-C C2/C2</td>
<td>22</td>
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<td>65</td>
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<td>HLA-A Bw6/Bw6</td>
<td>65</td>
<td>21.0</td>
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<td>HLA-A Bw6/Bw4</td>
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<td>32.0</td>
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<td>0.389</td>
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<tr>
<td>HLA-A Bw4/Bw4</td>
<td>21</td>
<td>43.0</td>
<td>70</td>
<td>0.609</td>
<td>1.57 (0.71 to 3.47) 0.28</td>
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</table>

† Incidence rate difference is shown in italic type.

* Patients who died from unrelated causes (n = 13) were excluded from the analysis.
KIR and HLA Genotypes of Uveal Melanoma Patients versus Those of Healthy Control Subjects

To test whether polymorphisms in NK receptors or their ligands contribute to resistance to the development of intraocular melanoma, we focused our attention on KIRs and HLA class I, since both loci are highly polymorphic. The KIR and HLA genotypes were determined in 154 patients with uveal melanoma patients and 222 healthy control subjects (Table 2). The frequency of individual KIR genes did not differ significantly between these two groups. Furthermore, the frequencies of HLA encoded ligands for KIR2DL1 (HLA-C group 2), KIR2DL2/3 (HLA-C group 1), and KIR3DL1 (HLA-Bw4) also did not differ between the patients and control subjects (Table 2). As the phenotype frequencies of KIR2DL1, KIR2DL2/3, and KIR3DL1 were >95% in both groups, the frequencies of inhibitory KIR–HLA pairs were virtually identical with the frequencies of the HLA-encoded ligands and therefore also did not differ between the two groups (data not shown). Thus, there was no apparent correlation between HLA/KIR genotype and susceptibility to uveal melanoma.

Influence of KIR and HLA Genotypes on Melanoma-Related Mortality

It has been hypothesized that NK cells can control hematogenic spread of uveal melanoma. Since the detection of metastases is invariably and rapidly (within months) followed by death of the patient, death from uveal melanoma metastases was chosen as the endpoint. We attempted to correlate the incidence rates of metastasis-related death with the KIR and HLA genotype (Table 3). Among the KIR genes, only the absence of the HLA-Bw4-specific inhibitory KIR3DL1 showed a weak association with longer survival (P = 0.04). KIR haplotypes can be classified according to the combinations of KIR genes. A haplotypes contain one or fewer activating KIRs, and B haplotypes possess more activating KIRs (http://www.ebi.ac.uk/ipd/kir/haplotypes.html/ European Bioinformatics Institute). AA homozygous patients (≤1 activating KIR) did not do significantly worse, and the number of activating KIRs did not correlate with improved survival (not shown). In summary, there was no clear effect of KIR genotype on melanoma-related mortality.

The large majority of the patients possessed inhibitory KIR specific for HLA-Bw4 (KIR3DL1, 95%), HLA-C group 1 (KIR2DL2 or KIR2DL3, 100%), and HLA-C group 2 (KIR2DL1, 98%), which made it difficult to find statistically significant correlations with these KIR genes. To probe indirectly the role of these KIR in tumor immunosurveillance, we made use of the fact that their HLA ligands are considerably less frequent in the population. The individuals who possessed KIR2DL1 ligands only (C2/C2) had a significantly (P = 0.04) higher incidence rate of metastasis-related mortality than did the C1/C2 heterozygotes (Table 3, Fig. 3A). The same trend was visible when comparing the individuals with KIR2DL2/KIR2DL3 ligands only (C1/C1) to the C1/C2 heterozygotes (Table 3, Fig. 3A). When comparing all the HLA-C homozygous patients (C1/C1 and C2/C2 together: n = 77; incidence: 33.8%; follow-up: 5226 months; incidence rate: 0.50) to the C1/C2 heterozygotes, the heterozygous patients displayed a twofold (95% CI 1.07–3.81-fold; P = 0.03) higher incidence rate of metastasis-related deaths than did the C1/C2 heterozygotes. No such effect was

selective loss of C2 from the cell surface expressed the C2-specific inhibitory receptor KIR2DL1. In the case of loss of expression of a single HLA-C allele, the other C2 allele was still present to inhibit NK cell activation via KIR2DL1 and NK cells would not attack the tumor.

FIGURE 3. HLA-C heterozygote advantage in uveal melanoma. (A) Metastasis-free survival of patients with uveal melanoma subdivided by HLA-C genotype. Vertical hash marks: censored events. For statistical analysis based on incidence rates of metastasis-related death, see Table 3. (B) A possible mechanism for HLA-C heterozygote advantage in uveal melanoma. Top: in HLA-C group 1 (C1C1) homozygous individuals, NK cells are educated to become sensitive to the absence of C1 but not C2. The NK cells sensitive to a selective loss of C1 from the cell surface express the C1-specific inhibitory receptors KIR2DL2 or -3. In the case of loss of expression of a single HLA-C allele, the other C1 allele is still present to inhibit NK cell activation via KIR2DL2 or -3 and NK cells will not attack the tumor. Middle: in C1C2 heterozygotes, one subset of NK cells is educated to become sensitive to the absence of C1 and another subset of NK cells is educated to become sensitive to the absence of C2. In this case, selective loss of C1 from the surface of the melanoma completely removes the KIR2DL2/3-mediated inhibition from the first NK subset, leading to its activation. In the same vein, tumor cells that lose expression of C2 become sensitive to lysis by the second NK subset that carries KIR2DL1. Bottom: in HLA-C group 2 (C2C2) homozygous individuals, NK cells were educated to become sensitive to the absence of C2 but not C1. The NK cells sensitive to a
observed for HLA-B. Thus, patients possessing ligands for both types of HLA-C-specific inhibitory KIR fared better than patients with only a single type of ligand.

**DISCUSSION**

Uveal melanoma is a rare but often lethal type of cancer. We present three novel lines of evidence indicating a role for NK cells in the immune control of uveal melanoma. First, uveal melanoma cell lines expressed ligands for activating and inhibitory NK receptors. Second, such cell lines were efficiently lysed by NK cells in vitro. Finally, polymorphisms in HLA molecules that regulate NK cell function influenced the risk of death by metastasis.

KIR and HLA genotypes did not differ between patients and healthy control subjects, but did appear to influence the risk of metastasis development in patients when HLA-C genotypes were categorized according to the inhibitory KIRs that they interact with. Individuals whose genotype provided ligands for both KIR2DL1 and KIR2DL2/3 (C1/C2) fared better than individuals whose genotype encoded ligands for either KIR2DL1 (C2/C2) or KIR2DL2/3 (C1/C1) alone. These data support a role for interactions between KIR and HLA in NK cell–mediated control of uveal melanoma metastasis formation. It must be stressed, however, that these associations were weak. This association study should therefore be considered preliminary evidence that awaits confirmation in independent cohorts. To our knowledge, such cohorts are presently unavailable.

How might HLA-C heterozygosity lead to a lower risk of metastasis? Because loss of heterozygosity on the short arm of chromosome 6 is a common feature in uveal melanoma,28 tumors usually lose expression of only one of two HLA class I alleles.38 Loss of a single allele in HLA-C group 1 (C1/C1) or group 2 (C2/C2) homozygous individuals is unlikely to be detected by NK cells, as the other allele is still there to bind the corresponding inhibitory KIR (Fig. 3B). In contrast, if the tumor cell looses a single HLA-C allele in a heterozygous (C1/C2) individual, this cell will then become susceptible to lysis by NK cells expressing the corresponding inhibitory KIR, as these NK cells no longer receive inhibitory signals via this NK receptor (Fig. 3B). Virtually all donors possess the relevant inhibitory KIRs, since the gene frequency of HLA-C1 (KIR2DL2 and KIR2DL3 together)- and HLA-C2 (KIR2DL1)-specific KIRs is 100% and 98% in the population, respectively. These data support a model in which class I loss variants of the tumor are associated with better prognosis according to some studies.43,44 Like uveal melanoma, colorectal cancer metastasizes via the blood to the liver. Thus, NK cells may act by removing micrometastases from the circulation and also by attacking metastases in the liver.

To substantiate a role for NK cells in the immune control of uveal melanoma, it is essential to test directly the ex vivo expression patterns of NK receptor ligands, including HLA class I, as well as the susceptibility to lysis by primary NK cells, as was recently done for ovarian carcinoma.45 Our current data suggest that loss of HLA-C heterozygosity by the primary tumor in HLA-C heterozygotes (C1/C2) but not homozygotes (C1/C1 or C2/C2) is associated with better survival. Ultimately, our findings pave the way for adoptive immunotherapy with allogeneic NK cells46 in metastasized uveal melanoma.

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**References**


