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KIR3DL1 Allelic Polymorphism and HLA-B Epitopes Modulate Response to Anti-GD2 Monoclonal Antibody in Patients With Neuroblastoma

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Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

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ABSTRACT

Purpose
In patients with neuroblastoma (NB), treatment with anti-GD2 monoclonal antibody (mAb) directs natural killer (NK) cell–mediated antibody-dependent cellular cytotoxicity (ADCC) against tumor cells. However, tumor cytotoxicity is attenuated by ligation of inhibitory killer immunoglobulin-like receptors (KIRs) by HLA class I molecules. *KIR3DL1* polymorphism influences its ability to engage HLA-Bw4 ligands. We tested the hypothesis that poorly interacting combinations of KIR3DL1 and HLA ligands are more permissive of mAb-mediated antitumor effect.

Methods
KIR3DL1 and *HLA-B* subtyping were performed with a multiplex intermediate-resolution polymerase chain reaction assay for a cohort of 245 patients who were treated with antibody 3F8 for high-risk NB. Patient outcomes were analyzed according to expected degree of interaction between KIR3DL1 and HLA-B subtypes and grouped as strong, weak, or noninteractors. A comparison of NK response to 3F8 mAb opsonized NB cells between strong- and noninteracting donors was performed by flow cytometry.

Results
KIR3DL1 and *HLA-B* subtype combinations associated with noninteraction as a result of lack of receptor expression [KIR3DL1(−)], failure of interaction with inhibitory ligands [KIR3DS1(+)], or absence of KIR ligands resulted in significantly improved overall and progression-free survival. Patients with *KIR3DL1* and *HLA-B* subtype combinations that were predictive of weak interaction had superior outcomes compared with those that were predictive of strong interaction; however, both groups were inferior to those with noninteracting subtype combinations. In vitro analysis of 3F8-mediated ADCC showed that KIR3DL1(−) and 3DS1(+) NK cells were insensitive to inhibition by HLA-Bw4–expressing NB targets.

Conclusion
We conclude that *KIR3LD1* and *HLA-B* allele combinations can have a prognostic impact on patient survival after treatment with anti-GD2 mAb that relies on NK-ADCC. The survival advantage seen in noninteracting combinations supports the therapeutic disinhibition of individuals with strongly interacting KIR and ligand pairs.

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INTRODUCTION

Of patients with neuroblastoma (NB), > 50% have high-risk disease at diagnosis, and despite intense multimodal therapy, long-term survival is poor.[1-6] Monoclonal antibody (mAb) therapies directed at disialoganglioside GD2 have been a major advancement in the treatment of NB.[7-10] Natural killer cells (NK cells), which mediate ADCC (antibody-dependent cellular cytotoxicity), are vital to the efficacy of anti-GD2 antibody therapy.[11-15]

NK cells are lymphocytes with innate antiviral and antitumor capacity whose potency is determined by the balance of various activating and inhibitory signals delivered via cell-surface receptors. The most well-studied receptors that are known to modulate NK activity are the killer immunoglobulin-like receptors (KIRs).[16] KIRs direct a process of education, whereby NK cells that express inhibitory KIR for self-HLA are

licensed and become armed with effector functions against target cells that lack self-HLA.[17] NK cells that fail to encounter cognate HLA as self are unlicensed; however, with a sufficient activating signal, these populations have the potential to mount an effector response.[14,18-20]

Among patients with NB who are treated with the anti-GD2 mAb 3F8, patients who lack HLA class I ligands for their inhibitory KIR (missing ligand) exhibit significantly improved outcomes compared with patients who possess all KIR ligands. Of note, the absence of the HLA-Bw4 ligand for KIR3DL1 is associated with the greatest survival benefit.[14,15] Considering the allelic diversity that exists for both *KIR3DL1* and *HLA-Bw4,* we hypothesized that the varying strengths of interaction between their protein products might influence the potency of NK response to 3F8.[21-24] The *KIR3DL1* locus encodes both activating (*KIR3DS1*) and inhibitory alleles.[16] On the basis of patterns of expression and homology, inhibitory *KIR3DL1* alleles can be categorized as high (*KIR3DL1-h*), low (*KIR3DL1-l*), and null (*KIR3DL1-n*) surface expression subtypes.[24-26] A dimorphism at position 80 of HLA-Bw4 between isoleucine (80I) or threonine (80T) results in variable binding specificity and the inhibition of different KIR3DL1 subtypes.[22,23,27]

We investigated whether subtype combinations of *KIR3DL1* and *HLA-B* with no, weak, and strong interaction could influence patient outcome after treatment with 3F8. Lack of interaction as a result of a lack of ligand or of cell-surface receptor was associated with greatest survival. Weak interaction was associated with intermediate survival, and strong interaction was associated with the poorest survival. These data may aid in the identification of patients with NB who are likely to benefit from treatment with 3F8 as well as those for whom alternative therapies should be explored.

METHODS

Patients

We performed a retrospective analysis of 245 patients with NB who were treated with 3F8 at Memorial Sloan Kettering Cancer Center (MSKCC) between 1994 and 2007. Antibody therapy was initiated after multimodal therapy that included chemotherapy (Appendix Table A1, online only), surgery, and radiation therapy,[28] with or without autologous stem cell transplantation. Patients treated in clinical trials NCT00072358, NCT00002560, and NCT00040872 received intravenous 3F8 plus granulocyte-macrophage colony-stimulating factor (GM-CSF; Appendix Table A2, online only).[9] Investigators obtained consent for therapy, specimen collection, and analysis as approved by the MSKCC Institutional Review Board.

Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) collected from patients or anonymous healthy volunteer donors from the New York Blood Center were isolated by Ficoll (New York, NY) density gradient centrifugation. Additional research consent for New York Blood Center samples was waived. PBMCs were cryopreserved in liquid nitrogen.

HLA Typing

Genomic DNA was extracted from PBMCs or marrow mononuclear cells by using the QIAmp DNA Blood Mini Kit according to the manufacturer instructions (Qiagen, Germantown, MD). Patient HLA typing was performed by a combination of HLA serology, sequence-specific polymerase chain reaction (PCR), and PCR-specific oligonucleotide probe. HLA genotyping for healthy donors was performed by HistoGenetics (Ossining, NY).

KIR3DL1 *and* HLA-B *Subtyping and Combined Group Assignments*

KIR3DL1 subtyping was performed by using an intermediate-resolution, multiplex PCR-based typing method, which categorizes alleles by surface density without assigning specific alleles.[26] *KIR3DL1* alleles were classified as *KIR3DL1-high* (*KIR3DL1-h*), *KIR3DL1-low* (*KIR3DL1-l*), *KIR3DL1-null* (*KIR3DL1-n*), and activating *KIR3DS1* (*KIR3DL1-s*) subtypes.[26] Compound *KIR3DL1* subtypes, whose known surface expression profiles and binding affinities provide predicted inhibitory strengths, were based on previously described classifications[22,23,25,29]: *KIR3DL1-N* (*KIR3DL1-n/n,* or *-n/s); KIR3DL1-L* (*KIR3DL1-l/l, -l/h, -n/l,* and *-l/s*); *KIR3DL1-H* (*KIR3DL1-h/h, -n/h,* and *-h/s*); and *3DS1 (-s/s)*.[29]

HLA-B alleles were assigned as *HLA-Bw6*, *Bw4-80I*, or *Bw4-80T* epitopes, per the Immuno Polymorphism database.[30] Compound epitopes were categorized as: group *Bw4-80I* (*Bw4-80I/Bw4-80I*, *Bw4-80I/Bw4-80T*, or *Bw4-80I/Bw6*); group *Bw4-80T* (*Bw4-80T/Bw4-80T* or *Bw4-80T/Bw6*); and group *Bw6 (Bw6/Bw6).*

Target Cells and Culture Conditions

The NB cell lines SKNBE(1)n and SKNMM were derived from patient tumors, and they express the KIR ligands HLA-C1, HLA-C2, and HLA-Bw4. NB cells were cultured in RPMI 1640 medium that was supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin and they were incubated at 37°C with 5% CO_2. To induce class I HLA expression in NB cell lines, recombinant human interferon-gamma (IFN-γ; PeproTech, Rocky Hill, NJ) was added at 1,000 U/mL per day for 72 hours. Frozen PBMCs from healthy donors were thawed, cultured in medium that was supplemented with human IL-2 (proleukin; Prometheus Laboratories, San Diego, CA) at 200 IU/mL, and incubated at 37°C with 5% CO_2 for 12 to 16 hours before experiments.

NK Activation and Flow Cytometry

NK cell activation was measured by including anti-LAMP1 antibody (CD107a, BD Biosciences, San Jose, CA) during NK:target cell cocultures. PBMCs (5×10^5 cells/well) were cultured with targets at a 5:1 ratio, with or without 1.0 μg/mL 3F8, in a 96-well V-bottom plate in complete RPMI 1640. After a 4-hour incubation, PBMCs were stained with live/dead fixable stain (Life Technologies, Grand Island, NY) and the following mAbs: anti-CD3 (OKT-3; BioLegend, San Diego, CA); anti-CD56 (N901; Beckman Coulter, Brea, CA); anti-KIR3DL1 (DX9; BioLegend); anti-3DL1/S1 (Z27; Beckman Coulter); anti-NKG2A (Z199; Beckman Coulter); anti-KIR2DL1/S1 (EB6B; Beckman Coulter); and anti-KIR2DL2/L3/S2 (GL183; Beckman Coulter). To identify NK that expresses intracellular KIR3DL1-n, PBMCs were fixed and permeabilized for 10 minutes (Fix & Perm; Life Technologies), then stained for 1 hour at room temperature with anti-KIR3DL1 clone 177407 (Beckman Coulter). HLA class I expression in NB cell lines was evaluated with anti–HLA-Bw4 (REA274; Miltenyi Biotec, San Diego, CA) and anti–HLA-C (DT-9; NCI, Frederick, MD).[24] The effect of disrupting KIR3DL1/HLA-B ligation was evaluated by using anti-3DL1 (DX9) and anti–HLA-B,C (clone 4E; MSKCC mAb core facility) antibodies. Multicolor flow cytometry was performed by using an LSR Fortessa (BD Biosciences). Data analysis was performed by using FlowJo 9.7 software (Treestar, Ashland, OR).

Statistical Methods

Overall survival (OS) was defined as the time interval between the start of 3F8 therapy and the date of last follow-up or death. Progression-free survival (PFS) was defined as the time interval between the start of 3F8 therapy and the earliest date of disease progression, death, or last follow-up when no event was observed in the patient. The Kaplan-Meier method and the log-rank test were used to estimate and compare OS and PFS for

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KIR3DL1 and *HLA-B* subtype interaction and clinical factors. The permutation log-rank test was used when the number of events was less than five.[31] Univariable estimates of hazard ratio (HR) and 95% CIs were computed by using the partial likelihood from the Cox proportional hazards regression model.

A weighted partial likelihood estimate of the average HR was computed to determine OS and PFS risks associated with *KIR3DL1/HLA-B* subtypes after adjustment for statistically significant clinical factors ($P \leq .05$) of age, LDH, disease status, and GM-CSF route (GM-CSF administration route for OS only). Variables that were found not to be statistically significant included the presence of bone marrow disease, metastatic bone disease, and MYCN amplification. The weighted partial likelihood was applied to account for the lack of proportional hazards in risk models.[32]

P values for paired comparisons of activation in NK that express specific KIRs were generated from a permutation distribution that was derived from the two-sample mean difference. An unpaired Student *t* test was used to compare NK inhibition. Statistical analyses were performed in software packages SAS (SAS/STAT User's Guide, Version 9.2; SAS Institute, Cary, NC), library clinfun, coxphw in R version 2.13 (The R Foundation for Statistical Computing), and Prism 6 (GraphPad Software, La Jolla, CA) for Mac.

RESULTS

Patient and Disease Characteristics

To investigate the impact of KIR3DL1 and HLA-B interactions in response to 3F8 in patients with NB, we analyzed *KIR3DL1* and *HLA-B* subtype combinations in a cohort of 245 patients with high-risk NB who were treated with 3F8 at MSKCC from October 1994 to December 2007. Patient and disease characteristics are listed in Table 1. Patients treated with and without autologous stem cell transplantation were analyzed as a single group as it was previously determined that KIR and HLA effects were similar in both treatment groups.[14] One hundred and seventeen patients were alive at the last follow-up, with a median follow-up of 102.9 months (95% CI, 96.6 to 111.8 months; range, 27.7 to 232.7 months).

HLA-B *Subtypes Impact Survival After Treatment With 3F8*

HLA typing revealed that 58% of patients (n = 141) possessed at least one allele that exhibited the Bw4 epitope (*HLA-Bw4*), whereas 42% of patients (n = 104) were homozygous for alleles that exhibited the Bw6 epitope (*Bw6*), which was consistent with published population frequencies.[33] Patients who were homozygous for *Bw6* alleles and who exhibited *KIR3DL1* are missing ligand for KIR3DL1, which is associated with superior outcomes.[14,15,34] Patients with *HLA-Bw4* were further segregated by amino acid at Bw4 position 80. In univariable analysis, patients with *Bw4-80T* had an observed reduction in OS and PFS compared with *Bw6* patients (OS: HR, 1.38; 95% CI, 0.89 to 2.14; *P* = .151; and PFS: HR, 1.36; 95% CI, 0.91 to 2.03; *P* = .133); and OS and PFS among patients with *Bw4-80I* were demonstrably worse compared with *Bw6* patients (OS: HR, 1.58; 95% CI, 1.05 to 2.38; *P* = .027; and PFS: HR, 1.58; 95% CI, 1.09 to 2.30; *P* = .016; Fig 1). On multivariable analysis, inferior outcomes observed for *Bw4-80T* (OS: HR, 1.63; 95% CI, 0.93 to 2.84; *P* = .085; and PFS: HR, 1.94; 95% CI, 1.14 to 3.30; *P* = .014) and *Bw4-80I* (OS: HR, 2.23; 95% CI, 1.34 to 3.71; *P* = .002; and PFS: HR, 2.43; 95% CI, 1.53 to 3.87; $P \leq .001$) were maintained.

KIR3DL1 *Subtypes Do Not Impact Survival Independent of* HLA-B

Among patients, 94% (n = 230) carried at least one allele of *KIR3DL1* and 6% (n = 15) were homozygous for *KIR3DS1*. *KIR3DL1* subtyping was completed on genomic DNA from all patients and revealed *KIR3DL1* subtype frequencies of 48.4% (n = 237), 15.9% (n = 78), 16.3% (n = 80), and 19.4% (n = 95) for *KIR3DL1-h*, *KIR3DL1-l*, *KIR3DL1-n*, and *KIR3DL1-s*, respectively, which was consistent with published frequencies.[26,35] Patients were then categorized on the basis of composite subtype, with *KIR3DL1-H*, *-L*, *-N*, and *KIR3DS1* representing 57.6% (n = 141), 26.9% (n = 66), 9.4% (n = 23), and 6.1% (n = 15), respectively. No one *KIR3DL1* subgroup was associated with either OS or PFS in patients who were treated with 3F8 (data not shown).

Patients With KIR3DL1-N, KIR3DS1*, or* HLA-Bw6 *Experience Similar Outcomes*

Activated by CD16 engagement but refractory to inhibition by induced HLA on NB cells, unlicensed NK cells, such as KIR3DL1+ cells in *Bw6* patients, seem to be the primary effectors for the antitumor benefit of anti-GD2 mAb.[14] We hypothesized that

Table 1. Patient and Disease Characteristics (N = 245)

Patient Characteristic	No. of Patients
INSS neuroblastoma disease stage	
4	243
3	2
Age, years	
< 1.5	21
> 1.5	224
MYCN status	
Nonamplified	142
Amplified	77
Unknown	26
LDH, U/mol	
< 1,500	128
> 1,500	66
Unknown	51
Bone marrow metastases	
No	24
Yes	221
Bone metastases	
No	56
Yes	189
Disease status at 3F8 therapy	
First CR/VGPR	108
Second CR/VGPR	19
Primary refractory	86
Secondary refractory	4
Other*	26
KIR3DL1 status	
Positive	230
Negative†	15
Treatment regimen	
ASCT + 3F8	168
3F8 alone	77

Abbreviations: ASCT, autologous stem cell transplantation; CR, complete response; INSS, International Staging System for Neuroblastoma; LDH, lactate dehydrogenase; VGPR, very good partial response.
*Patients who received 3F8 in combination with induction chemotherapy.
†*KIR3DL1*-negative patients were considered homozygous for *KIR3DS1*.

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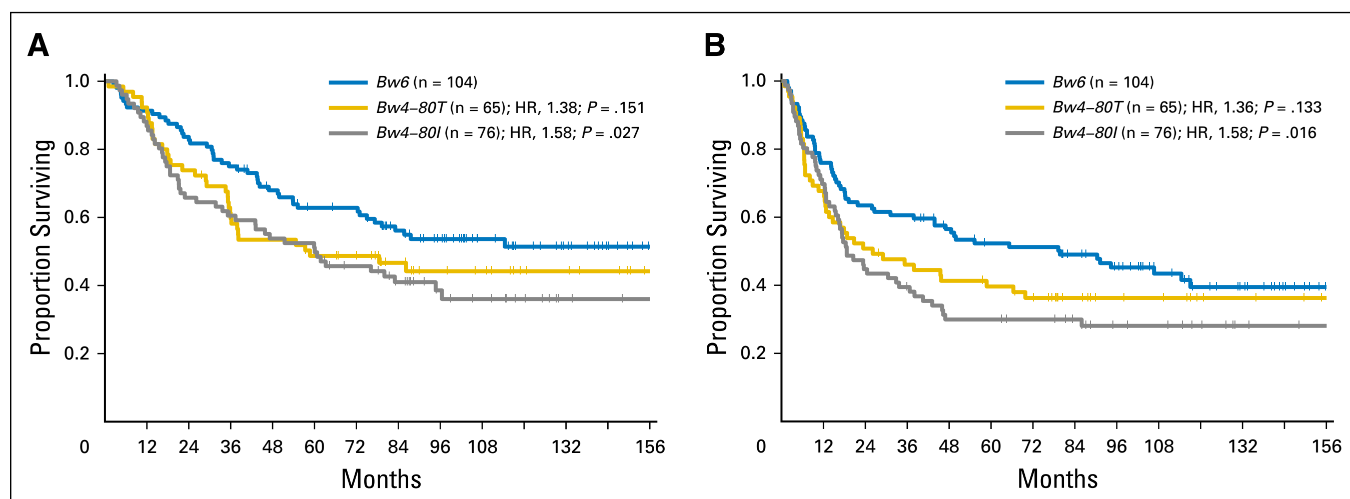

Fig 1. *HLA-B* subtypes influence outcomes in patients with neuroblastoma (NB) who are treated with 3F8. Among patients with NB, 245 who received treatment with 3F8 were segregated on the basis of HLA-B epitope groups. Kaplan-Meier curves for (A) overall survival and (B) progression-free survival among patients encoding *Bw6* (blue lines), *Bw4-80T* (gold lines), and *Bw4-80I* (gray lines) are shown. HR, hazard ratio.

KIR3DL1 subtypes that are unable to engage HLA-Bw4 on tumor cells might be also associated with favorable outcomes. The KIR3DL1-n protein is improperly folded and retained intracellularly,[36,37] and KIR3DS1 does not interact with HLA-Bw4.[27,38] A univariable comparison of *HLA-Bw4+* and *KIR3DL1-N* or *KIR3DS1* patients (n = 23) with *Bw6* patients (n = 104) revealed similar outcomes (OS: HR, 1.14; 95% CI, 0.56 to 2.33; *P* = .721; and PFS: HR, 1.12; 95% CI, 0.59 to 2.14; *P* = .724), which highlighted the potential benefit of the noninteracting receptor populations.

Strength of KIR3DL1 *and* HLA-B *Interactions Predict 3F8 Therapeutic Efficacy*

The high degree of polymorphism among *KIR3DL1* and *HLA-B* subtypes, when combined, produce a spectrum of NK response capacity.[22,39] Most notably, strongly interacting combinations are associated with superior protection against HIV progression[29] but greater susceptibility to leukemic relapse after hematopoietic stem cell transplantation (manuscript in submission). We examined whether subtype combinations might also titrate the sensitivity of NK cells to inhibition by HLA-Bw4 presented in NB cells and influence the efficacy of 3F8 therapy in patients.

Patients were segregated on the basis of the relative strengths with which their KIR3DL1 and HLA-B subtypes were predicted to interact: strong, weak, or noninteracting.[29] Strong interaction pairings were composed of *KIR3DL1-H + Bw4-80I* or *KIR3DL1-L + Bw4-80T*, and weak interacting pairings were composed of *KIR3DL1-H + Bw4-80T* or *KIR3DL1-L + Bw4-80I*. Because patients with *Bw6*, *Bw4 + KIR3DL1-N*, and *Bw4 + KIR3DS1* exhibited similar outcomes after 3F8 therapy, we grouped together these subtype configurations as noninteracting pairs (Appendix Table A3, online only).

Univariable analysis demonstrated that, compared with strongly interacting combinations (n = 63), the noninteracting combinations (n = 127) had the most favorable OS outcomes (HR, 0.57; 95% CI, 0.38 to 0.85; *P* = .007) and PFS outcomes (HR, 0.56; 95% CI, 0.39 to 0.81; *P* = .002; Fig 2). Even when the *Bw6* patients were excluded from the group of noninteractors, which left only patients with *KIR3DL1-N* and *KIR3DS1 + HLA-Bw4* (n = 23), similar trends toward benefit were observed (OS: HR, 0.51; 95% CI, 0.25 to 1.06; *P* = .070; and PFS: HR, 0.52 95% CI, 0.27 to 1.00; *P* = .052). Patients with weakly interacting pairs (n = 55) demonstrated a trend toward intermediate protection, with lower mortality and relapse compared with the strongly interacting group (OS: HR, 0.89; 95% CI, 0.56 to 1.42; *P* = .627; and PFS: HR, 0.85; 95% CI, 0.55 to 1.30; *P* = .450).

On multivariable analysis, the benefit of noninteracting *KIR3DL1/HLA-Bw4* pairs versus strongly interacting pairs was maintained for both OS (HR, 0.41; 95% CI, 0.25 to 0.65; $P \leq .001$) and PFS (HR, 0.43; 95% CI, 0.28 to 0.66; $P \leq .001$), and the weakly interacting pairs were again associated with a trend toward intermediate outcomes (OS: HR, 0.55; 95% CI, 0.32 to 0.96; *P* = .036; and PFS: HR, 0.68; 95% CI, 0.40 to 1.15; *P* = .153; Table 2). Because of the heterogeneity of this cohort, we performed a similar analysis on a more uniform subset of International Neuroblastoma Staging System stage 4 patients, age > 18 months, and in first complete response or very good partial response (n = 66). The findings observed within this subset recapitulated the above findings, as noninteractors demonstrated superior OS (HR, 0.40; 95% CI, 0.18 to 0.85; *P* = .018) and PFS (HR, 0.46; 95% CI, 0.22 to 0.97; *P* = .040), and weak interactors displayed a trend toward intermediate outcomes (OS: HR, 0.53; 95% CI, 0.19 to 1.43; *P* = .209; and PFS: HR, 0.61; 95% CI, 0.25 to 1.48; *P* = .275) compared with strong interactors (Appendix Table A4, online only).

KIR3DL1-n and KIR3DS1(+) NK Cells Are Activated by 3F8 and Resist Inhibition by HLA-Bw4

NB primary tumor cells and cell lines typically express low surface HLA at rest[40,41]; however, treatment-induced inflammation results in upregulation of HLA class I molecules, which provides ligand for the inhibition of licensed NK cells.[14,42] We hypothesized that this would negatively impact the activity of cells that bear KIR3DL1 on the NK surface but would minimally impact the activity of noninteracting populations. To test this hypothesis, we

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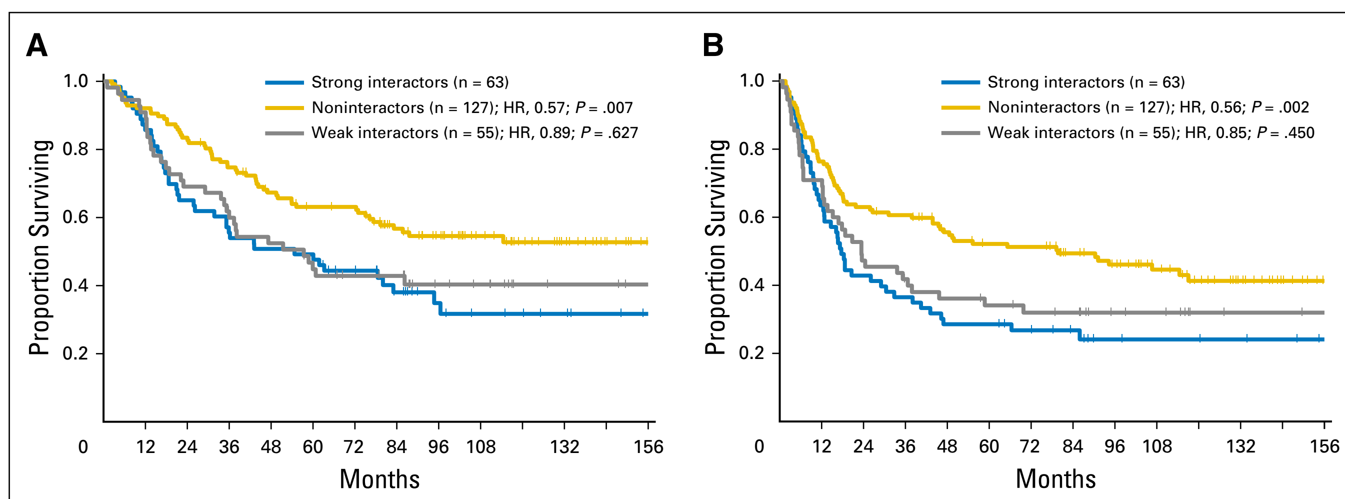

Fig 2. *KIR3DL1* and *HLA-B* subtype combinations predict outcomes in patients with neuroblastoma (NB) who are treated with 3F8 on the basis of strength of interaction. Among patients with NB, 245 who received treatment with 3F8 were grouped according to expected strength of interaction of KIR3DL1 and HLA-B into strongly interacting pairs (blue lines), weakly interacting pairs (gray lines), and noninteracting pairs (gold lines). Kaplan-Meier curves for (A) overall survival and (B) progression-free survival are shown. HR, hazard ratio.

compared the responsiveness of NK cells that express KIR3DL1-n, KIR3DS1, or KIR3DL1-h toward the Bw4-80I+ NB cell line SKNBE(1)n. To mimic an autologous setting, *Bw4-80I+* donors were selected for study. To induce expression of HLA class I, we treated the cell line with IFN-γ (Fig 3A).[14]

Multiparametric flow cytometry enabled the evaluation of NK cells that express KIR3DL1-h, 3DS1, or intracellular KIR3DL1-n, exclusive of all other inhibitory KIRs and NKG2A. CD107a was used to quantitate degranulation in response to 3F8-opsonized NB cells. In the presence of 3F8, KIR3DL1-h(+) NK cells were highly activated against resting NB cells but were inhibited by IFN-γ–treated, HLA-Bw4–expressing NB cells (Figs 3B-3D). Similarly, upregulation of HLA-C dramatically interfered with the reactivity of NK cells that express KIR2DL1, KIR2DL2, or KIR2DL3, the inhibitory KIR specific for the HLA-C ligand (Figs 3B and 3C). Of note, KIR3DL1-n(+) and 3DS1(+) NK populations reacted to NB cells in the presence of 3F8 and were only mildly inhibited by IFN-γ–treated target cells (Figs 3C and 3D). Inhibition was released by the addition of anti-HLA mAb but not anti-KIR3DL1 (DX9), which suggested that their inhibition may be mediated by other HLA-specific receptors, such as the leukocyte immunoglobulin-like receptor family of receptors (Fig 4).[43] In contrast, KIR3DL1-h(+) cells were more dramatically inhibited, and the addition of either DX9 or anti-HLA antibodies rescued the functional response of KIR3DL1-h(+) NK, which indicated that KIR3DL1/HLA-Bw4 interactions were the primary source of inhibition of KIR3DL1-h(+) NK cells (Fig 4). Similar results were seen with the *Bw4-80I+* target NB cell line SKNMM (Appendix Fig A1, online only).

DISCUSSION

Treatment of NB with anti-GD2 mAb is most successful in patients who lack HLA ligands for at least one inhibitory KIR,[14,15] with the greatest benefit observed among those who lack HLA-Bw4 ligands for KIR3DL1.[14] KIR3DL1 and HLA-B ligands mediate NK cell inhibition with varying efficiency,[22,23,27] which supports the hypothesis that the strength with which KIR3DL1 and HLA-B subtypes interact determines the extent of NK inhibition and, consequently, the antitumor benefit of 3F8 therapy. We demonstrate that individuals with *KIR3DL1* and *HLA-B* subtypes that do not interact at the cell surface exhibit the greatest OS and PFS and that those with weakly and strongly interacting combinations were associated with intermediate and poor 3F8 antitumor effect,

Table 2. OS and PFS Associated With *KIR3DL1* and *HLA-B* Subtype Pairs Among Patients Who Received 3F8 for High-Risk Neuroblastoma

	No.	Univariable Analysis				Multivariable Analysis*			
		OS		PFS		OS		PFS	
KIR3DL1/HLA-B Pairs	OS/PFS	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Strong interacting	63/63	1		1		1		1	
Weak interacting	55/55	0.89 (0.56 to 1.42)	.627	0.85 (0.55 to 1.30)	.450	0.55 (0.32 to 0.96)	.036	0.68 (0.40 to 1.15)	.153
Noninteracting	127/127	0.57 (0.38 to 0.85)	.007	0.56 (0.39 to 0.81)	.002	0.41 (0.25 to 0.65)	< .001	0.43 (0.28 to 0.66)	< .001

NOTE. Strong interacting: *KIR3DL1-H* + *Bw4-80I* or *KIR3DL1-L* + *Bw4-80T*; weak interacting: *KIR3DL1-H* + *Bw4-80T* or *KIR3DL1-L* + *Bw4-80I*; and noninteracting: *KIR3DL1-N* + any *HLA-B*, *3DS1* + any *HLA-B*, and *Bw6* + any *KIR3DL1*.
Abbreviations: HR, hazard ratio; OS, overall survival; PFS, progression-free survival.
*Adjusted for age, lactate dehydrogenase, granulocyte-macrophage colony-stimulating factor route (in OS only) and disease status at 3F8.

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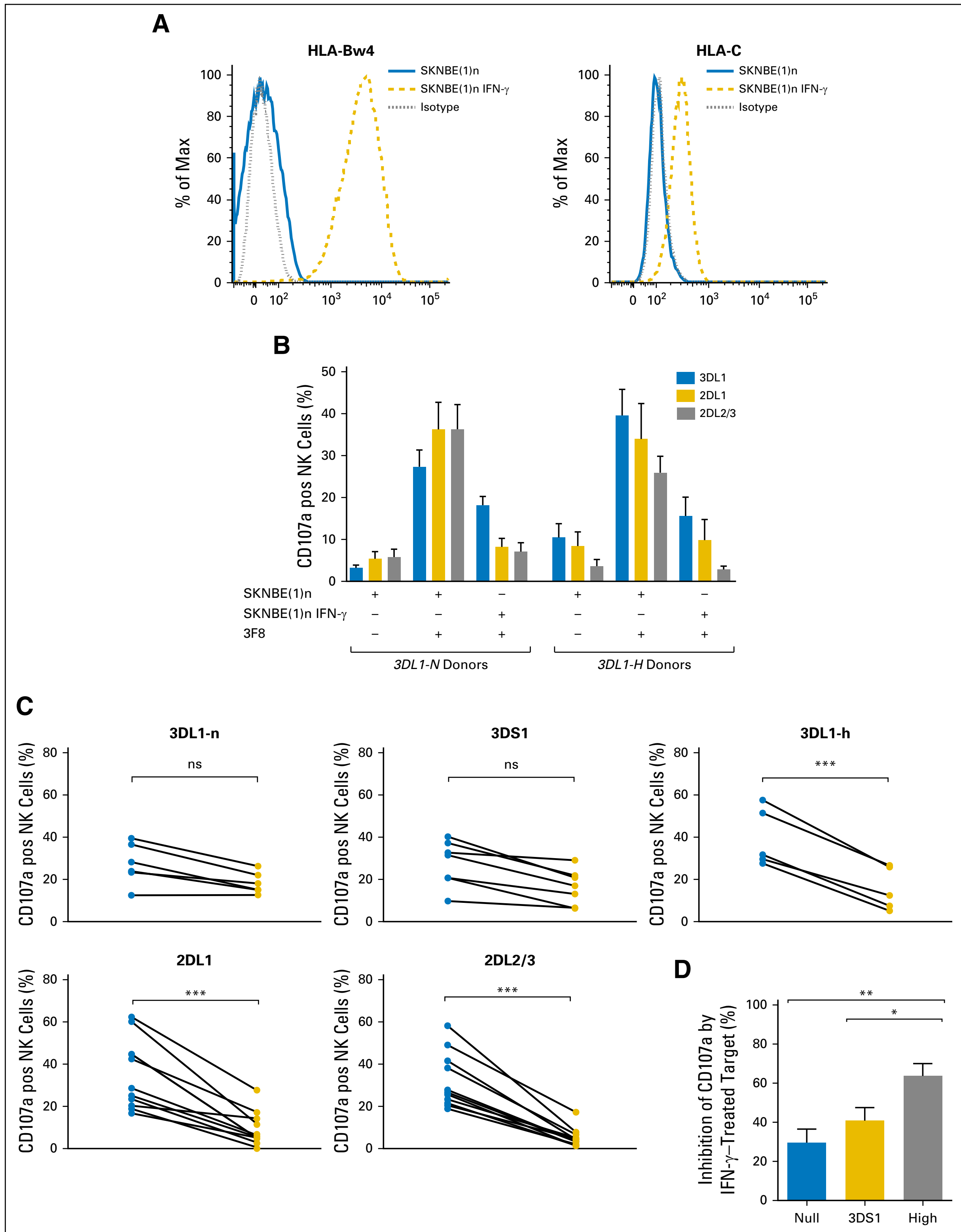

Fig 3. KIR3DL1-n+ and KIR3DS1+ natural killer cells (NK cells) are resistant to inhibition by neuroblastoma (NB) cells that express HLA-Bw4-80I. (A) Incubation of SKNBE(1)n with interferon-gamma (IFN-γ) results in increased expression of HLA-Bw4 and HLA-C. (B) Peripheral blood mononuclear cells from healthy *Bw4-80I+* donors were stimulated with 3F8 and the KIR3DL1 ligand-matched NB cell line SKNBE(1)n with or without IFN-γ pretreatment. CD107a degranulation of NK cells that exclusively express KIR2DL1 (gold bars), KIR2DL2/3 (gray bars), or KIR3DL1 (blue bars) was compared between *KIR3DL1-N* (n = 6) and *3DL1-H* (n = 5) donors. (C) Paired comparison of CD107a degranulation against IFN-γ–untreated (blue circles) and IFN-γ–treated (gold circles) SKNBE(1)n among NK populations that express specific killer immunoglobulin-like receptors with *P* value generated from a permutation distribution. (D) Percent inhibition among NK cells that exclusively express KIR3DL1-n (blue bars), KIR3DS1 (gold bars), or KIR3DL1-h (gray bars) was calculated by determining the change in CD107a degranulation in response to 3F8 against IFN-γ–untreated and –treated SKNBE(1)n targets and compared by unpaired Student *t* test. Data represent three independent trials with four to six donors per group, per trial. ns, nonsignificant. $*P < .05$; $**P \leq .01$; $***P \leq .001$.

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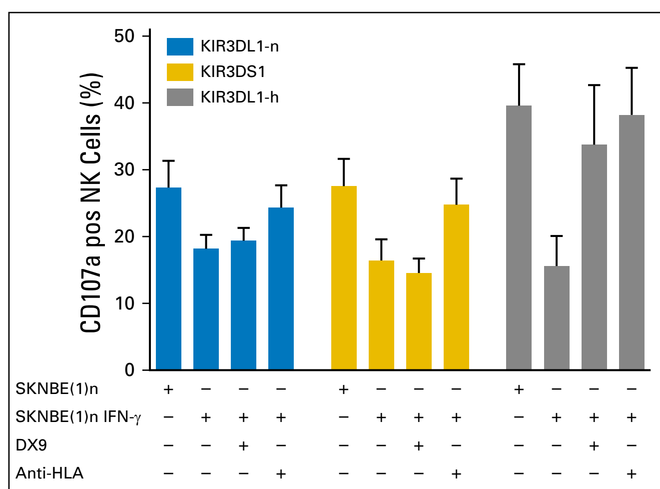

Fig 4. Blocking KIR3DL1-h interaction with Bw4-80I rescues natural killer cells (NK cells) from inhibition. Peripheral blood mononuclear cells from healthy donors were stimulated with 3F8 and KIR3DL1 ligand-matched cell line SKNBE(1)n with or without interferon-gamma (IFN-γ) pretreatment. Effect of addition of KIR3DL1 monoclonal antibody (DX9) and anti–HLA-B-C antibody on CD107a degranulation was evaluated in NK cells that exclusively express KIR3DL1-n (blue bars), KIR3DS1 (gold bars), and KIR3DL1-h (gray bars). Data represent two independent trials with four to six donors per group, per trial.

respectively. Thus, although maximizing the NK cell activation for ADCC should enhance NB tumor clearance,[44-46] considerations should also be given to specific KIR and HLA interactions, which could influence the efficacy of mAb therapy. In this study, we demonstrate that NK effectiveness against cancer may be tuned at the allotype level and not necessarily abolished by tumor expression of HLA-Bw4. Comprehensive in vitro studies are still needed, however, to model inhibitory strengths of receptor–ligand allotypes.

KIR3DL1-n(+) NK cells have been associated with NK cell education[36,37] and delayed HIV progression,[29] which suggests that intracellular sequestration of the receptor does not impede the ability of these NK cells to recognize and respond to target cells. Similarly, despite its sequence homology to KIR3DL1 and expression on the NK cell surface, KIR3DS1 does not interact with HLA-Bw4 molecules.[27,38] Nevertheless, KIR3DS1(+) cells respond to stimulation with target cells[27,47,48] and have been associated with protection from graft-versus-host disease,[49] HIV[29,50] and hepatitis C virus,[51] which suggests that either the presence of KIR3DS1 or the absence of KIR3DL1 is relevant to disease control. Here, we extend these findings to the setting of mAb treatment of NB, for which the ability of these NK cells to respond to activating signals, but not inhibitory ligands, results in increased disease control and survival among patients with NB who receive 3F8. The effect of *KIR3DL1* and *HLA-B* subtypes and anti-GD2 therapy should be confirmed in a prospective multicenter trial of a homogeneous cohort, and future studies should examine these interactions in patients who are treated without mAb.

Because other therapeutic antibodies engage NK cells for ADCC,[52] including rituximab and trastuzumab, *KIR3DL1* and *HLA-B* subtyping may have a broader use. Indeed, the lack of NK inhibition in patients with lymphoma who are treated with rituximab is associated with higher survival, making *KIR3DL1* and *HLA-B* subtyping potentially relevant.[53,54] For patients with an unfavorable genomic profile, alternative treatment options could be considered to improve outcomes. Strategies to augment NK-ADCC can also be considered via adoptive cell therapies or allogeneic hematopoietic cell transplantation with donors selected for NK cells not easily inhibited by recipient HLA. Therapeutic antibodies designed to disrupt KIR and HLA interactions may enhance NK-mediated ADCC in patients with strongly interacting KIR3DL1 and HLA-B pairs, as evidenced *in vitro* by the restoration of the effector function in the KIR3DL1-h expressing NK cells after antibody blockade during the KIR3DL1 and Bw4 interaction. These antibodies are an area of ongoing clinical interest.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at www.jco.org.

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Provision of study materials or patients: Nai-Kong V. Cheung, Katharine C. Hsu
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Manuscript writing: All authors
Final approval of manuscript: All authors

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■■■

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GLOSSARY TERMS

ADCC (antibody-dependent cell-mediated cytotoxicity): a mechanism of cell-mediated immunity whereby an effector cell of the immune system actively lyses a target cell that has been bound by specific antibodies.

CD16: a component of the low-affinity F_c receptor involved in phagocytosis and antibody-dependent cellular cytotoxicity. CD16 is found on neutrophils, natural killer cells, and macrophages.

natural killer cells (NK cells): cells that belong to the innate immune system and are specialized to kill target cells that are either infected with viruses or host cells that have become cancerous. CD56 is a surface marker specific to NK cells.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

KIR3DL1 Allelic Polymorphism and HLA-B Epitopes Modulate Response to Anti-GD2 Monoclonal Antibody in Patients With Neuroblastoma

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No relationship to disclose

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Patents, Royalties, Other Intellectual Property: Patent pending on the KIR3DL1 multiplex PCR assay that is used for subtyping in this manuscript

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Patents, Royalties, Other Intellectual Property: ScFv constructs of anti-GD2 antibodies (Inst), therapy-enhancing glucan (Inst), use of mAb 8H9 (Inst), methods for preparing and using scFv (Inst), GD2 peptide mimics (Inst), methods for detecting MRD (Inst), anti-GD2 antibodies (Inst), generation and use of HLA-A2–restricted peptide-specific mAbs and CARs (Inst), high-affinity anti-GD2 antibodies (Inst), multimerization technologies (Inst), bispecific HER2 and CD3 binding molecules (Inst), affinity matured hu8H9 (Inst), anti-chondroitin sulfate proteoglycan 4 antibodies and uses thereof (Inst), ROR2 antibodies (Inst), T-cell receptor–like antibody agents specific for EBV latent membrane protein 2A peptide presented by human HLA (Inst)

Katharine C. Hsu
Patents, Royalties, Other Intellectual Property: Patent application on the KIR3DL1 multiplex PCR assay that is used for subtyping in this manuscript

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Appendix

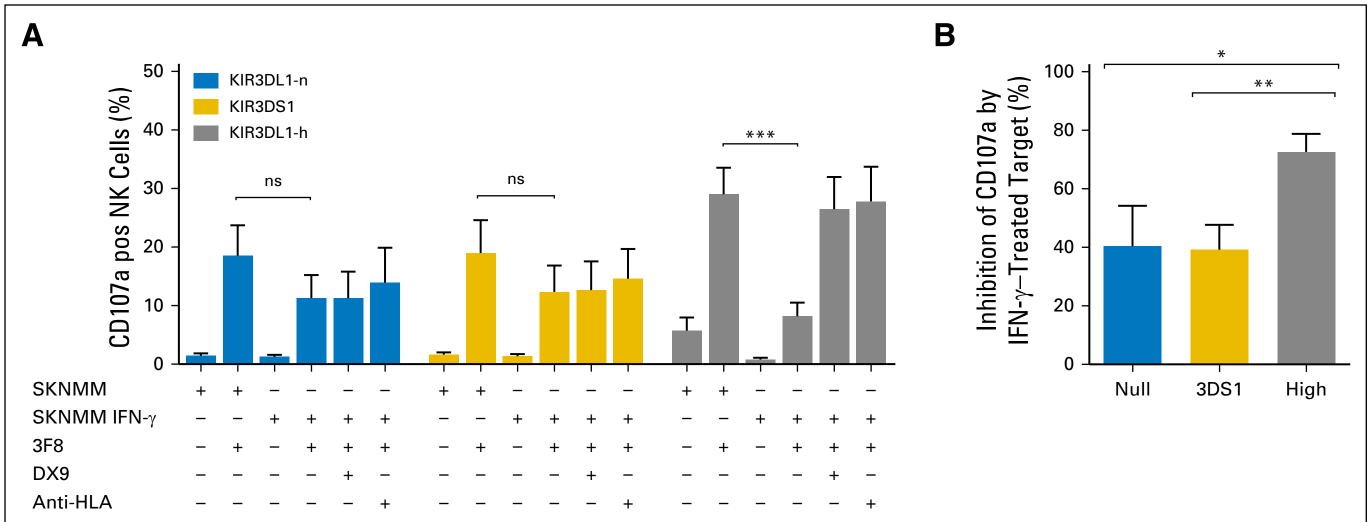

Fig A1. KIR3DL1-n+ and KIR3DS1+ natural killer cells (NK cells) are resistant to inhibition by the HLA-Bw4-80I–expressing neuroblastoma (NB) cell line, SKNMM. (A) CD107a degranulation to the NB cell line, SKNMM, among NK cells that exclusively express KIR3DL1-n (blue bars), KIR3DS1 (gold bars), and KIR3DL1-h (gray bars). Conditions included presence and/or absence of 3F8, pretreatment of tumor with interferon-gamma (IFN-γ), and presence and/or absence of anti-HLA or anti-KIR3DL1 (DX9) mAb. Comparison of NK degranulation against IFN-γ–untreated and –treated SKNMM among NK populations that express specific KIR with *P* value generated from a permutation distribution. (B) Percent inhibition among NK cells that exclusively express KIR3DL1-n (blue bars), KIR3DS1 (gold bars), or KIR3DL1-h (gray bars) was calculated by determining the change in CD107a degranulation in response to 3F8 against IFN-γ–untreated and –treated SKNMM targets and compared by unpaired Student *t* test. Data represent two independent trials with four to six donors per group, per trial. ns, nonsignificant. $^*P < .05$; $^{**}P \leq .01$; $^{***}P \leq .001$.

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Table A1. Induction Chemotherapy Before 3F8 Therapy

Regimen	No. of Patients
High-risk induction: COGA3973-based therapy[10]	186
High-risk induction: POG9640-based therapy*	40
High-risk induction: per European Group Protocols (United Kingdom, Germany)†[1]	7
Other	12

NOTE. In addition to induction chemotherapy, all patients received maximal surgical resection and radiotherapy to primary sites and bulky sites of disease.
*Granger M, et al: Pediatr Blood Cancer 59:902-907, 2012.
†Pearson AD, et al: Lancet Oncol 9:247-256, 2008.

Table A2. Comparison of 3F8 Dosing and Supportive Treatment as Determined by Protocol

3F8 Protocol	No.	3F8 Dose/Cycle, mg/m^2	GM-CSF	Isotretinoin 160 mg/m^2/d*	Other
NCT00002560†	104	100	IV‡	14 d × 6 cycles	N/A
NCT00072358[7]	115	100	SQ	14 d × 6 cycles	N/A
NCT00040872[28]	26	50	SQ	14 d × 6 cycles	Oral etoposide§

Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; IV, intravenous; N/A, not applicable; SQ, subcutaneous.
*Matthay KK, et al: N Engl J Med 341:1165-1173, 1999.
†Kushner BH, et al: J Clin Oncol 19:4189-4194, 2001.
‡Patients received IV GM-CSF during weeks of 3F8 infusion.
§Patients treated in NCT00040872 received adjuvant oral etoposide 50 mg/m^2/d for 21 days alternating with cycles of 3F8 for four cycles.

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Table A3. Summary of Composite *KIR3DL1* and *HLA-B* Subtype Pairs

KIR3DL1 + *HLA-B* Subtype Pair	No.
Strong interaction	63
h/h + *Bw4-80I*	24
h/s + *Bw4-80I*	12
n/h + *Bw4-80I*	11
l/l + *Bw4-80T*	4
l/s + *Bw4-80T*	4
l/h + *Bw4-80T*	8
Weak interaction	55
h/h + *Bw4-80T*	15
h/s + *Bw4-80T*	6
l/l + *Bw4-80I*	3
l/s + *Bw4-80I*	6
l/h + *Bw4-80I*	9
n/h + *Bw4-80T*	13
n/l + *Bw4-80I*	3
No interaction	127
n/n + *Bw4-80I*	1
n/s + *Bw4-80I*	3
n/n + *Bw4-80T*	7
n/s + *Bw4-80T*	4
n/n + *Bw6*	2
n/s + *Bw6*	6
h/h + *Bw6*	25
h/s + *Bw6*	18
n/h + *Bw6*	17
l/l + *Bw6*	5
l/s + *Bw6*	6
l/h + *Bw6*	3
n/l + *Bw6*	15
3DS1+ *Bw6*	7
3DS1 + *Bw4-80I*	4
3DS1 + *Bw4-80T*	4

Table A4. OS and PFS Associated With *KIR3DL1* and *HLA-B* Subtype Pairs Among Patients International Staging System for Neuroblastoma Stage 4, > 18 Months, and in First Complete Response/Very Good Partial Response When Receiving 3F8 for High-Risk Neuroblastoma

		OS Multivariable Analysis		PFS Multivariable Analysis	
KIR3DL1/HLA-B Pairs	No.	HR (95% CI)	*P*	HR (95% CI)	*P*
Strong interacting	24	1		1	
Weak interacting	16	0.53 (0.19 to 1.43)	.209	0.61 (0.25 to 1.48)	.275
Noninteracting	36	0.40 (0.18 to 0.85)	.018	0.46 (0.22 to 0.97)	.040

NOTE. Multivariable analyses adjusted for age, lactate dehydrogenase only, granulocyte-macrophage colony-stimulating factor route of administration not significant on univariable analysis. Strong interacting: *KIR3DL1-H* + *Bw4-80I* or *KIR3DL1-L* + *Bw4-80T*; weak interacting: *KIR3DL1-H* + *Bw4-80T* or *KIR3DL1-L* + *Bw4-80I*; and noninteracting: *KIR3DL1-N* + any *HLA-B*, *3DS1* + any *HLA-B*, and *Bw6* + any *KIR3DL1*.
Abbreviations: HR, hazard ratio; OS, overall survival; PFS, progression-free survival.

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