

Research Article

CALGB 150905 (Alliance): Rituximab Broadens the Antilymphoma Response by Activating Unlicensed NK Cells

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Abstract

Natural killer (NK) cells contribute to clinical responses in patients treated with rituximab, but the rules determining NK-cell responsiveness to mAb therapies are poorly defined. A deeper understanding of the mechanisms responsible for antibody-dependent cellular cytotoxicity (ADCC) could yield useful biomarkers for predicting clinical responses in patients. Unlicensed NK cells, defined as NK cells lacking expression of an inhibitory KIR for self-HLA class I ligands, are hyporesponsive in steady state, but are potent effectors in inflammatory conditions. We hypothesized that antitumor antibodies such as rituximab can overcome NK-cell dependence on licensing, making unlicensed NK cells important for clinical responses. Here, we examined the influences of variations in KIR and HLA class I alleles on *in vitro* responses to rituximab. We tested the clinical significance in a cohort of patients with follicular lymphoma treated with rituximab-containing mAb combinations, and show that rituximab triggers responses from all NK-cell populations regardless of licensing. Neither IL2 nor accessory cells are required for activating unlicensed NK cells, but both can augment rituximab-mediated ADCC. Moreover, in 101 patients with follicular lymphoma treated with rituximab-containing mAb combinations, a "missing ligand" genotype (predictive of unlicensed NK cells) is associated with a higher rate of progression-free survival. Our data suggest that the clinical efficacy of rituximab may be driven, in part, by its ability to broaden the NK-cell repertoire to include previously hyporesponsive, unlicensed NK cells. A "missing ligand" KIR and HLA class I genotype may be predictive of this benefit and useful for personalizing treatment decisions in lymphomas and other tumors. *Cancer Immunol Res*; 2(9): 878–89. ©2014 AACR.

Introduction

Curing patients while sparing unnecessary toxicity remains the ultimate goal in cancer treatment. Monoclonal antibodies (mAb) hold this potential by recruiting a patient's immune system to destroy cancer cells with minimal toxicity, but responses are unpredictable and rarely curative (1–5). Identifying the genetic and cellular factors that drive immune responses in mAb therapy may make clinical responses more predictable and allow the identification of strategies for augmenting mAb-induced responses (6). Natural killer (NK) cells contribute to clinical responses in patients treated with ritux-

imab, but the rules governing how NK cells respond to rituximab are unclear, thus limiting our ability to clinically manipulate and predict NK-cell behavior in rituximab-treated patients (3, 7–9).

The number and ligand specificity of inhibitory receptors expressed by an NK-cell clone have been shown to modify NK-cell-mediated cytotoxic responses (10–13). NK cells lacking inhibitory receptors specific for self-MHC class I are weakly responsive compared with fully competent (i.e., licensed) NK cells expressing one or more inhibitory receptors for self-MHC class I (10–14). However, these same inhibitory receptors can suppress the licensed NK cells through interactions with MHC class I ligands expressed by tumors. In human hematopoietic stem cell transplantation and mouse cytomegalovirus (CMV) infection, limitations of NK-cell licensing can be overcome by harnessing hyporesponsive NK cells lacking inhibitory receptors for self-MHC class I that may contribute to clinical responses, although data are conflicting (15–20). It is unknown whether rules of licensing similarly govern NK-cell responses to antibody-coated hematologic malignancies and whether mAb alone can trigger these responses (12, 21). Because rituximab activates NK cells through CD16, and CD16 does not require coactivating signals to trigger NK-cell responses (22), we hypothesize that rituximab can convert tolerant human NK cells lacking inhibitory killer cell Ig-like receptors (KIR) for self-MHC class I into potent killers. This may be a

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Table 1. Characteristics of patients from CALGB 50402 and CALGB 50701

Characteristics	Treatment protocol		P ^a
	Rituximab + epratuzimab (n = 55)	Rituximab + galiximab (n = 46)	
Ethnicity			1.00
Caucasian	50/53 (94.3%)	45 (97.8%)	
Non-Caucasian	3/53 (5.7%)	1 (2.2%)	
Median age, y (range)	54 (32–90)	58 (22–84)	0.75
Female gender	32 (58.1%)	16 (34.8%)	0.03
LDH > ULN	5 (9.0%)	7 (15.2%)	0.37
"B" symptoms	4/53 (7.5%)	4/43 (9.3%)	1.00
Follicular lymphoma stage			0.74
I	—	—	
II	2 (3.6%)	3/45 (6.7%)	
III	18 (32.7%)	16/45 (35.6%)	
IV	35 (63.3%)	26/45 (57.8%)	
FLIPI			0.97
0–1	12/54 (22.2%)	9/45 (20.0%)	
2	24/54 (44.4%)	20/45 (44.4%)	
≥3	18/54 (33.3%)	16/45 (45.6%)	
Histologic grade			0.82
Grade 1	30/53 (56.6%)	27/45 (60.0%)	
Grade 2	20/53 (37.7%)	17/45 (37.8%)	
Grade 3a	3/53 (5.7%)	1/45 (2.2%)	
Bone marrow			0.69
Positive	30 (54.6%)	23 (50.0%)	
Negative	25 (45.4%)	23 (50.0%)	
Bulky disease	11/55 (20.0%)	8/40 (20.0%)	1.00
ECOG performance status			0.05
0	33 (60.0%)	33/45 (73.3%)	
1	22 (40.0%)	10/45 (22.2%)	
2	0 (0.0%)	2/45 (4.4%)	

Abbreviations: FLIPI, Follicular Lymphoma International Prognostic Index; LDH, lactic dehydrogenase; ULN, upper limit of normal.
^aP values for the categorical variables were computed using the Fisher exact test; the Wilcoxon rank-sum test was used to compute age differences between groups.

fundamental and potentially exploitable mechanism contributing to antitumor antibody clinical responses.

In the present study, we examined the influences of variations in human *KIR* and *HLA* class I alleles on *in vitro* responses to rituximab. We further tested the clinical significance of our *in vitro* findings in a cohort of patients with follicular lymphoma treated with rituximab-containing antibody combinations (23, 24).

Materials and Methods

Follicular lymphoma patients

One hundred and two patients with previously untreated follicular lymphoma provided Institutional Review Board (IRB)-approved informed consent for collection of blood and biospecimens to be used for research related to his or her cancer, such as the correlative science aims of Cancer and Leukemia Group B (CALGB) protocol #150905 (NCT01057459; NCT01749969). Eligible patients with follicular lymphoma had previously untreated, stage III, IV, or bulky stage II disease and World Health Organization tumor grade 1, 2, or 3a. Patients were treated

with a noncytotoxic strategy of rituximab-containing antibody combinations on CALGB protocols 50402 (rituximab with galiximab) and 50701 (rituximab with epratuzumab) from 2005 through 2009 (23, 24). Forty-six of 62 patients (74%) from CALGB 50402 consented and had samples available, although 1 patient never began treatment and was excluded from the analysis. Fifty-six of 60 patients (93%) from CALGB 50701 consented and had samples available. One patient from 50701 was determined to have stage I disease at baseline during final chart review and was excluded from analysis. In all, 101 patients are included in this analysis (Table 1, Supplementary Fig. S6).

KIR genotyping, *HLA* genotyping, and *KIR* ligand assignment

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMC) by using a QIAmp DNA Blood Mini Kit (Qiagen). *KIR* genotyping was performed as previously described (25, 26). *HLA* class I genotyping was performed by PCR using a combination of sequence-specific primers and

PCR-specific oligonucleotide probes. The CLIA-approved Immunogenetics Laboratory at the University of California, San Francisco, performed the genotyping. *HLA-C* and *HLA-B* alleles were segregated into KIR ligand groups: C1 (*HLA-C^{Asn80}*), C2 (*HLA-C^{Lys80}*), and HLA-Bw4 or HLA-Bw6, and *KIR* haplotypes were assigned as previously described (25).

***In vitro* culture conditions**

For functional experiments, blood was obtained from healthy volunteer deidentified leukocyte reduction filters (Blood Centers of the Pacific, San Francisco, CA). PBMCs were separated by density gradient centrifugation (Histopaque-1077; Sigma) and were suspended in 10% dimethyl sulfoxide (DMSO; Fisher Scientific) and 90% fetal bovine serum (FBS; Omega), and then stored in liquid nitrogen. For NK-cell recovery, cryovials of PBMCs were transferred to a 37°C water bath, thawed quickly in RPMI-1640 media (with 20% FBS, warmed to 37°C), and then washed in complete cell culture media (RPMI-1640 with 10% FBS, 2 mmol/L glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin; Cell Culture Facility, University of California, San Francisco, San Francisco, CA). Cells were counted and viability was confirmed using a Vi-Cell XR (Beckman Coulter Inc.). Cells were cultured overnight at 37°C with 5% CO₂ in a 24-well plate at a concentration of 3 × 10⁶/mL of RPMI-1640 media. Exogenous IL2 was not routinely added to the culture conditions, but only added (1,000 U/mL of IL2; BioVision) in experiments testing the specific contribution of IL2. NK cells were isolated from PBMCs using a MACS NK Isolation Kit (Miltenyi Biotec Inc.).

Tumor cells

Three cell lines were used: human CD20⁺ HLA class I-deficient EBV-transformed B lymphoblastoid cell line 721.221, Raji Burkitt lymphoma cell line, and the human erythroleukemia cell line K562. All cell lines were cultured in complete RPMI-1640 media. CD20 expression was confirmed by staining with Brilliant Violet 421-conjugated anti-human CD20 (clone 2H7; BioLegend). HLA-A, HLA-B, HLA-C expression was monitored weekly, and before each experiment, by using FITC-conjugated anti-human HLA-A, HLA-B, HLA-C antibody (clone G46-2.6; BD Biosciences). The FITC-conjugated anti-human HLA-Bw4 mAb (One Lambda) was used to evaluate cell-surface expression of HLA-Bw4. All three cell lines were validated to be mycoplasma free; except for the expression of CD20 and other cell-surface markers, no other authentication assays were performed.

Antibody-induced NK-cell activation assays

To model the physiologic interaction of NK cells with antibody-coated tumors, we developed a coculture system using resting human PBMCs from *KIR*- and *HLA*-genotyped subjects added to antibody-coated tumor cell lines. To assess individual NK-cell responses, we measured both degranulation of NK cells by staining for LAMP-1 (Pacific Blue-conjugated anti-CD107a; clone H4A3; BioLegend; refs. 15, 27), the lysosome-associated membrane protein upregulated on the NK-cell surface after NK-cell stimulation, and intracellular IFNγ (V450-conjugated anti-IFNγ, clone B27; BD Biosciences) expression as a marker of cytokine secretion, which both

correlate with target cell death (12, 27). We initially determined the percentage of CD3⁻CD56⁺ NK cells in the PBMCs using antibodies for CD3 (APC-eFluor 780-conjugated anti-CD3, clone SK7; eBioscience), and CD56 (Brilliant Violet 605-conjugated anti-CD56, clone HCD56; BioLegend). On the basis of the percentage of CD3⁻CD56⁺ NK cells, PBMCs containing 1.2 to 3.0 × 10⁵ NK cells were mixed with tumor cells coated with 0.1 to 1,000 µg/mL of rituximab at a NK cell-to-target ratio (1:2) in sterile 24-well plates with RPMI-1640 + 10% FBS. Cells were incubated for 4 hours at 37°C, 5% CO₂. For CD107a detection, 5 µL/mL of Pacific Blue-conjugated anti-CD107a was added to the mixture of PBMCs and antibody-coated tumor cells in each well at the beginning of the incubation. For intracellular IFNγ staining, brefeldin A (BioLegend) was added to the mixture of PBMCs and antibody-coated tumor cells in each well after 1 hour of incubation. Fixation was performed after staining cell-surface markers, and permeabilization was performed at the time of IFNγ staining, according to the manufacturer's instructions (Fix & Perm Cell Permeabilization Kit; Invitrogen Life Technologies).

NK-cell subset analysis

To interrogate the relative responsiveness of individual NK-cell subsets expressing a single KIR (spKIR), we designed a panel of fluorochrome-conjugated antibodies specific for individual NK-cell receptors. To evaluate and control for the influence of cognate KIR and HLA interactions endowing effector function to individual NK-cell subsets, we used *HLA* genotyping and HLA class I KIR ligand assignment to identify the presence or absence of the ligand for the inhibitory KIR. NK cells exclusively expressing an inhibitory KIR from a subject lacking the cognate HLA class I ligand were considered "unlicensed" spKIR-expressing NK cells. For NK-cell phenotyping, a combination of the following antibodies was used: APC-eFluor 780-conjugated anti-CD3 (clone SK7; eBioscience), PerCp-conjugated anti-CD3 (clone SK7; BioLegend), Brilliant Violet 605-conjugated anti-CD56 (clone HCD56; BioLegend), APC-Cy7-conjugated anti-CD16 (clone 3G8; BioLegend), FITC-conjugated anti-KIR2DL1 (clone 143211; R&D Systems), Alexa Fluor 700-conjugated anti-KIR3DL1 (clone DX9; BioLegend), PE-Cy7-conjugated anti-KIR2DL2/L3/S2 (clone GL183; Beckman Coulter), APC-conjugated anti-NKG2A (clone Z199; Beckman Coulter), and PE-conjugated anti-KIR3DL2 (clone DX31; UCSF Hybridoma Core). Unstained controls, isotype-matched Ig controls, single-color controls (BD Compbeads), and FMO controls were used for multicolor compensation and gating. The isotype-matched Ig control for CD107a is Pacific Blue-conjugated mouse IgG1 (MOPC-21; BioLegend); IFNγ staining was performed by using an isotype-matched Ig control or anti-IFNγ antibody combined with anti-CD3, anti-CD56, and anti-KIR antibodies, allowing precise gating of IFNγ⁺ cells.

Gating strategy

Cells were analyzed on an LSRFortessa flow cytometer (BD Biosciences) using FACSDiva software. Data were further processed with FlowJo software (v9.5.2; Tree Star, Inc). Lymphocytes were gated on forward and side light scatter parameters after excluding doublets. NK-cell subsets exclusively

Table 2. Frequency of NK-cell populations and HLA KIR ligand status in healthy study subjects

Subject number	Percentages of single-positive NK-cell subsets (%)					HLA KIR ligands		
	KIR2DL2/L3	KIR2DL1	KIR3DL1	KIR3DL2	KIR ⁻	HLA-B	HLA-C	HLA-A
1	3.23	2.82	1.97	3.06	8.22	Bw4/Bw6	C2C2	24,32
2	6.56	5.30	5.80	2.98	18.20	Bw4/Bw6	C1C1	03,26
3	5.52	0.41	0.78	1.52	2.78	Bw6/Bw6	C1C1	02,02
4	7.56	5.06	1.06	3.69	11.40	Bw4/Bw6	C2C2	03,26
5	22.4	4.78	0.00	2.75	17.30	Bw6/Bw6	C1C1	02,24
6	6.85	1.48	0.71	1.89	5.49	Bw4/Bw6	C1C2	02,11
7	9.45	1.43	0.97	3.27	6.13	Bw4/Bw6	C1C1	03,29
8	4.97	2.91	1.96	7.10	11.20	Bw6/Bw6	C1C1	02,24
9	4.41	1.81	3.85	1.37	12.70	Bw4/Bw6	C2C2	02,30
10	9.17	6.70	2.58	1.61	14.40	Bw6/Bw6	C1C1	02,11
11	2.32	1.24	1.54	0.44	2.30	Bw6/Bw6	C1C2	11,31
12	3.00	1.21	2.58	2.74	7.54	Bw4/Bw6	C1C2	02,31
13	5.78	9.56	0.00	4.11	16.70	Bw4/Bw6	C1C2	03,30
14	3.76	4.81	0.00	1.88	4.94	Bw4/Bw6	C1C2	02,68
15	8.01	1.23	1.02	1.09	5.17	Bw4/Bw6	C1C2	02,11
16	1.77	1.45	0.62	1.22	3.57	Bw4/Bw4	C1C2	01,02
17	9.51	5.20	5.61	1.53	16.80	Bw4/Bw6	C1C1	03,26
18	8.24	1.01	4.09	3.05	10.10	Bw4/Bw6	C1C1	03,24
19	5.12	2.01	1.44	3.61	9.35	Bw4/Bw6	C1C1	02,24

NOTE: KIR ligands are assigned on the basis of HLA class I genotyping. NK-cell subsets are NKG2A⁻.

expressing a single inhibitory KIR were detected after gating on the CD3⁻CD56⁺ NK-cell population (Supplementary Fig. S1).

Cytotoxicity assay

We performed cytotoxicity assays using sorted NK-cell populations and PKH-26-labeled 721.221 lymphoblasts to validate the degranulation and cytokine measurements, as previously described (12, 28). Both direct killing and antibody-dependent cellular cytotoxicity (ADCC) were tested, in triplicates, using labeled target cells coated with or without 10 µg/mL of rituximab and mixed with unlicensed NK cells at an effector:target cell ratio of 1:1, 2:1, and 5:1. Dead 721.221 lymphoblast target cells were defined as TO-PRO3 + PKH⁻26⁺, after subtracting spontaneous death of 721.221 cells cultured without NK cells.

Statistical analysis

Patients were classified on the basis of the presence or absence of cognate HLA class I ligand (determined by HLA genotype) for inhibitory KIR (determined by KIR genotype). HLA-A and HLA-B alleles were grouped on the basis of the presence or absence of the Bw4 epitope; HLA-C status was characterized by the presence of HLA-C1 alleles only, HLA-C2 alleles only, or both. "Missing ligand" was defined as the absence of cognate HLA class I ligand in the presence of the gene encoding its corresponding inhibitory KIR. The primary outcomes for this study were complete response and overall best response (29). The secondary endpoint was progression-free survival (PFS), defined as time from study entry to pro-

gression, relapse, or death, whichever occurred first. Probabilities of PFS were estimated using the Kaplan–Meier method, and the log-rank test was used to evaluate differences in survival distributions based on ligand status. To investigate statistically significant differences in NK-cell function, NK-cell subsets were compared using the Mann–Whitney, Wilcoxon, or Kruskal–Wallis tests. All *P* values reported in this article are two sided. Statistical analyses were conducted by the Alliance Statistics and Data Center, using data collected through November 2012.

Results

Estimation of the size of NK-cell subpopulations

We used an exclusion gating technique to enumerate specific NK-cell populations expressing a single KIR (Supplementary Fig. S1). We focused on five NK-cell subsets: spKIR2DL2/3, spKIR2DL1, spKIR3DL1, spKIR3DL2, and KIR⁻. We operationally defined "KIR⁻" NK cells as lacking KIR2DL1, 2DL2/S2, 2DL3, 3DL1, 3DL2, and NKG2A. We used HLA class I genotyping and KIR ligand assignment to further characterize spKIR⁺ NK cells (NKG2A⁻) as either "licensed" (cognate HLA class I ligand present in the subject) or "unlicensed" (subjects lacking cognate HLA class I ligand). The frequency of spKIR⁺ and KIR⁻ NK cells in the 19 study subjects was variable (Table 2), consistent with prior reports (21, 30, 31). The proportion of immature CD56^{bright}CD16^{dim} and mature CD56^{dim}CD16^{bright} NK cells within KIR⁻, unlicensed, and licensed NK-cell subsets was comparable (Supplementary Fig. S4). To assess and compare NK-cell subset responses within each subject, we

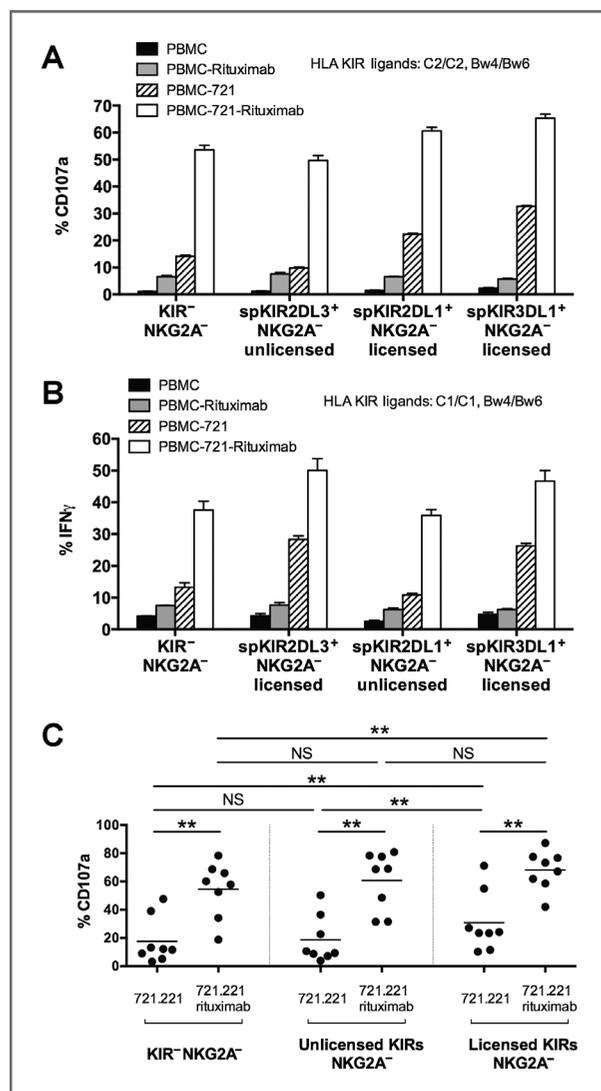


Figure 1. Rituximab triggers degranulation and IFN γ release from hypo-responsive KIR⁻NKG2A⁻ and unlicensed spKIR⁺NKG2A⁻ NK cells. A, the vertical axis depicts the percentage of NK cells expressing CD107a (a marker of degranulation) after 4 hours of incubation with CD20⁺ B-cell lymphoblasts (721.221) \pm rituximab. The KIR⁻NKG2A⁻ and unlicensed spKIR2DL3⁺NKG2A⁻ NK-cell subsets responded poorly to CD20⁺ lymphoblasts alone, but coating the lymphoblasts with 10 μ g/mL of rituximab for 30 minutes activated these hypo-responsive NK-cell subsets. B, intracellular IFN γ responses between KIR⁻NKG2A⁻ and unlicensed spKIR2DL1⁺NKG2A⁻ NK cells from subject #2 (HLA-C1/C1, Bw4/Bw6) were consistent with the degranulation responses, with a 2- to 3-fold increase in the percentage of hypo-responsive, KIR⁻NKG2A⁻, and spKIR2DL1⁺NKG2A⁻ NK-cell subsets releasing IFN γ after the addition of rituximab. C, aggregate degranulation responses of NK cells exclusively expressing an unlicensed KIR, licensed KIR, or KIR⁻NKG2A⁻ from 8 subjects with distinct KIR-HLA genotypes and circulating NK-cell repertoires. Data were combined into three functional groups: NK cells lacking KIR2DL1, 2DL2, 2DL3, 3DL1, and NKG2A (KIR⁻NKG2A⁻); NK cells expressing unlicensed KIR (spKIR3DL1⁺HLA-Bw6/6, KIR2DL1⁺HLA-C1/C1, or KIR2DL2/3⁺HLA-C2/C2); and NK cells expressing licensed KIR (spKIR3DL1⁺HLA-Bw4, KIR2DL1⁺HLA-C2, or KIR2DL2/3⁺HLA-C1).

(Legend continued in the following column.)

measured tumor-triggered degranulation (CD107a) and cytokine production (IFN γ) of spKIR⁺ NK-cell populations after excluding NKG2A and other KIR (Supplementary Fig. S1).

Rituximab stimulates robust degranulation and IFN γ secretion from KIR⁻NKG2A⁻ and unlicensed spKIR⁺NKG2A⁻ NK cells

To control for interindividual variability in NK-cell responses, we compared NK-cell responses within subjects possessing both licensed and unlicensed spKIR⁺ populations. For the functional experiments, we used KIR and HLA class I genotyping to deliberately select subjects lacking a self-HLA class I ligand for an inhibitory KIR. As expected, the NK-cell repertoire had variable responses to the CD20-expressing, HLA class I-deficient B-cell lymphoblast 721.221 cell line (and K562 cell line), consistent with a role for cognate NK-cell KIR-HLA interactions in endowing a "missing self" response to HLA class I-deficient lymphoid and myeloid tumors (Fig. 1A-C and Supplementary Fig. S2). In direct 721.221 cell killing assays, licensed spKIR⁺ NK cells showed stronger responses than unlicensed spKIR⁺NKG2A⁻ NK cells and KIR⁻NKG2A⁻ NK cells. Adding rituximab to CD20⁺ 721.221 cells, however, allowed all NK cells to become activated, triggering NK-cell degranulation from the hypo-responsive subsets of unlicensed NK cells expressing a spKIR2DL3 receptor (NKG2A⁻) and KIR⁻NKG2A⁻ NK cells (Fig. 1A). Confirming the NK-cell degranulation responses, rituximab-coated B-lymphoblasts similarly triggered IFN γ release from hypo-responsive KIR⁻NKG2A⁻ and unlicensed spKIR⁺NKG2A⁻ NK cells, and increased cytotoxicity mediated by unlicensed NK cells (Fig. 1B and Supplementary Fig. S3). The presence of NKG2A minimally enhanced the responsiveness of NK cells to rituximab-coated 721.221 (data not shown). Aggregating the analysis for 8 subjects, rituximab consistently augmented the activity of previously tolerant, hypo-responsive NK cells and abolished the functional hierarchy observed when stimulating NK cells with tumor alone (Fig. 1C). These data suggest that coating hematologic tumors with antitumor antibody may overcome intrinsic mechanisms of NK-cell tolerance, unleashing the full repertoire of NK cells for a therapeutic benefit.

Rituximab augments the activity of hypo-responsive NK cells against the Raji Burkitt lymphoma cell line

To test whether the antibody-mediated triggering of hypo-responsive NK cells was restricted to rituximab-coated HLA class I-deficient 721.221 B lymphoblasts, we evaluated NK-cell activity in KIR-ligand compatible HLA-Bw6/6 subjects against the patient-derived, CD20-expressing, HLA-Bw6/6 Raji Burkitt lymphoma cell line, which we confirmed lacks expression of HLA-Bw4 by staining with anti-HLA-Bw4-specific mAb (data not shown). Consistent with results using the 721.221 cell line, unlicensed spKIR3DL1⁺NKG2A⁻ NK cells from an HLA-Bw6/

Statistical comparisons were made using the Wilcoxon test; **, $P < 0.01$. In subjects with more than one unlicensed or licensed KIR⁺ NK-cell subset, the dot represents the total number of CD107a⁺ spKIR NK cells divided by the number of spKIR NK cells. Experiments were performed in triplicate; horizontal bar represents mean, with standard errors of mean.

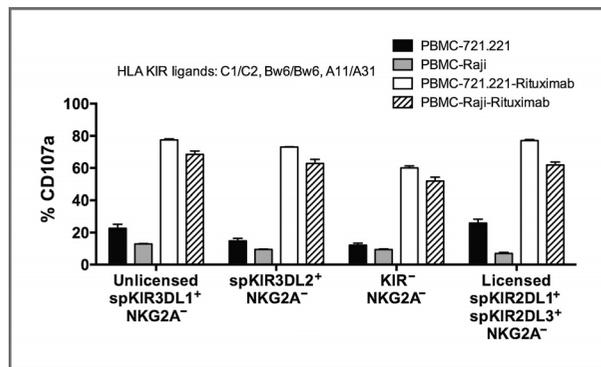


Figure 2. Coating Raji B-cell lymphoblasts with rituximab overcomes intrinsic NK-cell tolerance of the unlicensed spKIR3DL1⁺NKG2A⁻, KIR⁻NKG2A⁻, and hyporesponsive spKIR3DL2⁺NKG2A⁻ NK-cell subsets. Percentage of NK cells expressing cell-surface CD107a exclusively expressing spKIR3DL1, spKIR3DL2, and NK cells lacking inhibitory KIR expression for self-HLA using subject #11 (*HLA-C1/C2, Bw6/6, A11/A31*) after 4 hours of incubation with a different CD20-expressing B-lymphoblast cell line (Raji) confirmed to match the HLA-B KIR ligand status of subject 11. Unlicensed spKIR3DL1⁺NKG2A⁻, spKIR3DL2⁺NKG2A⁻, and KIR⁻NKG2A⁻ NK cells have minimal "missing self" activity against CD20⁺ HLA-class I-deficient 721.221 and HLA-Bw6/6 Raji B lymphoblasts. Incubating both CD20⁺ B lymphoblasts with 10 μg/mL of rituximab stimulates robust degranulation from these hyporesponsive unlicensed NK-cell subsets.

Bw6, C1/C2 subject were tolerant of Raji B-lymphoblast cells (Fig. 2). In contrast, coating Raji tumors with rituximab stimulated robust degranulation by the unlicensed spKIR3DL1⁺NKG2A⁻ NK cells (Fig. 2). Unlicensed spKIR3DL2⁺NKG2A⁻ NK cells had a similar response. This antibody-induced activation of hyporesponsive NK cells by rituximab likely reflects the uniquely potent calcium flux and signaling cascade triggered by CD16 ligation (22).

Unlicensed NK-cell ADCC activity does not require accessory cells, and is modified by antibody dose and IL2

Cytokines can overcome NK-cell requirements for licensing and may independently augment the effector function of hyporesponsive NK cells (11, 13, 32). To examine the requirement of cytokine signaling, and to eliminate the confounding influence of cytokine production by accessory cells, we sorted NK cells before incubation with antibody-coated tumors and found similar rituximab-mediated triggering of unlicensed spKIR2DL1⁺NKG2A⁻, unlicensed spKIR3DL1⁺NKG2A⁻, and KIR⁻NKG2A⁻ NK cells (Fig. 3A). To address the specific contribution of IL2, and to ask whether cytokines can enhance the antibody-dependent effector function of previously tolerant NK cells, PBMCs were primed overnight with 1,000 U/mL of IL2 before the addition of tumor targets. Hyporesponsive NK cells expressing KIR without self-MHC class I ligands did not require IL2 to degranulate in response to rituximab-coated Raji tumor cells, but IL2 augmented the unlicensed NK-cell ADCC activity compared with resting unlicensed NK cells (Fig. 3B). IL2 alone was sufficient to endow a degranulation response of hyporesponsive NK cells to direct stimulation by CD20⁺ Raji tumors, in the absence of rituximab (Supplementary Fig. S5). Titration of rituximab revealed that the majority (≥55%) of

both licensed and unlicensed NK cells can be activated with low and subtherapeutic concentrations of rituximab (Fig. 3C), but higher rituximab concentrations were required to generate comparable responses from unlicensed and licensed NK cells (Fig. 3D; refs. 33, 34). These data confirm that cytokines and accessory cells are not required for rituximab-dependent activation of unlicensed NK cells, and that activation of unlicensed NK cells may contribute to the clinical dose-response seen in patients treated with rituximab (35).

KIR3DL2⁺ NK cells are activated by rituximab

KIR3DL2 is encoded by a framework KIR gene present in all humans, expressed on 18% to 27% of NK cells, and reported to bind HLA-A3 and HLA-A11 ligands in the presence of specific peptides (36). The function and contribution of spKIR3DL2⁺ NK cells, however, are unclear (36, 37). NK cells exclusively expressing KIR3DL2 from HLA-A3 or HLA-A11 healthy individuals are minimally responsive (37, 38). To determine whether KIR3DL2⁺ NK cells contribute to the ADCC response, we performed exclusion gating to isolate the function of spKIR3DL2⁺NKG2A⁻ NK cells lacking KIR3DL1, KIR2DL1, KIR2DL2/3/S2, and NKG2A using NK cells from subjects with and without the cognate HLA-A3 or HLA-A11 ligand. Similar to KIR⁻NKG2A⁻ and unlicensed spKIR⁺NKG2A⁻ NK cells, the 721.221 B lymphoblasts alone triggered minimal degranulation, but the addition of rituximab activated the hyporesponsive spKIR3DL2⁺NKG2A⁻ NK-cell subset independent of HLA-A3 or HLA-A11 (Fig. 4). The expression of KIR3DL2 on unlicensed NK cells expressing another KIR without a self-MHC class I ligand did not significantly alter the responsiveness (data not shown). The abundant KIR3DL2⁺ NK-cell subset, therefore, may require potent stimulation through CD16 for activation, and may contribute to the aggregate NK-cell response in patients treated with rituximab.

"Missing KIR ligand" is associated with PFS in patients with follicular lymphoma treated with rituximab

To test whether the ability of rituximab to activate unlicensed NK cells (without cytokines, transplantation, or chemotherapy) is clinically significant, we genotyped 101 patients with follicular lymphoma treated with mAb alone [CALGB protocols 50402 (rituximab with galiximab) and 50701 (rituximab with epratuzumab); refs. 23, 24] and compared PFS between patients with and without a "missing KIR ligand" genotype (Table 1; Supplementary Fig. S6). The median PFS estimate for all patients was 3.74 years. We hypothesized that patients with a "missing KIR ligand" genotype will uniquely benefit from unlicensed NK-cell ADCC. Consistent with this hypothesis, we found a statistically significant difference in PFS among the KIR ligand groups (Fig. 5). Patients with follicular lymphoma lacking the *HLA-A11* ligand for *KIR3DL2* had a higher probability of remaining alive and progression free compared with patients possessing *HLA-A11* for the inhibitory *KIR3DL2* [Fig. 5B; HR, 0.29; *P* < 0.01]. We observed a similar association for patients lacking the *HLA-C2* ligand for *KIR2DL1* (Fig. 5C; HR, 0.48; *P* = 0.04). A difference in PFS among patients lacking the *HLA-Bw4* ligand for *KIR3DL1* was marginally statistically significant (PFS, 74% vs. 47%; HR, 0.55;

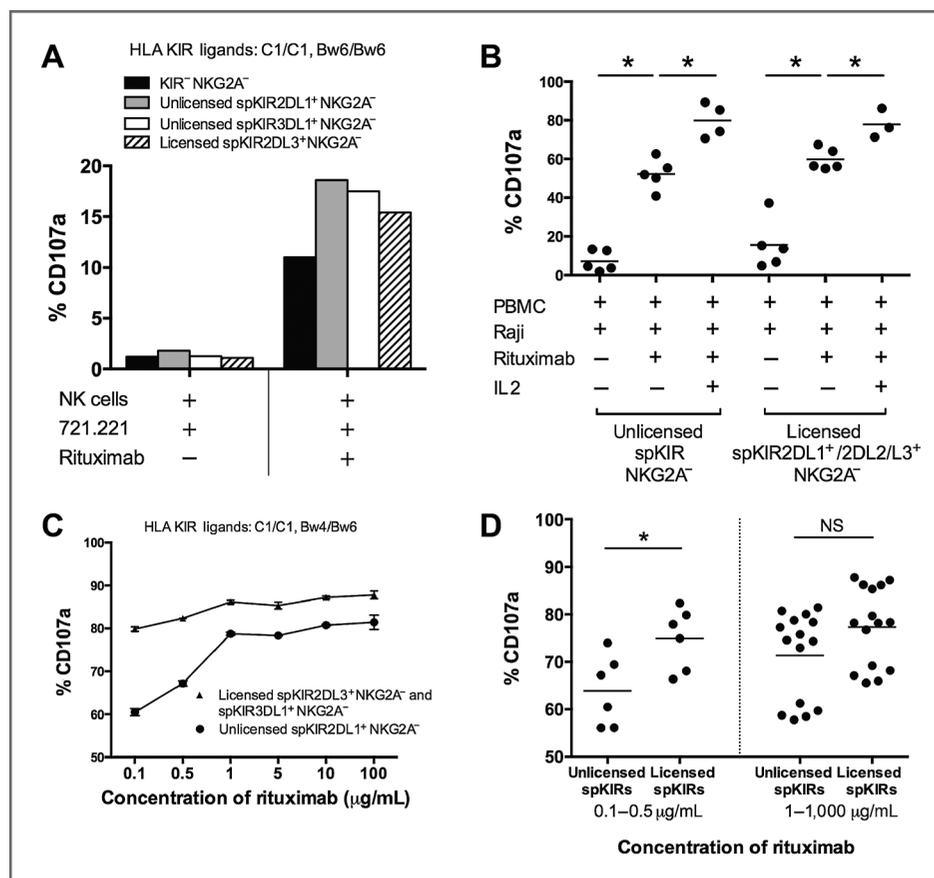


Figure 3. Rituximab alone is sufficient to activate unlicensed NK cells, which are augmented by IL2 and higher doses of rituximab. **A**, to determine whether cytokines secreted from other mononuclear cells within the PBMC population are required for rituximab-dependent activation of unlicensed NK cells, NK cells from subject #8 (*HLA-C1/C1, Bw6/Bw6*) were purified by antibody-coated magnetic bead sorting and incubated for 4 hours with rituximab-coated 721.221 B lymphoblasts. The CD107a response of NK-cell subsets is shown; similar results were seen using subject #2 (data not shown). **B**, to evaluate requirements for IL2, PBMCs from subjects #2, #1, #8, and #11 were incubated overnight with or without 1,000 U/mL of IL2 before coculture with rituximab-coated Raji cell lines. **C**, escalation of rituximab concentration demonstrates dose-dependent activation of unlicensed NK cells (subject #17). **D**, NK cells from 3 study subjects (#17, #18, and #19) were stimulated *in vitro* with increasing concentrations of rituximab and a fixed NK-cell to 721.221 cell ratio of 1:2. CD107a was measured on individual NK-cell subsets and licensing was determined on the basis of KIR expression and HLA class I genotyping. Statistical significance was defined as $P < 0.05$. Experiments were performed in duplicate for each study subject.

$P = 0.09$; Fig. 5D). Patients lacking both the *HLA-C2* and *HLA-Bw4* ligands had the highest probability of remaining alive and progression free (PFS, 88%; HR, 0.75; $P = 0.02$; Fig. 5E). We observed an association of PFS with *HLA-C2* gene dose, consistent with prior reports (39–41), such that *HLA-C1/C1* homozygosity is associated with longer PFS, followed by *HLA-C1/C2* heterozygosity, and *HLA-C2* homozygosity was associated with the highest probability of disease progression (76% vs. 49% vs. 37%; HR, 0.60; $P = 0.04$). Differences in response rates between the different groups were not statistically significant (data not shown).

Discussion

ADCC is one of several mechanisms contributing to clinical responses in patients treated with rituximab, but the immunogenetic factors modulating cellular responses to rituximab remain unclear. A deeper understanding of the functional impact of KIR and HLA diversity on NK-cell

responses to rituximab-coated tumors may help predict clinical responses to rituximab and other mAbs. We specifically sought to determine how the rules of NK-cell licensing modulate the responsiveness of NK-cell subsets to rituximab. The licensing model predicts a hierarchical NK cellular response, dominated by the NK-cell subset expressing an inhibitory KIR for self-HLA, which we confirmed in the absence of rituximab, when NK cells encounter HLA class I-deficient cells, consistent with prior studies (11–14, 42). Coating CD20-expressing transformed B cells with rituximab, however, activates NK cells lacking an inhibitory KIR for self-HLA. Activation of these unlicensed NK cells may contribute to the "missing KIR ligand" benefit that we observed in patients with follicular lymphoma treated with rituximab-containing mAb combinations.

Rituximab and other antitumor antibodies provide a potent NK-cell stimulus through CD16 (FcγRIII), the low-affinity IgG receptor, and unlike other activating NK-cell receptors, CD16 ligation alone triggers fast calcium flux, cytokine secretion, and

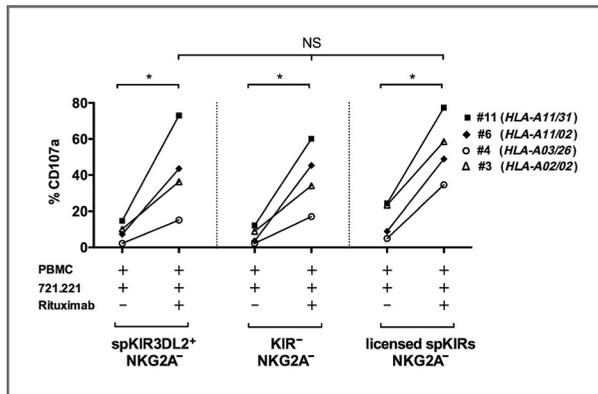


Figure 4. Hyporesponsive NK cells exclusively expressing KIR3DL2 are activated by rituximab. Shown is the degranulation (CD107a) response by NK cells exclusively expressing KIR3DL2 induced by 721.221 B lymphoblasts with or without rituximab. CD107a results from 4 subjects were aggregated: subject #11 (HLA-A11/31), #6 (HLA-A11/02), #4 (HLA-A3/26), and #3 (HLA-A02/02). Similar to the KIR⁻ NKG2A⁻ (shown) and unlicensed spKIR⁺ NKG2A⁻ (not shown) NK cells, NK cells exclusively expressing KIR3DL2 were hyporesponsive to 721.221 B lymphoblast tumor cells alone, but degranulated after the addition of rituximab, comparable with the licensed NK-cell subset. Statistical comparisons were made using the Mann–Whitney and Kruskal–Wallis tests; *, $P < 0.05$. The presence of the HLA-A11 (subjects #11 and #6) or HLA-A3 (subject #4) ligand did not influence the antibody-dependent activation of the spKIR3DL2⁺ NK-cell subset.

cytotoxicity of resting NK cells (22). We, therefore, hypothesized that rituximab exploits this unique signaling feature of CD16. We show that IL2 is not required for mAb to activate unlicensed NK cells as unlicensed NK cells sorted from supportive accessory cells can mediate a similar effect, and that antitumor ADCC mediated by unlicensed KIR3DL2⁺ NK cells may contribute to antibody responses. These data shed light on the licensing model, and suggest that the rules governing NK-cell responses to antibody-coated tumors may differ from the rules governing direct antitumor responses.

Because the population of KIR⁻ and unlicensed NK cells within the total NK-cell repertoire is large, rituximab's ability to activate these previously tolerant, hyporesponsive NK cells may be a fundamental mechanism contributing to the rituximab clinical responses. Our analysis of the NK-cell repertoire is consistent with the literature, demonstrating significant interindividual variability in spKIR⁺ and KIR⁻ NKG2A⁻ NK-cell populations (11, 12, 21, 31, 38, 43). By combining single-cell exclusion gating with KIR phenotyping, HLA genotyping, and HLA class I KIR ligand assignment, we also demonstrate that licensed NK cells may comprise a minority of the NK-cell population, depending on an individual's HLA genotype. In the absence of rituximab, therefore, NK-cell responses to CD20⁺ tumors may be limited by the small subpopulation of licensed NK cells that degranulate after direct exposure to tumors. Rituximab's ability to increase the number of activated NK cells by circumventing a licensing requirement may augment the magnitude of NK-cell responses to CD20⁺ tumors, similar to the role of naive T-cell frequencies in modulating the immune response to viruses and vaccines (44–47). Moreover, the potential for KIR⁻ NKG2A⁻ and unlicensed NK cells to

evade inhibition by HLA class I ligand expression on tumors may result in superior clinical responses in patients with greater numbers of these uninhibited NK-cell subsets, as suggested in other model systems (16, 21, 48).

Little is known about the functional role of spKIR3DL2⁺ NK cells, and published data suggest that spKIR3DL2⁺ NK cells are hyporesponsive regardless of the presence of the HLA-A3 or HLA-A11 ligand (36–38). The ability of rituximab to activate NK cells expressing KIR3DL2 and the clinical association of a "missing KIR3DL2 ligand" with improved PFS suggest that KIR3DL2⁺ NK cells are competent, do not require cognate HLA-A3 or HLA-A11 ligand recognition for antibody-dependent activation, and may mediate clinically meaningful responses in patients treated with rituximab. Expression of HLA-A ligands on tumors may impair KIR3DL2 NK-cell function (38), such that patients lacking HLA-A ligands for KIR3DL2 may benefit from a lack of KIR3DL2 inhibition. Because we used MHC class I–low or MHC class I–negative tumor targets in our *in vitro* experiments, we were unable to specifically address the role of HLA expression on tumors. Moreover, whether differences in HLA-A3 and HLA-A11 expression or binding to KIR3DL2 explain different clinical responses among HLA-A3⁺ and HLA-A11⁺ follicular lymphoma patients merits further investigation.

The *in vivo* NK-cell response to rituximab likely occurs in phases, similar to T-cell immune responses (49), with NK-cell expansion, contraction, and memory formation after initial activation (50). Our studies focus on the early activation phase, in which unlicensed and licensed NK cells appear to have comparable early degranulation and IFN γ responses to rituximab-coated transformed cells. Whether an advantage of unlicensed NK cells emerges in later phases of the *in vivo* response to rituximab is unclear, but suggested by prior studies (16, 21). Our experiments, performed in the absence of cytokines and validated with sorted NK cells, suggest that a highly inflammatory environment like mouse CMV infection or combination chemotherapy with mAb therapy may not be necessary for circumventing the requirement of MHC education (15, 16, 21). Antitumor antibodies, therefore, may be a uniquely targeted strategy for breaking tolerance and triggering activity from hyporesponsive NK cells, while avoiding toxicities of cytokines and inflammation.

Our finding that rituximab activates a large population of NK cells by overcoming a requirement for HLA-dependent NK-cell licensing may inform strategies for augmenting clinical remissions. Cognate inhibitory KIR–HLA binding plays a dual, paradoxical role in controlling NK-cell responses: endowing effector function and inhibiting NK-cell responses (12, 14, 16, 51). Because the presence of an HLA class I ligand for inhibitory KIR is not required for rituximab-dependent NK-cell responses, binding cognate ligand may actually lead to NK-cell exhaustion and impede clinical responses mediated by NK cells (52). In neuroblastoma patients treated with combination anti-GD2 chemioimmunotherapy, licensed NK-cell inhibition is the dominant result of cognate KIR–HLA binding (21). Blocking inhibitory KIR–HLA interactions to trigger licensed NK-cell responses is feasible in the clinic (53–55), and a rationale augmentation strategy for patients treated with rituximab,

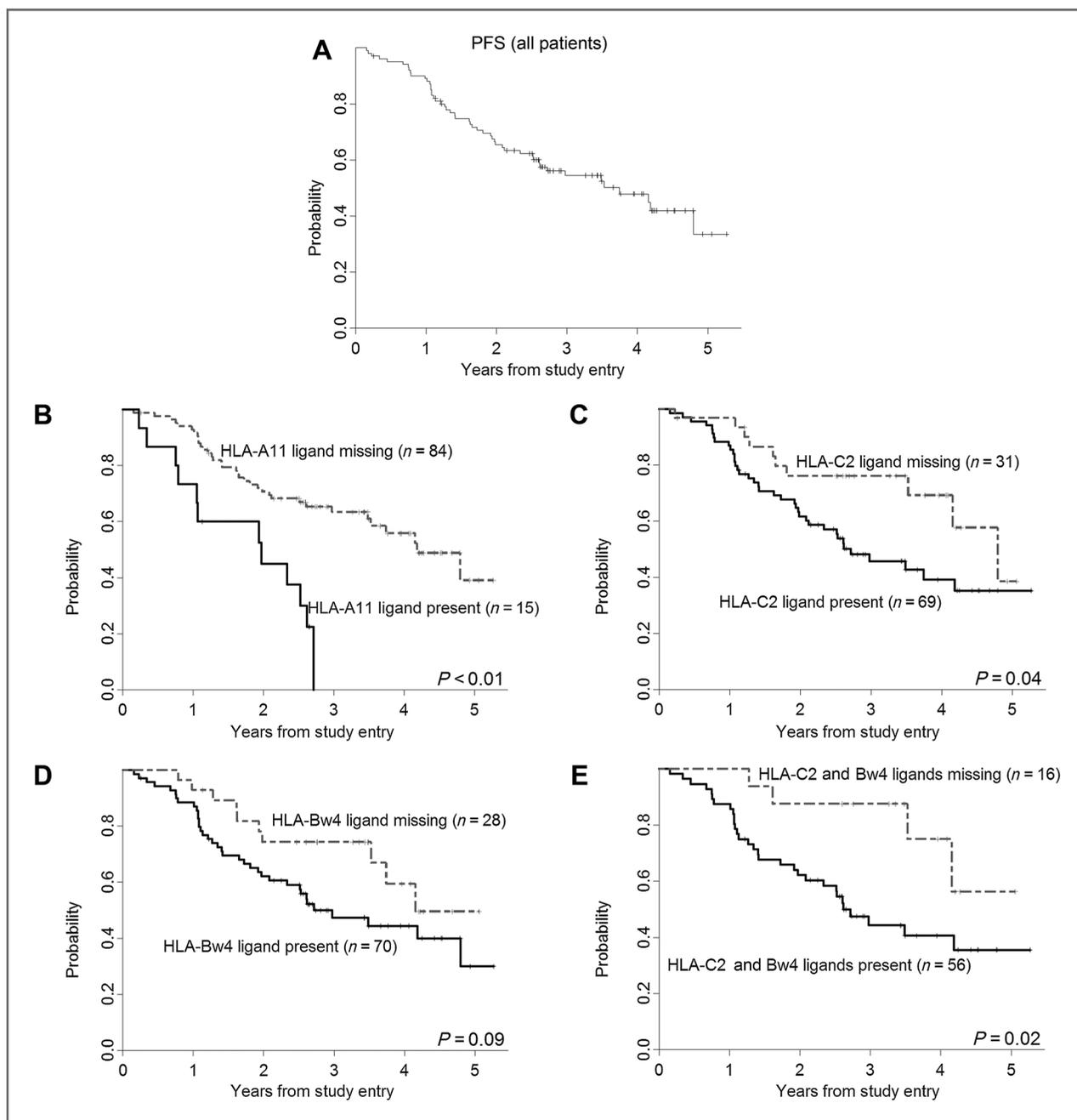


Figure 5. Influence of a "missing ligand" genotype on PFS in patients with follicular lymphoma treated with rituximab antibody combinations. A, PFS among patients with follicular lymphoma treated with rituximab-containing mAb combinations, and stratified according to the presence or absence of HLA-A11 (B), missing the HLA-C2 ligand for KIR2DL1 (C), missing the HLA-Bw4 ligand for KIR3DL1 (D), and the presence or absence of both HLA-C2 and HLA-Bw4 ligands (E).

although responses likely depend on the frequency of licensed NK cells in patients, which was highly variable in our study subjects. IL2 might also augment rituximab NK-cell responses, as demonstrated previously (56), although prevention of regulatory T-cell expansion may be required for optimal clinical benefit (57, 58). In aggregate, these data highlight the importance and feasibility of understanding the requirements for capturing the antitumor potential of the NK-cell repertoire,

which may facilitate safer, more effective, and personalized treatment decisions for patients with cancer.

Disclosure of Potential Conflicts of Interest

L.L. Lanier is a consultant/advisory board member for Bristol-Myers Squibb, Merck, Novo Nordisk, Ascend, Five Prime Therapeutics, Avidbiotics, and SBI Biotech. J.M. Venstrom reports receiving a commercial research grant from Sanofi Oncology and has received speakers bureau honoraria from Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Du, B.N. Pitcher, J. Johnson, S.-H. Jung, L. Zhou, L.L. Lanier, J.M. Venstrom
Writing, review, and/or revision of the manuscript: J. Du, S. Lopez-Verges, B.N. Pitcher, J. Johnson, S.-H. Jung, L. Zhou, K. Hsu, M.S. Czuczman, B. Cheson, L. Kaplan, L.L. Lanier, J.M. Venstrom
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Zhou, J.M. Venstrom
Study supervision: S.-H. Jung, J.M. Venstrom

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