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); 1–12. ©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 rituximab, 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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Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

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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. Forty-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).

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

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<tr>
<td></td>
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<td>Rituximab + galiximab (n = 46)</td>
<td>P&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
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<td>Ethnicity</td>
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<td>Caucasian</td>
<td>50/53 (94.3%)</td>
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<tr>
<td>Non-Caucasian</td>
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<td>1 (2.2%)</td>
<td></td>
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<tr>
<td>Median age, y (range)</td>
<td>54 (32–90)</td>
<td>58 (22–84)</td>
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<tr>
<td>Female gender</td>
<td>32 (58.1%)</td>
<td>16 (34.8%)</td>
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</tr>
<tr>
<td>LDH &gt; ULN</td>
<td>5 (9.0%)</td>
<td>7 (15.2%)</td>
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</tr>
<tr>
<td>&quot;B&quot; symptoms</td>
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</tr>
<tr>
<td>I</td>
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<td>20/45 (44.4%)</td>
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</tr>
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<td>23 (50.0%)</td>
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<td>Negative</td>
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<td>23 (50.0%)</td>
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<td>8/40 (20.0%)</td>
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<td>10/45 (22.2%)</td>
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Abbreviations: FLIPI, Follicular Lymphoma International Prognostic Index; LDH, lactic dehydrogenase; ULN, upper limit of normal.

<sup>a</sup>Pvalues for the categorical variables were computed using the Fisher exact test; the Wilcoxon rank-sum test was used to compute age differences between groups.
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\textsuperscript{CA}), C2 (HLA-C\textsuperscript{Cy\textsubscript{200}}), 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% CO\textsubscript{2} in a 24-well plate at a concentration of 3 × 10\textsuperscript{5}/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\textsuperscript{+} 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 H4A8; 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\textsuperscript{+} CD56\textsuperscript{+} 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\textsuperscript{+} CD56\textsuperscript{+} NK cells, PBMCs containing 1.2 to 3.0 × 10\textsuperscript{5} 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% CO\textsubscript{2}. 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\textsuperscript{+} 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
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 progression, 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/L3, spKIR2DL1, spKIR3DL1, spKIR3DL2, and KIR⁺/C0. We operationally defined "KIR⁺/C0" 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⁺CD16⁻NK cells and mature CD56⁺CD16⁺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

<table>
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<th>Subject number</th>
<th>KIR2DL2/L3</th>
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<th>KIR3DL2</th>
<th>KIR⁺/C0</th>
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<td>3.61</td>
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**NOTE:** KIR ligands are assigned on the basis of HLA class I genotyping. NK-cell subsets are NKG2A⁻.
Rituximab activates unlicensed NK cells

Figure 1. Rituximab triggers degranulation and IFNγ secretion from hyporesponsive KIR NK cells and unlicensed spKIR NK cells.

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.
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^+ NK cells lacking KIR3DL1, KIR2DL1, KIR2DL2/3/5, and KIR2DL3 using NK cells from subjects with and without the cognate HLA-A3 or HLA-A11 ligand. Similar to KIR 'NKGA^+ and unlicensed spKIR 'NKGA^+ NK cells, the 721.221 B lymphoblasts alone triggered minimal degranulation, but the addition of rituximab activated the hyporesponsive spKIR3DL2^+ 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;
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
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 antigen 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 IFNγ 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 chemoimmunotherapy, 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,
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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References


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