Rituximab infusion induces NK activation in lymphoma patients with the high affinity
CD16 polymorphism

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CD16 polymorphism in mAb-induced NK activation

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Abstract:
NK cell mediated antibody-dependent cellular cytotoxicity involving FcγRIIIa (CD16) likely contributes to the clinical efficacy of rituximab. To assess the in vivo effects of CD16 polymorphisms on rituximab-induced NK activation in vivo, blood was evaluated before and 4 hours after initiation of the initial dose of rituximab in 21 lymphoma subjects. Rituximab induced NK activation and a drop in circulating NK cell percentage in subjects with the high affinity [158(VF/VV)] but not the low affinity [158(FF)] CD16 polymorphism. There was no correlation between NK cell activation or NK cell percentage and polymorphisms in CD32A, C1q, or CH50. We conclude that NK activation occurs within 4 hours of rituximab infusion in subjects with the high affinity CD16 polymorphism, but not those with low affinity CD16 polymorphism. This finding may help explain the superior clinical outcome seen in the subset of high affinity CD16 polymorphism lymphoma patients treated with single agent rituximab.
**Introduction:**

Despite the remarkable success of rituximab in treating CD20⁺ malignancies¹,², there is still much we do not know about why patients respond, or do not respond, to therapy. Evidence that antibody-dependent cellular cytotoxicity (ADCC) plays a major role in the clinical activity of rituximab comes from a number of sources, including data exploring the impact of genetic polymorphisms in FcγR on rituximab effects. CD16 with valine at codon 158 (V) binds with higher affinity to human IgG1 than does CD16 with phenylalanine at codon 158 (F)³,⁴. In vitro, rituximab-coated target cells activate NK cells from subjects with the V polymorphism (VV/VF) at lower rituximab concentrations than (FF) subjects⁵. The higher affinity polymorphism also correlates with a better clinical response rate to single agent rituximab⁶-⁹. However, it was not known whether rituximab-induced NK cell activation varies as a function of CD16 polymorphisms in vivo. In the current study, we evaluated NK cells from lymphoma subjects before and 4 hours after initiation of their first dose of rituximab therapy, and assessed how CD16 polymorphisms impact on NK cell number and NK activation phenotype.
Methods:

Subject eligibility:
Subjects meeting following criteria were eligible for enrollment.
1. B cell proliferative disorder with less than 5,000 B cells per mm$^3$ in blood.
2. No rituximab therapy in the past 6 months.
3. Scheduled to receive rituximab at the standard dose (375 mg/M$^2$), either as a single agent or as part of combination therapy.
4. If the patient is to receive combination therapy, the regimen allows rituximab to be given prior to other anti-lymphoma drugs during the first course of therapy.
5. Provided informed consent as approved by the University of Iowa Institutional Review Board in accordance with the Declaration of Helsinki.

Subject characteristics are summarized in Supplementary Table 1

Sample collection and analysis:
Blood was obtained immediately prior to and 4 hours after initiation of rituximab infusion, administered by the standard procedure followed at the University of Iowa. Analysis included,
1. Complete blood cell count (CBC),
2. NK cell percentage and NK activation based on surface expression of CD56, CD16 and CD54 as described previously$^{5,10,11}$,
3. Genetic polymorphisms in CD16 (position 158)$^{5,7}$, C1q (position 276)$^{12,13}$ and CD32A (position 131)$^{7,14}$ by PCR with genomic DNA (pre-therapy sample only), and
4. CH50 (Diamedix, Miami, FL).

Statistical analysis:
Means and standard errors were computed for changes in NK cell activation, and reported separately for high and low affinity CD16 polymorphisms. Significance of mean changes and associations between markers were evaluated by paired t-tests and Pearson’s correlation coefficients, respectively. All statistical tests were two-sided and assessed for significance at 0.05 levels using the SAS 9.2 software package.
Results and Discussion:
Rituximab-induced NK cell activation was evaluated in twenty-one subjects with various B cell disorders. Only one subject was CD16 homozygous for V (VV) that was grouped with VF subjects for analysis. Clinical signs of infusion reaction\textsuperscript{15} were noticed in eight subjects (Supplementary Table 1), but did not correlate with the measured parameters. The majority of subjects had both the pre-therapy and 4 hours post-rituximab samples obtained prior to any other treatment. Four subjects had chemotherapy prior to rituximab, and three subjects had decadron premedication prior to rituximab. There was no significant difference in any of the parameters measured between subjects that received chemotherapy or decadron prior to rituximab and those that did not.

Rituximab treatment decreased total lymphocyte count within 4 hours compared to baseline in the majority of subjects (p<0.0001) and was similar for VF/VV and FF subjects (VF/VV vs FF p=0.8837) (Figure 1A and Supplementary Figure 1A). In contrast, the percent of NK cells decreased in VF/VV subjects (p<0.0001) but not FF subjects (p=0.70). The difference between VF/VV and FF subjects in the drop in NK cell percentage was statistically significant (p=0.035) (Figure 1B and Supplementary Figure 1B).

We previously demonstrated a strong in vitro correlation between rituximab-induced CD16 down modulation, CD54 (ICAM-1) upregulation, and NK-mediated ADCC\textsuperscript{5}. We therefore evaluated NK cell CD16 and CD54 expression following rituximab therapy. A trend towards CD16 down modulation was seen in VF/VV subjects (p=0.08), but not in FF subjects (p=0.83). The drop in CD16 expression for VF/VV versus FF subjects was of borderline significance (p=0.078) (Figure 1C and Supplemental Figure 1C). Rituximab induced a 3-fold increase in the percent of CD54\textsuperscript{bright} NK cells (p=0.029) in VF/VV subjects but no significant increase in CD54\textsuperscript{bright} NK cells in FF subjects (p=0.51). The difference between VF/VV subjects and FF subjects in fold-change in CD54\textsuperscript{bright} NK cell percentage was statistically significant (p=0.023) (Figure 1D and Supplemental Figure 1D).

When all subjects were considered, and when FF subjects were considered separately, there was a significant correlation between CD16 down modulation and CD54 upregulation (FF subjects. R=-0.72; p=0.018) (Table 1), which indicates mAb-induced CD16 down modulation can result in NK cell activation even in subjects with the low affinity CD16 polymorphism. There was also a trend towards a correlation between CD54 upregulation and a drop in lymphocyte
count, and CD16 down modulation and a decrease in NK cell percentage. No significant association was seen between NK activation and different lymphoma subtypes (data not shown). Additionally, NK activation did not correlate with other biomarkers, including CD32A and C1q polymorphisms or serum complement levels (data not shown).

Based on these results, we hypothesize CD54 upregulation alters NK cell trafficking to the tumor and this, along with enhanced NK cell activation, contributes to the enhanced therapeutic response as reported in subjects with the VF/VV polymorphism. Indeed, we previously reported in preliminary studies that rituximab treatment may lead to trafficking of mononuclear cells into involved lymph nodes.\(^{16}\)

There are limitations to our study. The lack of enough VV subjects prevented us from assessing whether this group of subjects responds differently to rituximab. We evaluated NK cell response just 4 hours after initiation of rituximab, but did not evaluate later time points because of possible late effects of chemotherapy. The ability to correlate the effects of CD16 polymorphisms on longer term changes in NK cell number, NK cell activation, or most importantly, clinical response and outcome, will be an important next step, even if it is complicated by concomitant chemotherapy. In addition, in the absence of more rigorous imaging studies or actual biopsies to test NK activation in the tumor per se, it is not possible to know whether the NK cells that left the circulation actually trafficked to involved lymph nodes, got activated at the site of tumor, or contributed to the anti-tumor effects. Finally, these findings may not be relevant in circumstances where polymorphisms in Fc receptors have not been shown to impact on prognosis such as Chronic Lymphocytic Leukemia.

Several next generation anti-CD20 antibodies are in various stages of development.\(^{17-20}\) A number of these have been modified so they have enhanced affinity to CD16, and are more effective than rituximab in vitro at activating NK cells and inducing ADCC irrespective of CD16 polymorphisms.\(^{17,20,21}\) It will be interesting to see whether these antibodies are able to induce rapid NK cell activation in vivo of individuals with the FF polymorphism, and most importantly, whether this translates into an improved clinical outcome.
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Authorship contributions:
S.V. – performed research, analyzed data and wrote the manuscript; S.Y.W., C.D., and S.B. – performed research; L.J. and T.K. – collected research specimens; A.B. – performed statistical analysis; B.L. – designed research; and G.J.W. – provided oversight of the research, designed research, analyzed data and wrote the manuscript.

Disclosure of conflicts of interest:
G.J.W. and B.L. serve as consultants for Genentech.
References:


Table 1: Correlation of various parameters changed by rituximab infusion:

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Figure Legends:

Figure 1: Fold change in the observed parameters at 4 hours after the initiation of rituximab infusion compared to the baseline (0 hours)

(A) Absolute lymphocyte count.
(B) Percent of NK cells in circulation.
(C) NK cell CD16 median fluorescence intensity
(D) Percent of CD54\textsuperscript{bright} NK cells

The single subject with a VV polymorphism is represented as an open square (☐).
Figure 1

(A) Absolute lymphocyte count

(B) NK cell percentage

(C) CD16 median fluorescence

(D) Percent CD54^bright^ NK cells
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