Increased natural killer cell expression of CD16, and augmented binding and ADCC activity to rituximab among individuals expressing the FcγRIIIA-158 V/V and V/F polymorphism.

Evdoxia Hatjiharissi1,2,4, Lian Xu1, Daniel Ditzel Santos1,2, Zachary R. Hunter1, Bryan T. Ciccarelli1, Sigitas Verselis3,2, Michael Modica3,2, Yang Cao1, Robert J. Manning1, Xavier Leleu1,2, Elizabeth A. Dimmock1, Alexandros Kortsaris4, Constantine Mitsiades1,5, Kenneth C. Anderson1,5, Edward A. Fox2,3 and Steven P. Treon1,2.

1 Bing Center for Waldenstrom’s Macroglobulinemia, Dana Farber Cancer Institute, 2 Harvard Medical School; Boston MA USA; 3 Molecular Diagnostics Laboratory, Dana Farber Cancer Institute; Boston MA USA; 4 School of Medicine, Democritus University of Thrace, Alexandroupolis, Greece; 5 Jerome Lipper Multiple Myeloma Center, Dana Farber Cancer Institute, Boston MA USA.

Running Title: FcγRIIIA-158 Variants and rituximab Activity

Corresponding author:
Steven P. Treon M.D., M.A., Ph.D.
Bing Center for Waldenstrom’s Macroglobulinemia
Dana Farber Cancer Institute
M548, 44 Binney Street, Boston MA 02115 USA
Tel: (617) 632-5880, Fax: (617) 632-4862
Email: steven_treon@dfci.harvard.edu

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Abstract

The presence of valine (V) at FcγRIIIa-158 (CD16) is known to improve clinical response to rituximab in indolent non-Hodgkin’s lymphoma (NHL). Little is known about the basic mechanisms for this observation. We examined natural killer (NK) cells from healthy donors representing the FcγRIIIa-158 polymorphic subgroups (V/V, V/F and F/F) for gene transcript and cell-surface CD16 expression, rituximab binding and rituximab-dependent NK-cell-mediated cytotoxicity (ADCC). We observed higher levels of FcγRIIIa transcripts among individuals with the FcγRIIIa-158 V/V versus -V/F or -F/F genotype (p<0.001); increased cell-surface CD16 expression by quantitative flow cytometry on NK-cells from individuals expressing at least one valine at FcγRIIIa-158 versus -F/F (p=0.029), as well as augmented rituximab binding and rituximab-dependent ADCC activity. These results suggest that individuals expressing at least one valine at FcγRIIIa-158 might, in part, have better clinical outcomes due to increased CD16 expression, rituximab binding, and rituximab-mediated ADCC.
Introduction

Rituximab is a CD20-directed, IgG₁-chimeric monoclonal antibody (mAb) used to treat patients with B-cell lymphomas, and various autoimmune disorders. Both quantitative as well as qualitative differences in NK-cell function may explain rituximab clinical activity. Higher circulating NK-cell levels and responses to rituximab have been reported in patients with indolent NHL suggesting that ADCC enacted by NK-cells may be a primary mechanism by which rituximab functions\(^1\)\(^2\). Moreover, responses to rituximab may depend upon polymorphisms present in the Fc\(\gamma\)RIIIA (CD16) receptor, a receptor mainly expressed on NK-cells\(^3\)\(^5\).

Polymorphisms in position 48 and 158 of the Fc\(\gamma\)RIIIA receptor expression have been reported to influence human IgG₁ binding and ADCC activity\(^6\)\(^9\). Polymorphisms at position 158 result in either valine (V) or phenylalanine (F) expression\(^6\)\(^9\), the former of which is associated with increased depletion of peripheral blood B-cells\(^10\) and response to rituximab in patients with indolent NHL\(^3\)\(^5\), but not CLL\(^11\). At position 48, polymorphisms of the Fc\(\gamma\)RIIIA receptor result in expression of either leucine, arginine or histidine, the first of which is linked to Fc\(\gamma\)RIIIA-158F, and the latter two with the Fc\(\gamma\)RIIIA-158V polymorphisms\(^5\)\(^8\)\(^9\). However, the binding of IgG₁ to Fc\(\gamma\)RIIIA appears to occur independent of position 48 polymorphisms most likely on the basis of tight genetic linkage to Fc\(\gamma\)RIIIA-158 polymorphisms\(^5\)\(^8\). Genetic linkage between polymorphisms in Fc\(\gamma\)RIIA (CD32), a receptor also implicated in predicting rituximab clinical response, and Fc\(\gamma\)RIIIA have recently been demonstrated by us, and point to the primacy of Fc\(\gamma\)RIIIA-158 polymorphisms in predicting rituximab response\(^12\).
While the above studies have suggested that variable responses to rituximab among FcγRIIIA-158 polymorphic groups are likely the result of qualitative (i.e. antibody affinity) differences, the possibility that quantitative differences in cell-surface CD16 expression, rituximab binding and ADCC activity have not been addressed. As such, we sought to delineate differences in FcγRIIIa gene expression; cell-surface CD16 expression; rituximab binding; and rituximab-dependent ADCC activity in NK-cells isolated from healthy individuals representing the three FcγRIIIa-158 polymorphic subgroups (V/V, V/F and F/F).

**Study Design**

**FcγRIIIa-158 Genotype Analysis**

We analyzed the genotype of 52 unrelated, healthy individuals by sequencing exon 4 of FcγRIIIa gene. FcγRIIIa-158 polymorphisms were determined by allele-specific RT-PCR and directed sequencing of genomic DNA, as we previously described. Genomic DNA was extracted from peripheral blood using a DNA isolation kit (Qiagen, CA, USA). The study was approved by the Dana Farber Cancer Institute’s Institutional Review Board and written consent was obtained from each donor.

**Cell Isolation and Culture**

Peripheral blood mononuclear cells (PBMN) were isolated using Ficoll-Paque (Amersham, Uppsala, Sweden). NK-cells were selected from PBMN using the NK-cell isolation kit II (Miltenyi, Auburn CA) resulting in > 95% purity (CD3−/CD56+). ARH-77 and Daudi cells were cultured as previously described.
RT-PCR Analysis

FcγRIIIa gene expression was determined by quantitative real-time RT-PCR (Applied Biosystems, Foster City CA). RNA was extracted from NK-cells. Primer sequences were as follows: FCGR3A sense (5’CCAAAAGCCACACTCAAAGAC 3’) and antisense (5’ ACCCAGGTGGAAA GAATGATG 3’), TaqMan probe (5’ AACATCACCATCACTCAA GGTTTG 3’). The quantity of FcγRIIIa mRNA in each sample was normalized to the relative quantity of HR-18S.

Quantitative Flow Cytometry

CD16 receptors were quantified using the QuantiBRITE system. NK-cells (2x10^5) were stained with 5µl (0.287mg/ml) of anti-CD16 PE bead-conjugated mAb for 20 min at 4°C (BD Biosciences, San Jose CA). After incubation, NK-cells were washed twice and resuspended in 1x PBS. Prior to each analysis, the flow cytometer was calibrated by QuantiBRITE PE calibration beads. CD16 receptors were assessed by gating 1X10^4 (CD3^-CD56^+) cells. Samples were analyzed using CellQuest™ software (BD Biosciences).

Rituximab Binding to NK-cells

Rituximab (Genentech BioOncology, San Francisco, CA) binding was determined using an indirect method as previously described, utilizing an anti-CD16 (3G8 clone) mAb. NK-cells (2x10^5) were incubated with rituximab at concentrations of 10, 50, 100 and 200µg/ml for 30 min at 4°C, followed by incubation with anti-CD16 FITC and anti-CD56 PE mAbs at 4°C for 20 minutes. After incubation, NK-cells were washed with PBS and analyzed by flow cytometry. CD16 median fluorescence intensity (MFI) was determined
by gating on CD3–CD56+ lymphocytes. Rituximab binding was defined as percentage of inhibition for binding of anti-CD16 mAb and calculated as follows: 
\[
\frac{(\text{MFI without Rituximab}) - (\text{MFI with Rituximab})}{(\text{MFI without Rituximab})} \times 100
\]

**ADCC Assays**

ADCC experiments were performed using NK-cell as effectors cells. To avoid KIR dependent ADCC, HLA class I expressing (ARH-77) and non-expressing (Daudi) cell lines (both of which are CD20+) were used as a target cells. Cells were incubated with 10µg/ml rituximab and human IgG1 (control) for 1 hour, washed twice and co-cultured (5x10^3/well) in varying ratios with effector cells for 4 hours at 37°C with 5% CO2. A colorimetric-based LDH assay (CytoTox 96®; Promega, Madison WI) was used and cytotoxicity calculated according to manufacturer’s instructions.

**Statistical Analysis**

Differences among polymorphic groups were compared by the Kruskal-Wallis and Mann-Whitney tests. The correlations between gene expression and CD16 receptors was assessed using the Pearson correlation coefficient.

**Results and Discussion**

We first analyzed the gene expression of FcγRIIIa by performing real-time RT-PCR analysis for 13 donors whose genotyping demonstrated FcγRIIIa-158 V/V (n=4), V/F (n=4), and F/F (n=5). Individuals with the FcγRIIIa-158 V/V genotype expressed higher FcγRIIIa transcript levels versus those individuals with the FcγRIIIa V/F and –F/F genotype (p<0.0001). However, no significant difference in FcγRIIIa transcripts was observed between individuals with the FcγRIIIa V/F and –F/F genotypes (Figure 1A). This observation is particularly intriguing in view of the fact that the absolute number of
CD16 receptors per NK-cell was significantly higher in donors who expressed at least one valine at FcγRIIIa-158 (i.e. were either V/V or V/F) versus F/F (p=0.029; Figure 1B). The basis for these discordant findings between FcγRIIIa transcript expression and cell-surface protein levels among individuals with FcγRIIIa-V/F remains to be clarified, but may reflect relative differences in transcript or protein stability, and/or recycling of CD16 at the cell surface imposed by the expression of valine.

We next evaluated the functional implications of FcγRIIIa-158 polymorphisms by studying rituximab binding and rituximab-dependent NK-cell-mediated cytotoxicity. Both rituximab binding (Figure 2A), as well as rituximab-mediated ADCC activity by NK-cells (Figures 2B, 2C) increased in a manner dependent on the degree of valine expression at FcγRIIIa-158: V/V>V/F>F/F at all concentrations (10-200 µg/ml) of rituximab studied. The cytotoxicity induced by the control (10µg huIgG1) was less than 10% (data not shown). These results are unlikely to be explained by KIR mismatching since HLA class I expressing (ARH-77) and non-expressing (Daudi) cell lines were used as a target cells. Independent of the FcγRIIIa-158 polymorphic subgroup, rituximab-mediated ADCC activity was observed to correlate with the number of cell-surface CD16 receptors (Figure 2D). These results are in agreement with previous studies demonstrating increased binding of IgG1-class antibodies, including rituximab among individuals expressing valine at FcγRIIIa-158 \(^6-9,13\), and suggest that the expression level of cell-surface CD16 may also contribute to augmented rituximab binding and ADCC activity, in addition to possible differences in binding affinity.
The results of these studies may help to explain augmented responses to rituximab observed with the addition of certain agents known to up-regulate CD16, including one study wherein the addition of IL-2 to rituximab appeared to selectively result in clinical responses among rituximab-refractory patients expressing FcγRIIIa-158F/F\textsuperscript{14}. Further exploration of agents aimed at augmenting CD16 expression, particularly in context with newer CD20-directed mAb bearing enhanced Fc binding and ADCC activity\textsuperscript{15,16} may lead to improved responses among patients with indolent NHL, including for those individuals expressing FcγRIIIa-158F/F.

In summary, the results of these studies suggest that individuals expressing at least one valine at FcγRIIIa-158 might in part have better clinical outcomes due to increased CD16 expression.

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**Authorship**

E.H. designed the study, performed research, analyzed the data and wrote the first draft of the manuscript. L.X., S.V., M.M. and E.A.F. designed, performed and analyzed experiments in molecular biology. D.D.S, B.T.C, Y.C. and X.L. performed various pertinent researches. C.M. contributed to the analysis and interpretation of the results. Z.R.H., R.J.M. and E.A.D collected samples and analyzed the data. A.K. and K.C.A.
contributed advice to the design and interpretation of the study. S.P.T. designed the study, oversaw the experiments and wrote the final draft of the manuscript.

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Figure Legends

Figure 1
FcγRIIIa transcript expression by real-time RT-PCR analysis (A) and cell-surface CD16 expression by quantitative flow cytometry (B) on NK cells for 13 donors whose genotyping demonstrated FcγRIIIa-158 V/V (n=4), V/F (n=4), and F/F (n=5). p=0.0001 for transcript levels for individuals with FcγRIIIa-158 V/V versus -V/F or –F/F genotype. p=0.029 for CD16 expression for individuals expressing at least one valine at FcγRIIIa-158 versus –F/F. Values represent the mean ± SE.

Figure 2
Characterization of NK cell rituximab (RTX) binding (A), and rituximab-mediated ADCC activity by NK cells against Daudi (B) and ARH-77 (C) CD20+ B-cells for 9 donors whose genotyping demonstrated FcγRIIIa-158 V/V (n=3), V/F (n=3), and F/F (n=3). Panel D depicts the correlation between cell-surface CD16 receptors and rituximab-dependent ADCC activity for these individuals.
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