Anti-RhD antibody therapy modulates human natural killer cell function

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Supplemental methods section

Cells

BW5147 cells overexpressing the extracellular domain of several NK receptors, including CD16, were generated as previously described.[1] The cells were grown in RPMI (Sigma-Aldrich) supplemented with 10% fetal calf serum (Sigma-Aldrich), 1% L-glutamine (Biological Industries (BI)), 1% sodium pyruvate (BI), 1% nonessential amino acids (BI), and 1% penicillin-streptomycin (BI).

For immature dendritic cell generation, isolated monocytes were grown in the presence of 1% autologous plasma, GMCSF (1000 U/mL), and IL-4 (500 U/mL). Every 2 days, 0.15 mL was removed, and 0.25 mL medium containing plasma and cytokines was added. LPS (10 ng/ml) was used to induce mature DCs (mDCs) in all experiments.

Antibodies

We used the following commercial polyclonal preparations: Human normal immunoglobulin (IVIG), Intratect (Biotest Pharma GMBH, Germany); human normal immunoglobulin (IVIG), KiovIg (Baxter Healthcare Corporation, USA); Rh_0(D) Immune Globulin, Rhophylac (CSL Behring, USA); Rh_0(D) Immune Globulin, KamRho (Kamada, Israel). Both anti-RhD preparations also contain non-specific antibodies. Since the anti-RhD antibodies are considered to be the active component of these compounds, the concentration given throughout the manuscript relates to the concentration of specific anti-RhD antibodies. As a control we used IVIG at a concentration which is equal to the concentration of the total antibodies of each of the anti-RhD preparations (or to the total antibody concentration of KamRho, which had the higher concentration of the two anti-RhD preparations). Unless otherwise stated, the IVIG preparation used was Intratect. We generally used an anti-RhD

concentration of 5µg/ml. For staining of the transfected BW cells with anti-RhD antibody preparations we used 0.01µg/ml of KamRho and 0.1µg/ml of Rhophylac.

We used the following antibodies for flow cytometry: APC anti-CD16 (cat. 360706, BioLegend), Non-conjugated anti-NTBA (cat. 317202, BioLegend), APC anti-NKp44 (cat. 325110, Biolegend), APC anti-DNAM-1 (cat. 338312, BioLegend), and PE conjugated anti-CD56 antibody (cat. 318306, BioLegend).

We used the following antibodies for all degranulation assays (including those involving human samples): APC conjugated anti-CD107a antibody (cat. 328620, BioLegend), PE conjugated anti-CD56 antibody (cat. 318306, BioLegend), and FITC conjugated anti-CD3 antibody (cat. 300306, BioLegend).

For secondary antibody staining we used: Alexa Fluor 647-conjugated AffiniPure F(ab')2 fragment goat anti-mouse IgG (H+L) (115-606-062, Jackson ImmunoResearch Laboratories), Alexa Fluor 647-conjugated AffiniPure F(ab')2 fragment donkey anti-human IgG, Fcγ fragment specific (709-606-098, Jackson ImmunoResearch Laboratories), and Allophycocyanin (APC)-conjugated AffiniPure F(ab')2 fragment donkey anti-human IgG, Fcγ fragment specific (709-136-098, Jackson ImmunoResearch Laboratories).

For blocking CD16, we used mouse IgG2a isotype control antibody at a concentration of 50µg/mL (cat. 400202, BioLegend).

Adsorption of anti-RhD antibodies and eluate preparation

Erythrocytes extracted from whole blood samples were used with the anti-RhD antibody preparation KamRho, which was diluted 1:2 with PBS prior to the experiment. For isolation of the unbound antibody fraction, equal volumes of erythrocytes and the 50% diluted KamRho were incubated at 37°C on a turning wheel. After 60 minutes, the erythrocytes were centrifuged, and the supernatant was collected. To increase the efficiency of adsorption, we

repeated the process twice, each time with new erythrocytes from the same donor. We performed this adsorption process in parallel using RhD^+ and RhD^- erythrocytes.

For isolation of the bound antibody fraction, we used the Gamma ELU-KIT II kit (cat. 0007861, Immucor Inc, USA). We incubated equal volumes of erythrocytes and the 50% diluted KamRho at 37°C on a turning wheel. After 60 minutes, the erythrocytes were centrifuged and washed several times with washing solution and PBS, while the supernatant was discarded. We then added an equal volume of eluting solution. Following mixing and centrifugation, the supernatant was transferred to a new tube to which we added an equal volume of buffering solution (the change of color indicates neutralization of the eluate solution). We then centrifuged this suspension and transferred the supernatant to a new tube. We performed this process in parallel with RhD^- and RhD^+ erythrocytes.

Human NK cells staining with RhD+ bound anti-RhD fraction

For the staining of human NK cells with anti-RhD antibodies, the bound RhD^+ fraction was first pre-incubated with a secondary anti-human antibody for 30 minutes on ice. Then NK cells were incubated for 1 hour on ice with these primary-secondary antibody complexes.

Fluorochrome conjugation of anti-RhD

The anti-RhD drug KamRho was conjugated to Alexa fluor 488 using the Conjugation Kit Lightning-Link (ab236553, Expedeon). The drug was purified and resuspended in PBS prior to conjugation, using Amicon® Ultra-15 Centrifugal Filter Unit (UFC903024, MERCK)

Identification of low and high-affinity CD16a-expressing donors

Genotyping of CD16a-158 polymorphism was determined by allele-specific reverse transcription polymerase chain reaction (RT-PCR) with TaqMan SNP assay probes (cat. AB-4351379, Thermofisher scientific).

F(ab')$_2$ preparation

F(ab')$_2$ segments were prepared with Pierce F(ab')$_2$ Preparation Kit (cat. TS-44988, Thermofisher scientific).

Human IgG subclass quantification

The human IgG subclasses 1, 2, 3, and 4 were quantified using the Human IgG subclasses single dilution BINDARID™ KIT (The Binding Site Group).

PNGase digestion

For deglycosylation of antibodies we used Peptide:N-glycosidase F (PNGase) (cat. P0704L, New England BioLabs) under non-denaturing reaction conditions. As a positive control, a parallel process was performed under denaturing conditions. The success of the deglycosylation was assessed by mobility shifts on SDS-PAGE.

B cell purification

B cells were isolated from the peripheral blood of healthy human volunteers, using a RosetteSep™ Human B Cell Enrichment Cocktail (cat. 15024, STEMCELL technologies). Cell purity of over 85% was determined by staining cells for CD19 (cat. BLG-302208, BioLegend) and CD45 (cat. BLG-368512, BioLegend).

Statistical analysis

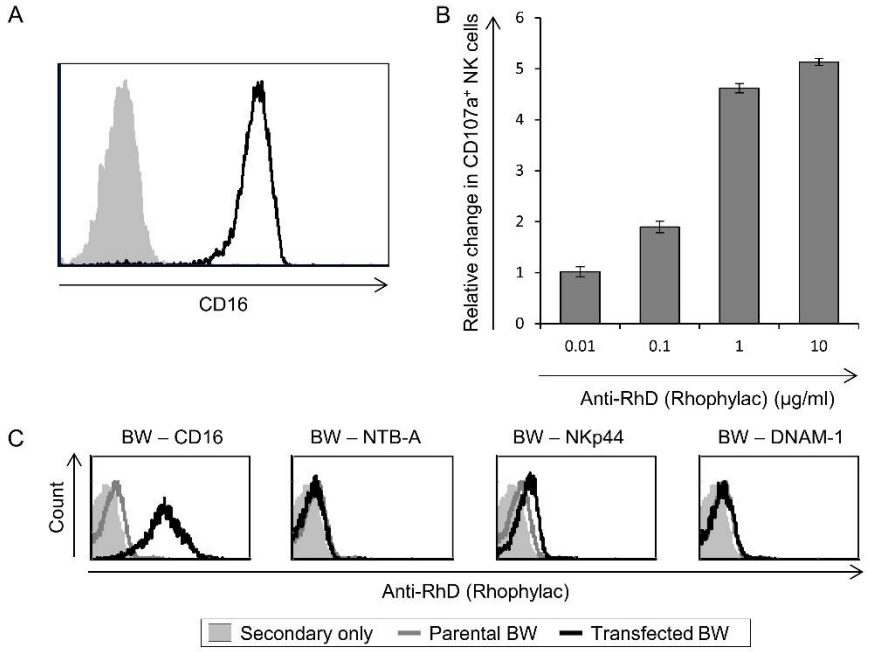
For statistical analysis we used Prism software version 7.02 (GraphPad, San Diego, CA). ANOVA was analyzed with *statpages.info/anova1sm.html* using Tukey's post-hoc analysis. Specific statistical tests are mentioned throughout the manuscript. A statistical test was considered significant when $p < 0.05$. All statistical tests were applied to raw data.

Supplemental figure legends:

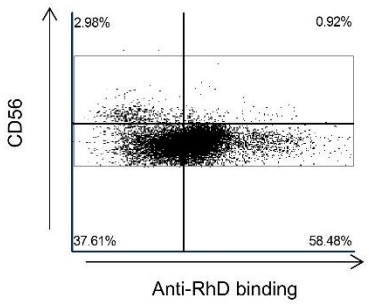
Sample	IgG 1	IgG 2	IgG 3	IgG 4
Rhophylac	80.2%	0	19.8%	0
KamRho	61.1%	31.1%	7.8%	0
IVIG	55.1%	37%	5.3%	2.6%

Supplementary Table 1. Abundance of IgG subclasses in anti-RhD and IVIG drugs. Quantification of the human IgG subclasses 1, 2, 3, and 4, in different antibody preparations. The results were obtained in mg/dl, and are presented as a percentage out of the total IgG

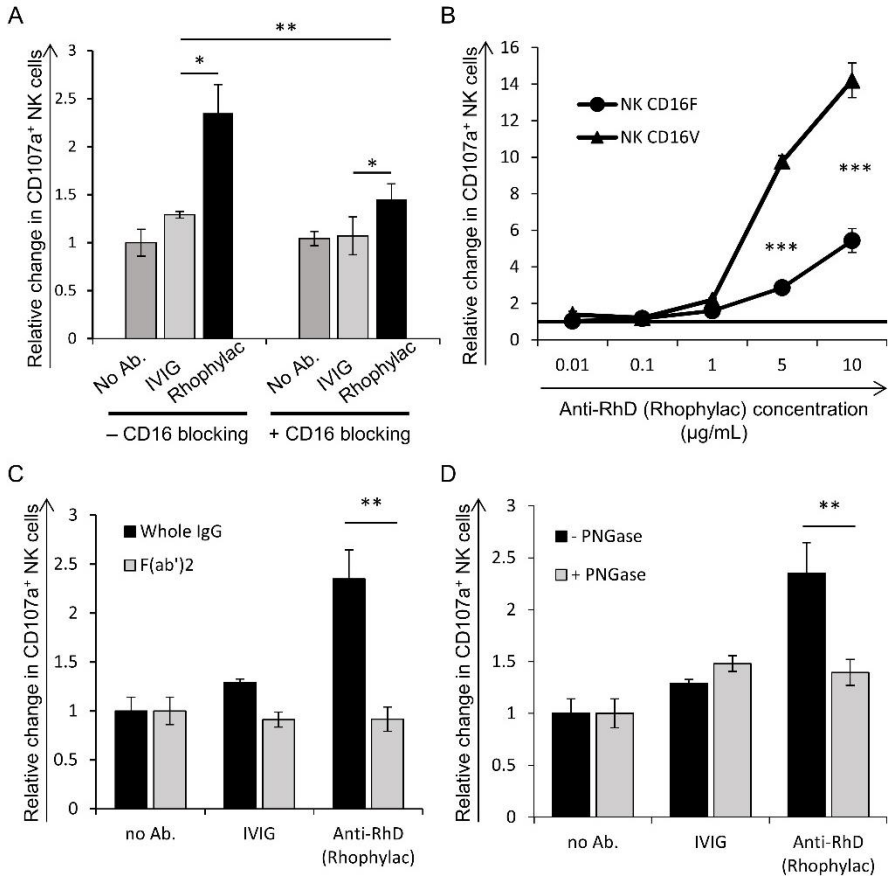
concentration in that sample.



Supplementary Figure S1. (A) Staining of primary activated natural killer cells (NKs) with an anti-CD16 antibody. Gating was on the $CD56^+$ cell population. (B) Degranulation of NK (natural killer) cells which were incubated with increasing doses of Rhophylac. Error bars represent standard deviation of triplicates. One representative experiment is shown out of three performed. (C) Staining of transfected BW cells with Rhophylac. The cells express the extracellular domain of specific NK cell receptors. The receptor which is expressed by each BW cell line is indicated above each histogram. Verification of receptor expression is shown in Figure 3A. Gray filled histograms represent staining with secondary antibody and gray histograms represent staining of the parental BW cells.

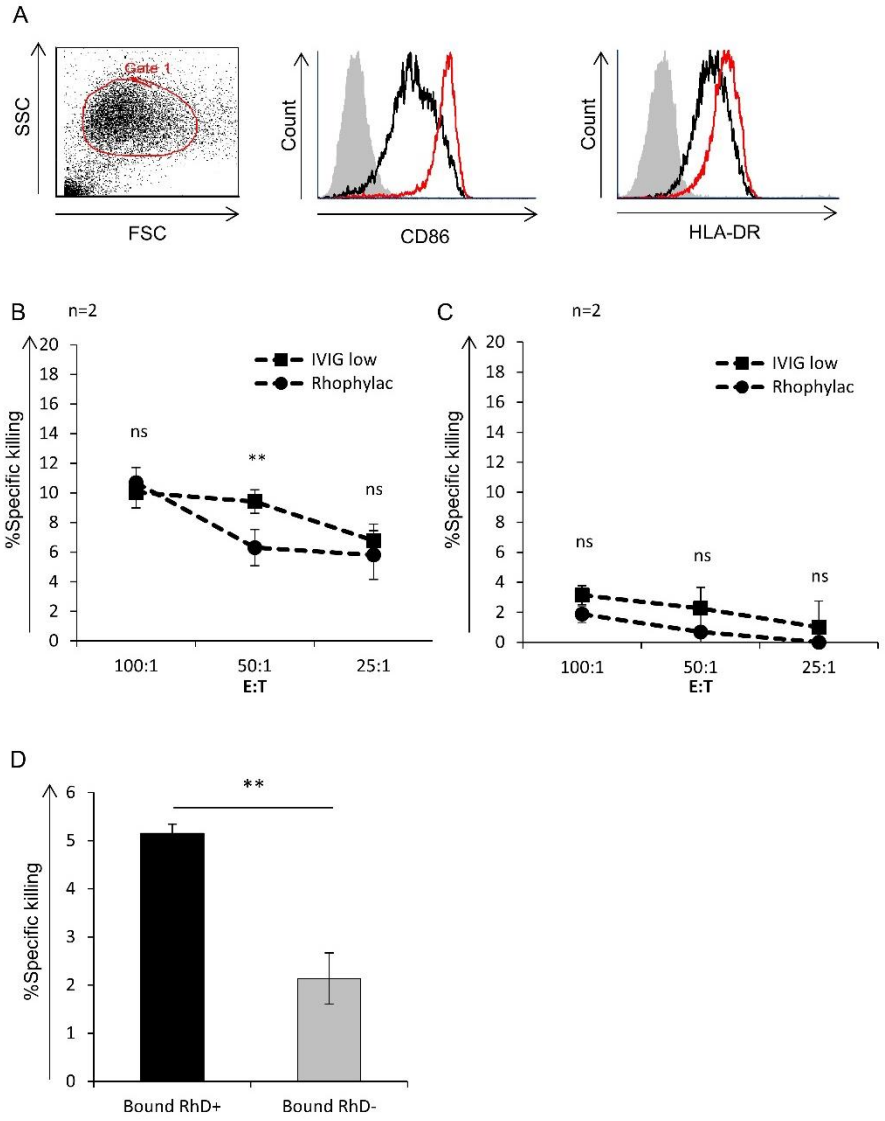


Supplementary Figure S2. Staining of freshly isolated NKs with Alexa fluor 488-conjugated KamRho. Gating was on the $CD56^{+}$ cell population (gray box). The y-axis represents the CD56 expression level; cells above the black line are $CD56^{bright}$ ($CD16^{-}$) and those below it are $CD56^{dim}$ ($CD16^{+}$).



Supplementary Figure S3. (A-D) NK cell degranulation assays. The NK cell degranulation level was normalized to the basal percentage of $CD107a^{+}$ NK cells without antibody (no Ab.). Error bars represent standard deviation of triplicates. *, $P<0.05$; **, $P < 0.01$; ***, $P < 0.001$; Student's *t* test. (A) NK cells were incubated with Rhophylac or IVIG, with or without blocking of CD16 by mIgG2a. (B) Primary NK cells were isolated from donors who express the low (158F) or high-affinity (158V) variants of CD16. These NK cells were incubated with increasing doses of Rhophylac and then the NK cell degranulation was assessed. (C) $F(ab')_2$ fragments were produced from Rhophylac and from IVIG. NK cells were incubated

with similar concentrations of the F(ab')$_2$ fragments or whole Rhophylac and IVIG. (D) Rhophylac and IVIG were deglycosylated with PNGase under non-denaturing conditions. NK cells were incubated with similar concentrations of the deglycosylated or the original antibodies.



Supplementary Figure S4. (A) Flow cytometry characterization of the dendritic cells (DCs) used for the cytotoxicity assay, showing a low expression of CD86 and HLA-DR in immature DCs (iDCs, black line) as compared with mature DCs (mDCs, red line). The staining was performed in parallel to the cytotoxicity assay. (B) Cytotoxicity assay. ^{35}S-labeled iDCs were incubated with activated NK cells and different polyclonal antibodies (IVIG or Rhophylac).

The effector to target (E:T) ratio is indicated on the x-axis. One representative experiment is shown out of two performed. **, P < 0.01; ns, non-significant; ANOVA with Tukey's HSD post-hoc test. Error bars represent standard deviation of triplicates. (C) Cytotoxicity assay was performed as in (B), with ^{35}S-labeled mDCs used as targets. One representative experiment is shown out of two performed. ns, non-significant; ANOVA with Tukey's HSD post-hoc test. Error bars represent standard deviation of triplicates. (D) Cytotoxicity assay. ^{35}S-labeled iDCs were incubated with activated NK cells and the bound anti-RhD fractions we generated. The effector to target (E:T) ratio is 25:1. **, P<0.01; student's *t* test. Error bars represent standard deviation of triplicates.

References

1. Elias S, Kahlon S, Duev-Cohen A, Mandelboim O. A BW Reporter System for Studying Receptor-Ligand Interactions. J Vis Exp. 2019(143).