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Characterization of rituximab-induced B cell depletion and infusion reactions in a human blood loop system

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Abstract

Introduction: Rituximab is a monoclonal antibody used to treat hematological malignancies. The antibody depletes CD20⁺ B cells via cytotoxic immune mechanisms, such as complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC), which is mainly induced by natural killer (NK) cells. Rituximab is mostly well-tolerated but has been reported to induce the release of large amounts of cytokines in blood, thus causing systemic inflammatory response. **Aim:** To study rituximab-induced B cell depletion and cytokine release in blood from healthy volunteers and how this was affected by Fc modified versions of the antibody. **Methods and materials:** Fresh blood from healthy donors (n=3) was incubated with rituximab and Fc modified versions that influence the antibody's target functions, namely ADCC and CDC, for 4 hours in a blood loop system. Results were measured using multicolor flow cytometry, except for cytokine release in plasma which was measured by enzyme-linked immunosorbent assay (ELISA). **Results:** Of all treatments, rituximab wild type (WT) showed superior B cell depletion than Fc mutant rituximab. The C1q knock-out variant (rituximab-P331S) and the variant with improved affinity to Fc receptor CD16 (rituximab-GASDALIE) did not differ in depletion. A cytokine release was not detected with the treatments, however, a cytokine stimulation in NK cells was observed. Rituximab-GASDALIE had the most prominent cytokine stimulation and CD107a (marker of NK cell functional activity) expression on NK cells. Rituximab-WT and rituximab-P331S had a minor and similar cytokine stimulation and CD107a expression between each other. Rituximab-IgG2 had minimal B cell depletion, CD107a expression and cytokine stimulation. **Conclusions:** Rituximab depleted B cells without inducing measurable cytokine release for healthy individuals. Among the treatments, Fc mutant rituximab seem to induce less B cell depletion. Moreover, rituximab-GASDALIE appear to elicit an enhanced NK cell activation. Further studies should include more donors as supplement and the results should be interpreted as complementary data to future data analyzed by performing the loop experiment using blood from patients.

Keywords: Rituximab, CDC, ADCC, infusion reactions, blood loop assay, Fc engineering

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1. Introduction

The human body is regularly attacked by pathogens or infectious agents that can cause severe and life-threatening diseases. The immune system is the body's defense system and it protects against diseases caused by harmful invaders. Immunoglobulins (Igs), also known as antibodies are large proteins that are produced by B cells which is a type of white blood cell. Antibodies have an important role in the immune system and in defending the body. They can identify and bind to foreign substances (antigens), and thereby elicit an immune response to eliminate pathogens that are carriers of these antigens. Today, genetically engineered antibodies (biologics) are developed for use in cancer immunotherapy. They target specific antigens on tumor cells and kill the cells by utilizing host immune response. Antibodies are divided according to their structure and function into five classes, IgG, IgA, IgE, IgM and IgD (1). The antibody therapy studied in this report is an IgG-antibody named rituximab. IgG is structurally Y-shaped and comprises of two long protein (heavy) chains and two short protein (light) chains joined by disulfide bonds. The protein chains are divided into two regions, called fragment crystallizable (Fc) region and fragment antigen binding (Fab) region (**Fig 1**). The Fab regions include the antigen binding site which particularly bind to antigens and the Fc region is accountable for the communication between the antibody and the immune system (1,2).

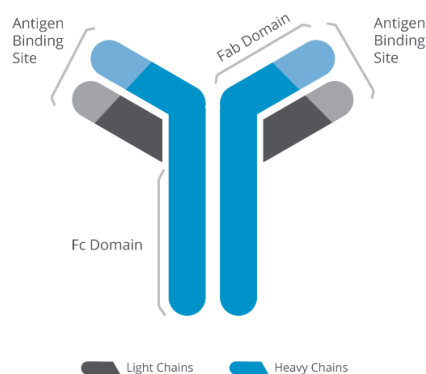


Figure 1. Schematic overview of an immunoglobulin G (IgG). An IgG-antibody consists of two heavy chains and two light chains linked by disulfide bonds. The antigen binding site in Fab domain connect to antigens and the Fc domain is identified by the host immune system (2).

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1.1. Rituximab

Rituximab (RTX) is an IgG1 chimeric monoclonal antibody (mAb) consisting of a human constant region (Fc), linked to antigen-binding murine variable regions.

Rituximab is used successfully in treatment of various B cell malignancies, such as chronic lymphocytic leukemia (CLL). In addition to cancer therapy, rituximab is used to treat autoimmune diseases. Rituximab targets only the antigen and transmembrane protein CD20 on B cells. The binding of CD20 on B cells leads to the killing of CD20⁺ B lymphocytes via effector cytotoxic immune mechanisms, such as antibody-dependent cell-mediated cytotoxicity (ADCC), complement dependent cytotoxicity (CDC) (3), antibody-dependent phagocytosis (ADP) (4) and direct apoptosis induction (5).

ADCC is an immune response included in the immune system and it is induced through engagement of effector immune cells, mainly natural killer (NK) cells, by the Fc part of rituximab (6). The mechanism is activated when low-affinity Fc gamma receptor IIIa (FcγRIIIa), also known as CD16, on NK cells and other effector immune cells for instance granulocytes and monocytes bind to antibody-coated target cells (7). The cell lysis is caused due to release of the cytolytic granules perforin and granzymes from effector cells (8,9). At the same time, effector immune cells also secrete cytokines such as interferon- γ (IFN γ), tumor necrosis factor α (TNF α) and interleukin-6 (IL-6) (10,11). On the other hand, CDC is another immune response that is initiated when the complement system and more specifically the classical complement pathway is activated, through for instance, the binding between the Fc portion of rituximab and the complement component 1q (C1q), which is one of the complement proteins in the complement system (3,12). The activation triggers the complement cascade which creates a membrane attack complex (MAC), from complement proteins, on the cell membrane of B cells. The MAC allows influx of ions and water which leads to cell lysis (13,14). In this report, rituximab-induced CDC and ADCC with the immune effector cell, NK cells, are mainly studied.

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1.2. Chronic lymphocytic leukemia (CLL)

CLL is a blood cancer form caused by tumor-transformed T and B lymphocytes. The disease causes an accumulation of abnormal lymphocytes in blood, bone marrow and lymph nodes due to the rapid division and prolonged survival of malignant cells (15). Between 2012-2016 in USA the number of deaths and the number of new cases were 1.2 per 100,000 and 4.9 per 100,000 for women and men respectively (16). The disease can be discovered from various tests such as blood tests, later symptoms are swelling of lymph nodes, abnormal bleeding, fatigue, anemia, fever, and recurrent infections. Available treatments are radiation therapy, targeted therapy, chemotherapy and immunotherapy (17).

The most common form of CLL is composed of mature CD5-positive B lymphocytes and one of the targeted therapies for B-CLL is rituximab. The disease is most common in older adults and median survival is 10 years, however the prognosis is individual since the disease can behave in different ways (18,19). Antigen CD5 is a protein expressed on clonal B cells that mainly characterize CLL and the expression can vary between patients (20). Beside from CD5, malignant B lymphocytes are phenotypically defined by markers such as CD23+, FMC-7- and low expression of surface Ig (21).

1.3. Cytokine release syndrome (CRS) with rituximab

Monoclonal antibodies have for a long time been successfully used in cancer therapy. However, the use of mAbs may lead to cytokine release syndrome (CRS), induced by a rapid release of massive amounts of inflammatory cytokines. CRS is a toxic condition that can cause systemic inflammatory response from a highly activated immune system and the source of cytokines are both from cells which the antibody targets or from non-target immune cells such as effector cells (22–24). Furthermore, cytokine production vary between therapeutic agents and their ability to activate the immune system (25). Infusion reactions caused by the syndrome can range from mild to severe or life threatening and are characterized by symptoms such as fever, tachycardia, rash,

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hypotension, hypoxia and tachypnea (26). It is known that CRS is initiated by activated immune cells, however, the pathophysiology of the syndrome is still not fully understood.

Rituximab is mostly well-tolerated by patients and is on the World Health Organization (WHO) Model List of Essential Medicines in 2019 for being one of the most safe, cost-effective and efficacious medicines in the health care system (27). However, rituximab is associated with CRS and all patients can suffer from toxic reactions during the infusion of the drug. The incidence and severity of the infusion reactions can vary between patients and have in lymphoma patients been reported to depend on the tumor burden at baseline. Patients with lower tumor burden experienced lower cytokine concentrations and milder adverse drug reactions than patients with higher tumor burden. At a second infusion of rituximab the cytokine release has been observed to not peak as much as after the first infusion because of a lowered tumor cell count. The B cell killing was also not as effective as in the first infusion (28). Furthermore, complement activation has been suggested to contribute to the toxicity by stimulating immune cells that produces cytokines. The severity of the toxicity correlated with a high complement activation and B cell count at baseline (29).

CRS is reported to be very rare ($< 1/10,000$) in the summary of product characteristics for the European approved drug MabThera®. For patients with a tumor count above $25 \times 10^9/L$ it is recommended to administer prednisolone before intravenous rituximab treatment to reduce the risk and severity of CRS (30).

1.4. Fc engineering

Fc engineering is a field in protein therapeutics where mAbs are engineered with amino acid substitutes at the Fc region to for example gain favorable qualities, such as improved efficacy by altering with effector functions (31,32) and extending the half-life leading to a reduced administration frequency (33).

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In this project, three rituximab variants with Fc modifications are studied. One of them was the G236A/S239D/A330L/I332E (GASDALIE) Fc mutation. The GASDALIE mutations have an improved affinity to CD16 through increased electrostatic interactions at the binding interface (34). An increased affinity to CD16 on NK cells have shown to improve the mediation of ADCC and the level of target cell depletion (31). The NK cell activation in regards to the concentration of rituximab was more successful with a Fc modification to enhance the affinity to CD16 compared to a Fab modification to the enhance the affinity to CD20 (7).

Another rituximab variant in the project was rituximab with the Fc mutation P331S (C1q knock-out). The mutation obliterates the antibodies ability to bind to the complement protein C1q and inhibits therefore CDC activity. The Fc mutation P331S has been reported to not interfere with the binding between the mAb and FcγR receptors on human NK cells or the efficacy of ADCC (35).

Immunoglobulin G is divided into four subclasses, IgG1, IgG2, IgG3 and IgG4. These isotypes have different structures and therefore different effector mechanisms. The third antibody in the project was rituximab-human IgG2. The only subclasses that have been detected to bind C1q and activate the complement cascade are IgG1 and IgG3 (36). In a study by Bruhns et al. (37) it was concluded that IgG3 had the highest affinity to CD16 and thereafter IgG1. IgG2 and IgG4 had a lower affinity (approximately 35-fold and 7-fold respectively) than IgG1 which was suggested to have an impact on the activation of CD16-expressing effector cells. Furthermore, in an another study IgG1 and IgG3 were the only subclasses with an effective ADCC activity (38).

1.5. Blood loop assay

Cytokine release assays (CRAs) have for many years been used to predict cytokine release in humans. CRAs are requested by the regulatory agencies for new therapeutic agents and there are several methods available were the adverse reaction can be

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predicted, such as in vivo assays, whole blood (WB), peripheral blood mononuclear cells (PBMCs) and blood loop (25). WB is the most extensively used assay (39).

The method used in this project was the blood loop assay. This *ex-vivo* human assay with the use of blood can predict mode of action and infusions reactions induced by immunotherapeutic agents when infused in humans. Blood is very sensitive and prone to coagulate. The main mechanism to prevent clotting in the loop assay is to keep the blood in movement. A secondary mechanism and a way to keep the complement system intact is to have a low free heparin (anti-coagulant) concentration in the circulated loops. In addition, contact coagulation is prevented by the coating of heparin-conjugate to the inner surface of plastic tubes and metal connectors (25). The complement system is an important mechanism to provide a true prediction of the B cell depletion induced by rituximab and was therefore remained intact in the blood loop system.

Fresh blood from healthy donors was incubated in the blood loop system with rituximab wild type (WT) or with Fc modified versions of the mAb that influence the target functions of the mAb, especially ADCC and CDC (**Fig. 2**).

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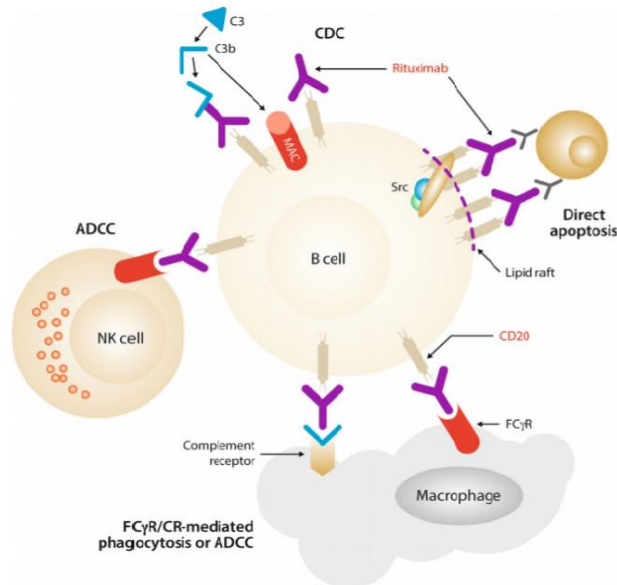


Figure 2. The four reported cytotoxic mechanisms mediated by rituximab (RTX) are direct apoptosis, phagocytosis, antibody dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC). In the blood loop mainly ADCC and CDC are studied. CDC is initiated by rituximab binding to CD20 on B cells, triggering the complement system to form a membrane attack complex (MAC) on B cells. Binding of rituximab to CD20 on B cells induce the cell killing immune mechanism ADCC, by in addition bind to Fc-gamma receptors ($Fc\gamma R$ s) on NK cells (40).

2. Aim

The aim of this project was to study rituximab-induced B cell depletion and cytokine release in blood from healthy volunteers and how this was affected by Fc modified versions of the antibody.

3. Methods and materials

All experiments in the project were performed at the department of Pharmaceutical Biosciences at Uppsala Biomedical Centre or at the blood lab facility at Rudbeck laboratory in Uppsala.

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3.1. Study population

In this study, fresh blood from healthy donors were acquired and analyzed. The inclusion criteria to participate where that they are healthy with no acute illness and above 40 years old. Donors did not, within 10 days prior to blood donation, take nonsteroidal anti-inflammatory drugs (NSAIDs), paracetamol or other drugs that influence the coagulation cascade and immune system.

The experiment was performed with fresh blood from three healthy donors in an age range of 45-55 years old. Results from the blood loop assay included a maximum of three human samples.

3.2. Reagents

The therapeutic reagents included in the blood loop experiment are presented in Table 1. Cetuximab (Merck, Germany) was the negative/isotype control and alemtuzumab (Sanofi, France) the positive control (25). The baseline in the project was blood from zero-time-point and negative control. The concentrations chosen for rituximab is based on the literature, where 10 µg/ml is the minimal reported rituximab concentration that induces both CDC and ADCC in whole blood (41).

Duplicated loops were run. One of the duplicates was intended for intracellular cytokine analysis and therefore the protein secretion inhibitor brefeldin-A (Sigma-Aldrich, USA) was added to block the cytokine release in these specific loops.

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Table 1. Therapeutic reagents included in the experiment and their final concentration in blood.

Reagent	Target	Isotype	Source	Final concentration in blood
PBS	-	-	-	-
Cetuximab ¹	EGFR	Chimeric IgG1	Merck, Germany	200 µg/ml
RTX WT	CD20	Chimeric IgG1	Roche, Switzerland	10 µg/ml
RTX (P331S)	CD20	Chimeric IgG1	University of Southampton, UK	10 µg/ml
RTX (GASDALIE)	CD20	Chimeric IgG1	University of Southampton, UK	10 µg/ml
RTX	CD20	Chimeric IgG2	University of Southampton, UK	10 µg/ml
RTX WT	CD20	Chimeric IgG1	Roche, Switzerland	100 µg/ml
RTX WT	CD20	Chimeric IgG1	Roche, Switzerland	400 µg/ml
Alemtuzumab ¹	CD52	IgG1 _k	Sanofi, France	3 µg/ml

3.3. Buffer exchange

The antibody buffer was changed from tris buffer to phosphate-buffered saline (PBS) using ZebaTM Spin Desalting Columns 7K MWCO, 0.5 mL following instructions from the manufacturer (cat no; 89882, Thermo Fisher Scientific). The exchange was performed in a laminar flow cabinet with spin columns containing size-exclusion chromatographic resin. The storage solution in the columns was removed and the remained resin bed was washed twice with PBS to equilibrate it to PBS. The antibody sample was added on top of the resin bed before the buffer exchange was completed.

¹ Fletcher EAK, Eltahir M, Lindqvist F, Rieth J, Törnqvist G, Leja-Jarblad J, m.fl. Extracorporeal human whole blood in motion, as a tool to predict first-infusion reactions and mechanism-of-action of immunotherapeutics. *International Immunopharmacology*. 2018;54:1–11

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PBS was a more suitable buffer when experimenting with living cells since it is non-toxic to them.

3.4. Heparinization of metal connectors

All materials (pipette tips, Falcon tubes, metal connectors) in contact with blood were coated with heparin-conjugate before use in the blood loop assay. Connectors pretreated with 5% APS (ammonium persulfate) solution were covered in different buffers. In between added buffer was an incubation period and thereafter a wash with deionized water. Buffers included in the procedure were 0.25 mg/ml PAV (polymeric amine compound) priming solution, prepared by diluting concentrated PAV priming formulation in priming buffer pH 9.0, 0.05 mg/ml corline heparin conjugate (CHC) coating buffer from diluting concentrated CHC coating formulation in coating buffer pH 4.0 and acetic anhydride (1:1000) mixed with acetylation buffer pH 10.5 (**Table 2**). The polyvinylchloride (PVC) tubings were purchased with surface coated heparin-conjugate (Corline, Sweden).

Table 2. Reagents included in heparinization.

Reagent	Cat. No	Manufacturer
Lab Site Heparin Coating kit	945-00	Corline, Sweden
Acetic anhydride	33214	Sigma Aldrich, USA

3.5. Ex-vivo whole human blood loop assay

Blood from healthy donors was collected in an open collection system and added into a Falcon tube with a low heparin concentration (Leo Pharma AB, Sweden). Whole blood was added in PVC tubings with a therapeutic reagent. Only the duplicated loops aimed for intracellular cytokine analysis included brefeldin-A. All loops were sealed with metal connectors, placed in a rotating wheel, and incubated for 4 hours at 37°C (**Fig. 3**).

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Blood collected before the start of the experiment (zero-time-point) was saved for analysis of baseline markers. After 4 hours incubation blood was taken from each loop and EDTA (Invitrogen, USA) was diluted in blood to a final concentration of 10 mM to stop further reactions. The samples were kept on ice until flow cytometry analysis.

Plasma was harvested by centrifugation (2000xg) for 20 minutes at 4 °C and stored at -80°C until cytokine analysis.

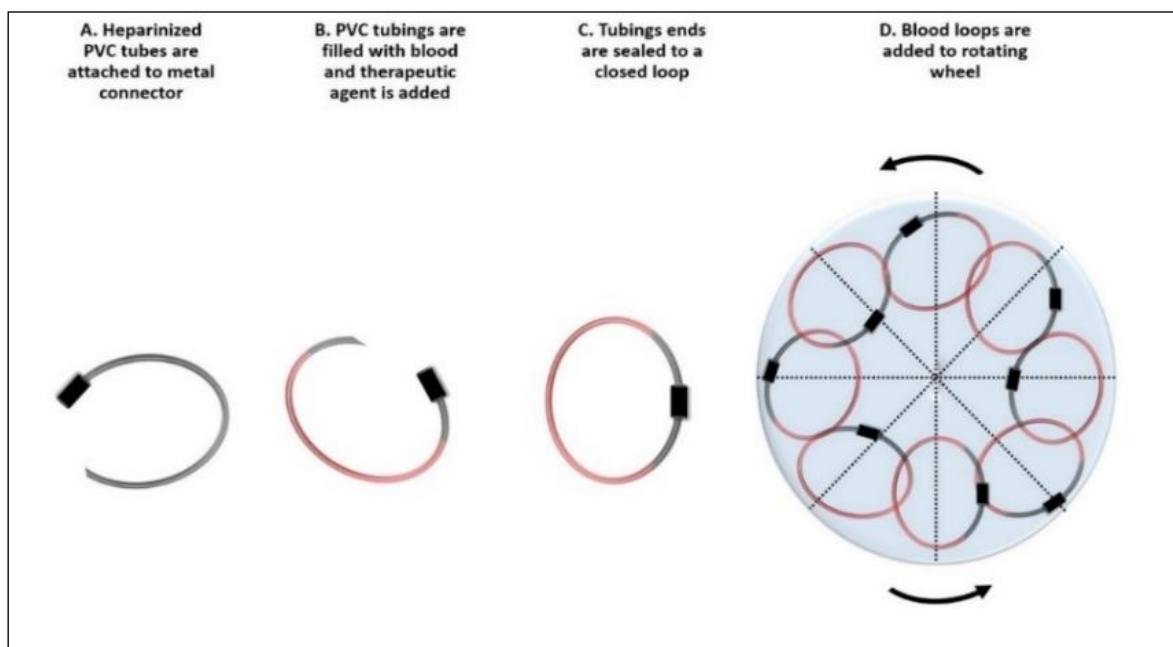


Figure 3. The image illustrates the preparation of blood loops (A-C), how loops are kept in motion in the incubator and how blood is circulating due to air in the tubes (D).

3.6. Blood cell counting

The Sysmex XP-300 automated hematology analyzer was used to assess the white blood cell (WBC) count and platelet count in blood at 0- and 4-hours incubation.

3.7. Multicolor flow cytometry (including intracellular staining)

The multicolor flow cytometry including intracellular staining were based on fluorochrome-labeled antibodies. The extracellular staining antibodies in the

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experiments were anti-CD3, anti-CD14, anti-CD19, anti-CD107a, anti-CD56 and anti-CD16. The intracellular staining's were anti-INF γ , anti-TNF α and anti-CD16. For zero-time-point blood samples (baseline) the extracellular staining's were anti-CD3, anti-CD19, anti-CD5, anti-HLA-E, anti-NKG2A, anti-CD16 and anti-CD20. The antibodies are presented in Table 3.

The fluorochrome-labeled antibodies were diluted in PBS (1% BSA) and mixed with blood in FACS tubes (BD, USA) to stain extracellular markers. Fixable viability dye eFlourTM 780 (cat no. 650865-14; Invitrogen, USA) was added in to label dead cells and analyze cell viability. After 30 minutes incubation at 4 °C, the red blood cells were lysed with 1x FACS Lysing solution (BD, USA) for 10 minutes before the cells were washed with PBS. Cytofix/Cytoperm (BD, USA) buffer solution was mixed in all tubes aimed for fixation and permeabilization to enable intracellular cytokine analysis, and then incubated for 20 minutes at 4 °C. The cells were after washed with PBS. Before and after adding the intracellular antibodies (diluted in Perm/Wash buffer) and the following 30 minutes incubation at 4°C, FACS permeabilization solution 1x Perm/Wash (BD, USA) was added. The Perm/Wash buffer was washed with PBS (1% BSA) and the cells were thereafter resuspended with PBS (1% BSA). The flow cytometry analysis was performed in Cytotflex (Beckman coulter).

Table 3. Anti-human fluorochrome-labelled antibodies applied in flow cytometry.

Antibody	Clone	Cat. No	Manufacturer
Anti-CD3	UCHT1	300448	BioLegend, USA
Anti-CD14	HCD14	325604	BioLegend, USA
Anti-CD19	HIB19	302208	BioLegend, USA
Anti-CD56	NCAM	318328	BioLegend, USA
Anti-CD16	3G8	302012	BioLegend, USA
Anti-CD107a	H4A3	328642	BioLegend, USA

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Anti-TNFα	Mab11	502528	BioLegend, USA
Anti-INFγ	4S.B3	502528	BioLegend, USA
Anti-CD20	2H7	302309	BioLegend, USA
Anti-CD5	L17F12	364029	BioLegend, USA
Anti-HLA-E	3D12	342607	BioLegend, USA
Anti-CD19a (NKG2A)	131411	747920	BD Biosciences, USA

3.8. Cytokine release analysis

Plasma from 0 and 4-hours were examined with enzyme-linked immunosorbent assay (ELISA) to quantify IFN γ and TNF α . Flat 96-well plates (Sarstedt, Germany) were coated with capture antibodies (TNF α : 1:200, INF γ : 1:50) diluted in 0.05M carbonate-bicarbonate buffer. The plates were blocked for unspecific binding with PBS (1% BSA). Samples diluted (1:5) in PBS (1% BSA) were pipetted in wells and on top of it diluted (TNF α : 1:200, INF γ : 1:50) detection antibody was added. Thereafter avidin-HRP (horseradish peroxidase) diluted 1:1000 in PBS (1% BSA) was applied (**Table 4**). The plates were incubated and washed three times with PBS (0.05% Tween) in between every added component. In addition, TMB was added and the color reaction between the HRP and TMB was stopped with H₂SO₄. Samples were read at an absorbance of 450nm with FLUOstar Omega (BMG Labtech). Standards were included in the analysis.

Table 4. Reagents applied in cytokine release analysis.

Reagent	Clone	Cat. No	Manufacturer
ELISA MAXTM TNFα kit	-	430201	BioLegend, USA

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IFN-γ coating / detection	MD-1 / 43.B3	502504 / 502504	BioLegend, USA
Carbonate- bicarbonate buffer	-	C3041-50CAP	Sigma-Aldrich, USA
1-Step™ Ultra TMB- ELISA Substrate solution	-	34028	Thermo Fisher Scientific, USA

3.9. Ethical approval

The human blood used in this project were collected from healthy donors under an ethical permit approved by the Regional Ethical Committee in Uppsala. Informed consent was signed prior to blood donation.

3.10. Statistics/data analysis

Flow cytometry data were analyzed with Kaluza Analysis Software version 2.1.1 (Beckman coulter). Statistics were performed in GraphPad Prism software version 8.2.1 (San Diego, CA) with a non-parametric, Kruskal-Wallis test with correction for multiple testing using Dunn's test.

4. Results

In this study, rituximab-induced B cell depletion and cytokine release in blood from healthy individuals were studied and how this was affected by Fc modified versions of the antibody in a blood loop system.

Platelet count was measured before the start of the experiment (zero-time-point) and at the end of the experiment (4 hours blood) to control for coagulation in the loop. Sample

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from zero-time-point was analyzed for WBC- and platelet count as a baseline before running the experiment. Platelet count for all donors (n=3) after 4 hours incubation with the different treatments compared to zero-time-point sample showed no deviant results. The figure is presented in Appendix A.

4.1. B cell viability

Blood from healthy donors (n=3) was examined with rituximab and rituximab variants to assess their B cell killing in blood loop system. The most prominent CD19⁺ B cell depletion was observed in donor 1 and donor 2 had minimal depletion (**Fig. 4a**).

Rituximab-WT showed superior depletion than the Fc mutant rituximab. A dose dependence of rituximab-induced depletion was observed with a drop in B cell count of 60.91%/85.53% and 22.26%/43.79% for rituximab [10/100 µg/ml] in donor 1 and donor 3, respectively. There was no difference in depletion between rituximab-GASDALIE and rituximab-P331S although donor 3 had a slightly better depletion for rituximab-GASDALIE. Rituximab-IgG2 had an observed B cell drop in donor 1 of ~ 25%.

Although a drop in B cell population was detected using flow cytometry, there was no change in WBC count (measured by hematology analyzer) after 4-hour incubation when measured in the loops that had brefeldin-A (**Fig. 4b**) or in the loops that did not have brefeldin-A (**Appendix B**). All treatments and their final concentration in blood are presented in Table 1.

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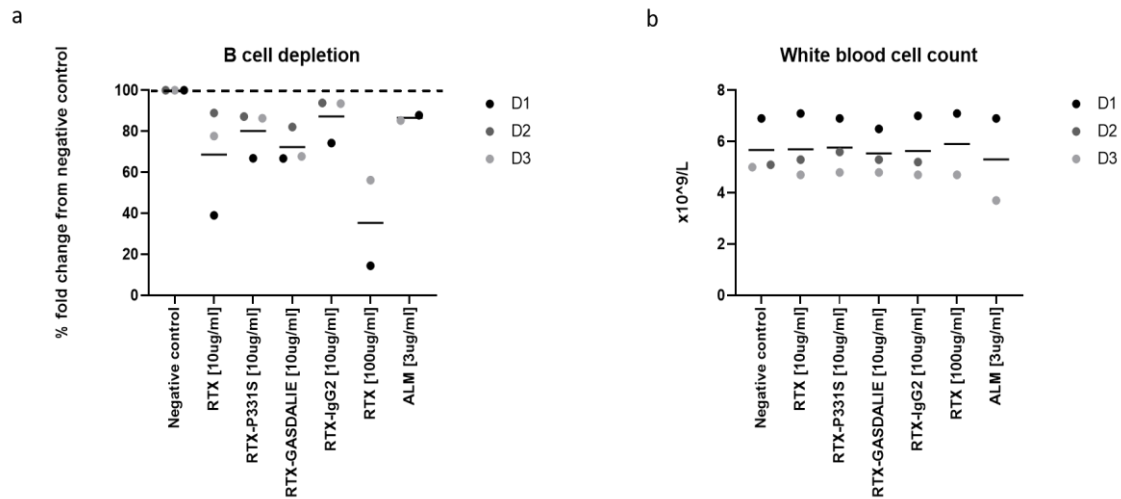


Figure 4. Rituximab-induced B cell depletion. Fresh blood from healthy donors ($n=3$) were incubated with different rituximab treatments for 4 hours using blood loop system. **A.** B cell depletion measured in flow cytometry as % viable B cells (CD19⁺). Data was normalized as fold change of negative control. **B.** WBC count was calculated with Sysmex XP-300 hemocytometer and plotted in $\times 10^9$ per liter. Final concentrations of all treatments are indicated in the figure. RTX: rituximab, ALM: Alemtuzumab, D1: donor 1, D2: donor 2, D3: donor 3.

4.2. Cytokine release in plasma

ELISAs were performed with plasma harvested from blood without the intracellular cytokine release inhibitor brefeldin-A at 0- and 4-hours incubation to analyze the release of TNF α and IFN γ in donor 1 and 3. The results showed a negative response, that is, no measurable cytokine release in plasma was observed in both donors (**Fig. 5**). The observed signal in the figures is background signal and at very low concentrations. No data is shown for TNF α in donor 1 due to experimental error.

Standards were used and the R values of the curves for TNF α and IFN γ was 0.98 and 0.99 respectively (data not shown).

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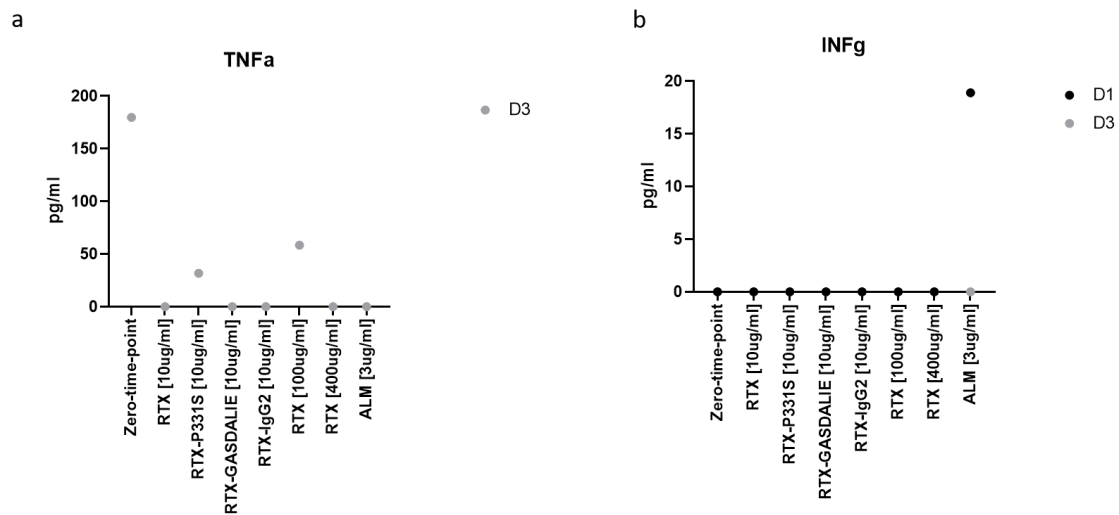


Figure 5. Cytokine release (TNF α and IFN γ) in plasma. Fresh healthy blood ($n=2$) was incubated for 4 hours with different rituximab treatments in blood loop system. After 4 hours incubation blood was mixed with EDTA to stop further reactions. Plasma from 0 and 4 hours was measured for cytokine release in ELISA and the absorbance was read at 450nm. **A.** The plot represents the release of TNF α in D3. Standard curve; R value 0.98. **B.** The plot represents the release of IFN γ in both D1 and D3. Standard curve; R value 0.99. Data are plotted in pg/ml. Antibodies concentrations are indicated in the figure. RTX: rituximab, ALM: alemtuzumab, D1: donor 1, D3: donor 3.

4.3. Intracellular cytokine stimulation

Intracellular cytokine (TNF α and IFN γ) stimulation in NK cells (CD56), T cells (CD3), B cells (CD19) and monocytes (CD14) with various rituximab treatments was measured in flow cytometry for samples ($n=3$) including brefeldin-A. A positive stimulation was only observed for NK cells (**Fig. 6**). The cytokine stimulation for all treatments was minor and similar pattern was observed between donors (**Fig. 7**). Rituximab-GASDALIE had superior cytokine stimulation and IFN γ was statistically significant compared to negative control. No difference in cytokine stimulation was observed between the studied rituximab-WT concentrations and a similar stimulation was shown for rituximab-P331S and rituximab-WT. Donor 1 had a stimulation for all treatments, except for rituximab-IgG2. Donor 3 had the least overall cytokine stimulation and no stimulation with rituximab-IgG2 either. In donor 2 there was no observed TNF α stimulation with rituximab-WT although there was an observed stimulation for

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rituximab-P331S. For $INF\gamma$ the opposite results were shown for the two treatments.
Alemtuzumab (ALM) has been used as positive control (data not shown).

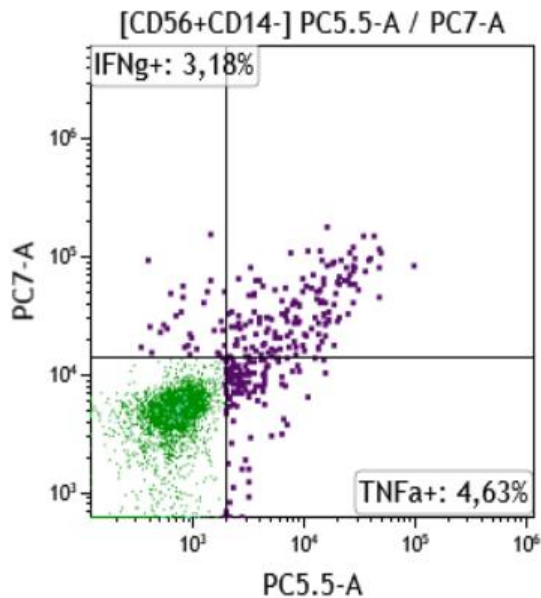


Figure 6. Intracellular cytokine stimulation in NK cells. Freshly acquired blood from healthy donors were incubated for 4 hours using a circulated blood loop system. The figure illustrates the dot plot and it represents the positive stimulation in % gated for $TNF\alpha$ and $INF\gamma$ stimulation in NK cells ($CD56+ CD3-$) for rituximab-GASDALIE in donor 1. The analysis was performed in Kaluza Analysis software. $PC7=INF\gamma-PE-Cy7$, $PC5.5=TNF\alpha-PerCP-Cy5.5$.

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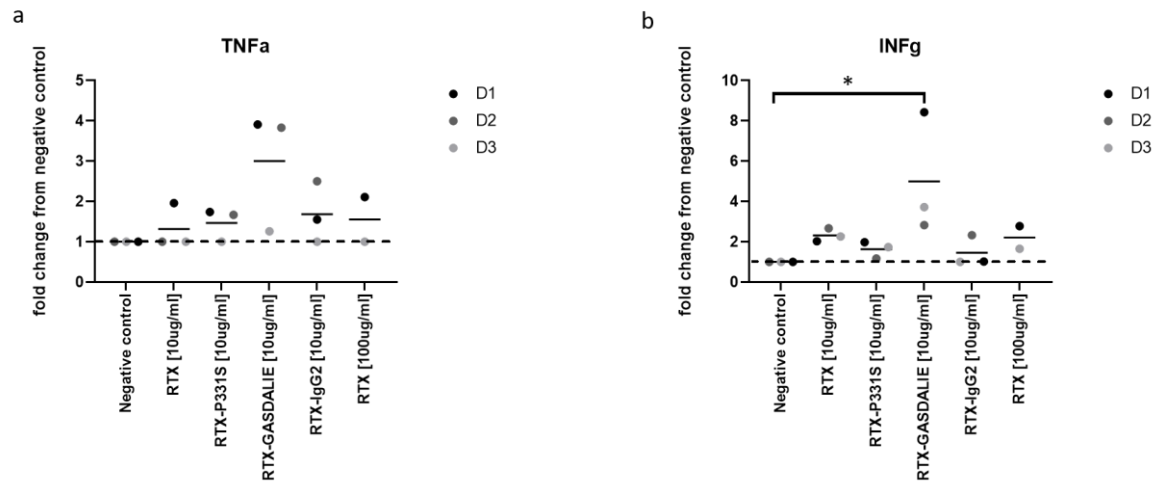


Figure 7. Intracellular (NK cells) cytokine stimulation by rituximab treatments. Freshly acquired blood from healthy donors were incubated for 4 hours using a circulated blood loop system. **A.** TNF α and **B.** INF γ stimulation was measured with multicolor flow cytometry including intracellular staining in NK cells (CD56+). Final concentrations of all treatments are indicated in the figure. Data were analyzed with a non-parametric, Kruskal-Wallis test with correction for multiple testing using Dunn's test * $p < 0.05$. RTX: rituximab, ALM: alemtuzumab, D1: donor 1, D2: donor 2, D3: donor 3.

4.4. Expression of CD16 receptor and CD107a

Extracellular CD16 receptor expression was measured with samples without brefeldin-A (n=2) since NK stimulated cells have shown to downregulate the surface expression of CD16 receptor (9). The figure in **Appendix C** show the extracellular expression of receptor CD16 on NK cells in mean fluorescence intensity (MFI) from flow cytometry. MFI is the mean of the fluorescence intensity and it measures the normalized expression of a marker in flow cytometry. No expression difference was observed for donor 3. Rituximab 10 μ g/ml, 400 μ g/ml and rituximab-GASDALIE had minimal downregulation in donor 1. The expression of CD16 was also measured intracellularly, however, the expression was only observed extracellular (data not shown).

An extracellular expression of CD16 on B cells (CD19+) was observed with rituximab-P331S and rituximab-GASDALIE in healthy donors (n=3) and the superior upregulation was observed for the latter rituximab variant. B cells normally only

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express surface receptor Fc γ RII, also known as CD32, hence CD16 was not expected to be found on B cells in a healthy individual. Therefore, an expression may have been induced by a protein transferred mechanism that potentially have occurred upon the binding between NK- and B cells (**Fig. 8**).

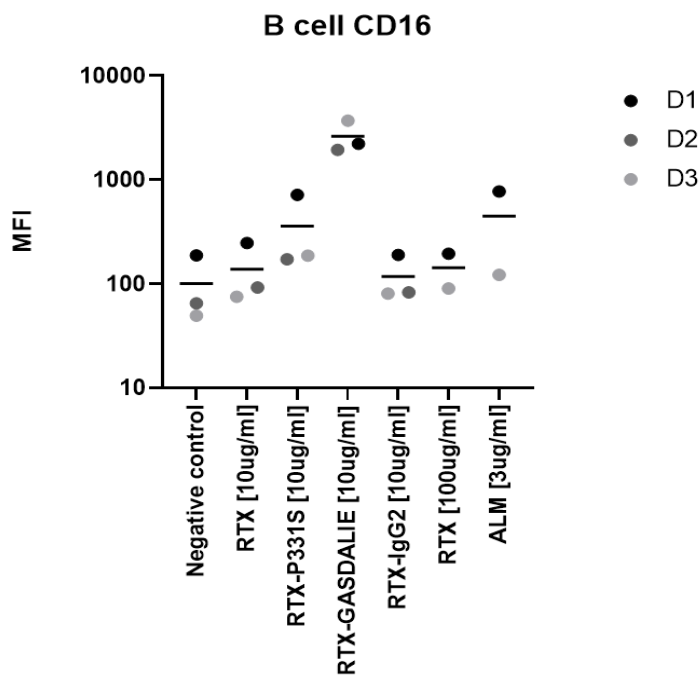


Figure 8. Extracellular CD16 expression on B cells. Fresh blood from healthy donors were collected and incubated for 4 hours in blood loop system. The figure illustrates CD16 expression on B cells for different rituximab treatments, measured in flow cytometry on B cells (CD19+). Data was measured in mean fluorescence intensity (MFI) and y-axis is presented in log scale. MFI describes the quantity of the expressed marker on every event. Final concentrations of all treatments are indicated in the figure. RTX: rituximab, ALM: alemtuzumab, MFI: mean fluorescence intensity, D1: donor 1, D2: donor 2, D3: donor 3.

Expression of CD107a (lysosome-associated membrane protein-1) on NK cells was studied from samples (n=2) without brefeldin-A to have a understanding of the ADCC activity, since it is externalized upon cytokine release and degranulation of perforin and granzymes (8,10). In donor 1 there was a clear upregulation for all treatments except for rituximab-IgG2 (**Fig. 9**). The highest upregulated expression of all rituximab treatments was shown with rituximab-GASDALIE (2.30%). Rituximab-WT 10 μ g/ml had a slightly higher upregulation from the studied WT concentrations. No difference in

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expression was observed between rituximab-WT 10 µg/ml and rituximab-P331S. Donor 3 showed no expression of CD107a for all treatments.

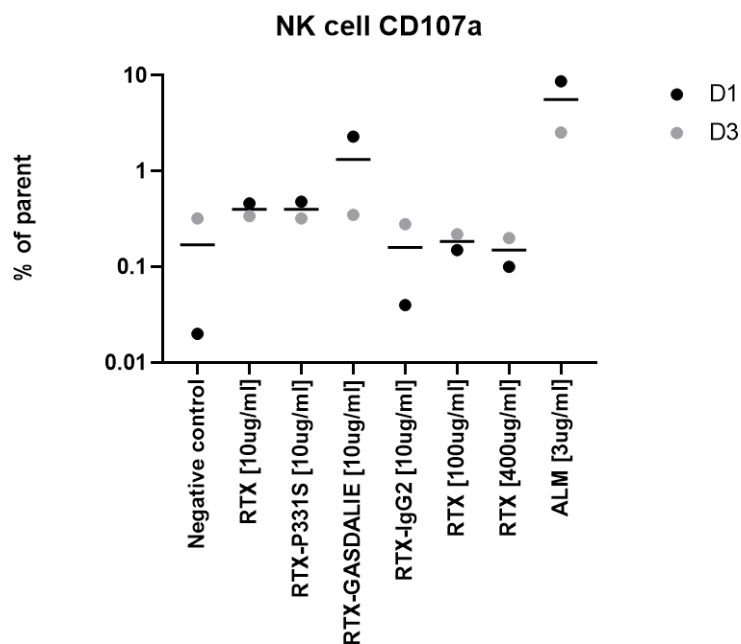


Figure 9. Expression of CD107a on NK cells with different rituximab treatments. Fresh blood was acquired and incubated for 4 hours in blood loop system. CD107a expression was measured in flow cytometry on NK cells (CD56+). Data are plotted as % of parent and y-axis is presented in log scale. Final concentrations of all treatments are indicated in the figure. RTX: rituximab, ALM: alemtuzumab, NK cell: natural killer cell, D1: donor 1, D3: donor 3.

4.5. Zero-time-point samples

Blood acquired before running the experiment (zero-time-point) was analyzed for the rituximab target marker CD20, B cells, NK group 2 member A (NKG2A), human leukocyte antigen E (HLA-E) and CD16 receptor. Both CD20 and B cells were measured for healthy donors since higher CD20 expression and larger B cell population have been reported to increase the cytotoxic activity of rituximab (12,28,29). The baseline expression of HLA-E and NKG2A was analyzed in donors because the binding between HLA-E on B cells and inhibitory heterodimeric CD94/NKG2A receptor on NK cells have been suggested to dampen NK cell activation (42). Furthermore, more CD16

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expression on cell surface have been stated to induce an enhanced NK cell mediated ADCC (43) and was therefore analyzed.

Percentage of B cells was highest in donor 3 (15.25%). Donor 1 and 2 had similar % CD19+ B cells, 11.66% and 11.14% respectively (**Fig. 10a**). Donor 1 had the strongest expression of both HLA-E and NKG2A of all donors (**Fig. 10b&c**). CD20 on B cells and the activation marker CD16 on NK cells showed superior expression in donor 2 (**Fig. 10d&e**). On the other hand, donor 1 had lowest CD20 expression and donor 3 had lowest expression of CD16.

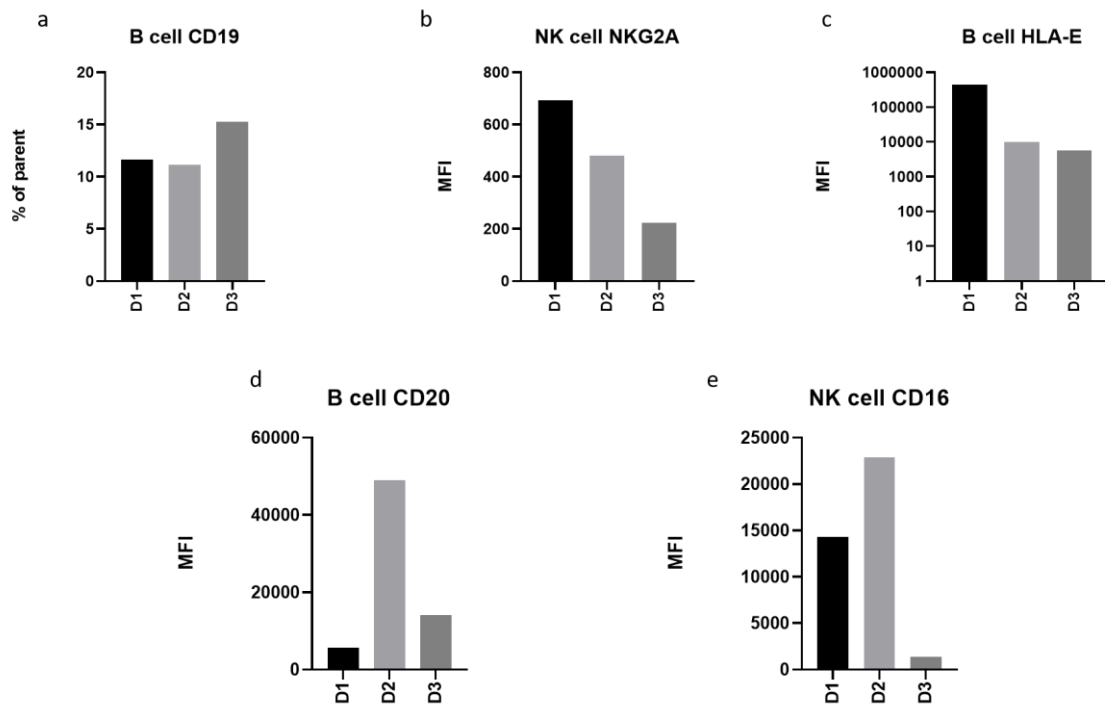


Figure 10. Zero-time-point analysis. Blood acquired from healthy donors (n=3) before starting the experiment in blood loop system was measured in flow cytometry for various baseline markers. A. Data on B cells (CD19+) are presented as % of parent. B. NKG2A (CD159a) was analyzed on NK cells (CD56+) and measured in MFI. C. HLA-E on B cells (CD19+) was measured in MFI and y-axis is presented in log scale. D. CD20 expression was analyzed on B cells (CD19+) and measured in MFI. E. CD16 receptor expression on NK cells (CD56+) was measured in MFI. MFI describes the quantity of the expressed marker on every event. MFI: mean fluorescence intensity, NK cell: natural killer cell, D1: donor 1, D2: donor 2, D3: donor 3.

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5. Discussion

The aim of the present study was to study rituximab-induced B cell depletion and cytokine release in healthy volunteers and how this was affected by Fc modified versions of the antibody that influence the target functions of rituximab, namely ADCC and CDC. This was studied in a blood loop system in order to predict the mode of action and infusion reactions in healthy individuals and to further improve the knowledge of the pathophysiology of infusion reactions.

Results from the measured response in the blood loop assay and zero-time-point samples showed inter-individual variability between donors. Variability is common with human samples and it can depend on various factors, such as inflammation in the body, infections, CD16 polymorphisms (6), responsiveness of immune cells and baseline markers. These factors can affect the outcome of how an individual respond to treatment.

5.1. Rituximab-induced B cell depletion and infusion reactions in healthy donors

The studied treatments had to some extent a cytokine stimulation and a drop in B cells observed in flow cytometry although most results were not statistically significant.

Complement-dependent cytotoxicity (CDC) seems to be an important mechanism since rituximab-WT where complement was active had a better killing than the C1q knock-out variant rituximab-P331S (**Fig. 4a**). This indicates on that both CDC and ADCC can work simultaneously which correspond with reported data for rituximab (12).

Rituximab-WT showed superior B cell depletion although rituximab-GASDALIE with its stronger affinity to CD16 was expected to have an enhanced ADCC, and therefore a superior B cell depletion with the addition of CDC. Why this did not occur could be due to the Fc modifications (G236A/S239D/A330L/I332E) in rituximab-GASDALIE. To Fc engineer a mAb and enhance NK cell activation have shown to increase NK cell mediated ADCC and to not affect CDC (31). However, the A330L mutation in

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rituximab-GASDALIE has been suggested to interfere with the C1q binding site and lead to a disconnected CDC activity (31). Based on the results, rituximab-GASDALIE seem to still have triggered an enhanced NK cell activation and ADCC activity since it showed superior upregulation of CD107a (**Fig. 9**) and cytokine stimulation. Although this was observed the B cell depletion between rituximab-P331S and rituximab-GASDALIE did not differ. A longer incubation period could perhaps have shown a clear difference in B cell drop between the rituximab variants. Ocaratuzumab (AME-133v) is a clinically used IgG1 humanized Fc-mutated anti-CD20 mAb with an improved binding to CD16. The Fc mutation elicits an enhanced ADCC activity and the mAb has been reported to have a more effective cytotoxicity than rituximab. The ADCC superiority of ocaratuzumab was observed at lower concentrations which could provide the possibility to administer such drug at lower concentrations than for a non-Fc mutated mAb and still have the same efficacy. Other mechanisms induced by the Ab that also are activated by rituximab, such as CDC, ADP and direct apoptosis showed similar cytotoxicity as rituximab (44). In the present study, a Fc mutant mAb which may enhance ADCC seem to also induce a stronger cytokine stimulation. Based on this observation ocaratuzumab would likely trigger a stronger cytokine release in blood and potentially cause more severe infusion reactions in patients than a non-Fc mutated mAb such as rituximab. No study was found where the safety profile of ocaratuzumab and rituximab was compared and should therefore be considered.

The lower CD107a expression observed with higher rituximab-WT (100 µg/ml) concentration even though the depletion was more effective could be because of less ADCC and superior CDC activity. The cytokine stimulation and CD107a expression were similar between rituximab-WT and rituximab-P331S which seem to indicate that the ADCC activity was not negatively affected by the C1q knock-out mutation. The lack of an upregulation of CD107a expression for rituximab-IgG2 is in line with that IgG2 does not provide Fc receptor cross-linking. Rituximab-IgG2 does not have an effective ADCC or CDC and minimal depletion and cytokine stimulation was therefore also expected. However, the observed depletion in donor 1 could be due to direct apoptosis if this were at all considered as depletion. Moreover, the intracellular cytokine

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stimulation observed for rituximab-IgG2 in donor 2 could have been triggered by unknown factors (**Fig. 7**). The unobserved signal of CD107a for donor 3 in flow cytometry with the treatments is likely due to less ADCC and the results correspond to the donor's response for having the least overall cytokine stimulation and a relatively low B cell depletion. Additionally, an upregulation of the CD107a marker have shown to downregulate the expression of CD16 on NK cells when stimulated (9,11). However, no clear downregulation or correlation was observed between CD16 and CD107a in the present study (**Appendix C**). One reason for this could be a too short incubation period which failed to observe the downregulation.

The observed difference in B cells at baseline between healthy donor were minor (**Fig. 10a**) and did probable not affect any results. However, the difference between healthy donors and patients with B cell malignancies is much more prominent. The B cell population in healthy donors constitute a minor population of the WBCs and may be the reason to the non-clear drop in white blood cell count with hemocytometer (**Fig. 4b**). Furthermore, the undetected cytokine release in plasma for healthy donors (**Fig. 5**) was anticipated due to the minor cytokine stimulation observed in flow cytometry. It is likely that without a high tumor load, the minor B cell population will not give rise to immune toxicity in the form of infusion reactions in healthy individuals. Furthermore, the larger B cell population in patients would most likely also induce a stronger activity of cytotoxic mechanisms.

The cell depletion profile for C1q knock out variant (rituximab-P331S) for donor 1 was superior of all donors (**Fig. 4**) which indicates on a strongly activated ADCC. However, the donor had a high baseline expression of NKG2A and HLA-E (**Fig. 10b&c**) which restrain NK mediated cell lysis and cytokine secretion. Despite this, donor 1 seemed to still have the most ADCC activity. However, the degree to which the binding occurs was not analyzed in the present study. Peptides presented on HLA-E are derived from peptide sequences from HLA class I molecules and recognized by CD94/NKG2A (42). CD94 has showed to have a significant role in the binding and therefore, an impaired interaction between the two due to a low-affinity peptide and/or mutations in CD94 or

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HLA-E (45,46) could be possible reasons for the observed ADCC activity. Furthermore, the incubation period in the blood loop could have been too short for this inhibition to occur. The expression of CD20 at baseline was expected to correlate with B cell depletion in the present study. In an *in vitro* study performed by Van Meerten et al. (9) they observed a correlation between low CD20-expression levels on B cells and less rituximab-induced CDC. Their results also showed that the two mechanisms operate complementary since an ADCC activity was still observed despite low CD20-expression. Donor 1 had the lowest CD20 density but the superior B cell depletion. Donor 2 had the highest CD20- and CD16 density on B and NK cells respectively (**Fig. 10d&e**) and was less responsive due to the relatively low depletion. These findings do not match with previous literature findings since no correlation between CD20, CD16 expression and depletion was observed. Additionally, there are CD16 receptor polymorphisms that have been reported to reduce the affinity to rituximab and its ADCC activity (6).

5.2. CD16 expression on B cells

The observed extracellular CD16 expression on B cells that was highly induced with rituximab-GASDALIE (**Fig. 8**) may have been caused by B cells capturing NK cell membrane fragments (including CD16) and then expressing CD16 on its own surface. This cell-cell contact dependent, inter-cellular protein transfer mechanism is in the literature called trogocytosis. Trogocytosis has been reported to occur between immune cells (e.g. T cells and FcyR-expressing antigen-presenting cells) for years and to be triggered through the stimulation of therapeutic agents (47). B cells are at baseline normally CD16-negative in healthy individuals and this observation is the first ever report that provides this information of CD16 on B cells with rituximab. The outcome of this phenomena is unknown such as how it could have affected the functional response of the therapeutic agents and what role CD16 receptors have on B cells. The transfer of CD16 could have for instance restrained the NK mediated ADCC activity for the treatment. In the present study, the mechanism appears to depend on the strength of

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the antibody to bind CD16 since the superior upregulation was observed with rituximab-GASDALIE. Then the mechanism should perhaps be investigated for the previously mentioned Fc modified Ab, ocaratuzumab.

5.3. Strengths and limitations of the present study

Cytokine release assays (CRAs) are essential for safety testing of acute infusion reactions. Today's most common CRAs lack one or more substantial blood component/s, such as complement and coagulation system. The blood loop assay retains these cascade systems and is a valuable method in situations when they affect the outcome. The assay also mimics the human blood circulation in the rotating wheel and prevents coagulation by keeping the blood in movement and is, in comparison to static whole blood plate assays a unique method to study infusion reactions in blood in motion. However, the drawback is the restricted gas exchange. A restricted oxygen exchange in the blood loop system can affect cellular responsiveness over time as pH may drop with long incubations. On the other hand, a too short incubation period can give an incomplete prediction of mode of action and infusion reactions if it develops over a long period of time. A four-hour incubation have been stated to be harmless for immune cells (25) and longer incubation periods (e.g. 48 hours) are reported in the literature (39). Another drawback with the blood loop assay compared to *in vivo* assay is the lack of endothelial cells, which may in the human body contribute to the adverse reactions (25).

The assay must start short after blood acquisition to avoid coagulation and for that reason the platelet count was measured pre- and post-experiment. Moreover, the method is dependent on fresh blood which was not tested for potential infectious agents. Appropriate personal protection equipment's were used as protection and the hepatitis B vaccines were taken before blood work.

The initial project plan was to include five healthy donors, but since the university was on a lock down due to the coronavirus (COVID-19) situation the experiment and the

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recruitment of donors was limited to a minimum of three donors. The second donor was dizzy during blood donation which prevented the collected of the total blood volume (40 mL) for the experiment. NK cell CD16 and CD107a expression, cytokine release in plasma, rituximab 100 µg/ml and positive control was therefore excluded from donor 2. The ability to do statistical analysis for these experiments were limited. For data where statistical analysis was performed most results did not reach statistical significance because of the low sample size and therefore all results are presented as trends of response.

An experiment on complement activation in blood loop assay was excluded in this study due to that the lack of cytokine release indicate that CRS will not be found in blood from healthy volunteers. Available complement ELISAs seem not to be sensitive enough to detect such a minor complement activation from rituximab in healthy donors although complement activation is detectable in CLL patients' blood with rituximab (verbal communication-Fletcher/Eltahir et al. unpublished data). The extent of the activation has been suggested to correlate with tumor count at baseline which is same as for cytokine release activation (28,29). ELISA was used to detect cytokine release in the present study and same as for complement the method seem to not be sensitive enough to detect such a minor cytokine release induced from killing B cells. Moreover, the ELISA for TNF α was not successful in donor 1 due to laboratory error. The assay failed twice, and it could be due to inadequate washing, contamination because of a reused plate sealer or imprecise dilutions.

5.4. Ethical and sustainability aspects

The safety profile has a crucial role in the approval of therapeutic agents, and it is therefore important that there are reliable and robust cytokine release assays available. The present project can besides predict efficacy and safety of rituximab, also further enhance knowledge on how to engineer the mAb with a favorable benefit-risk ratio.

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Human blood was the preferable option to study the aim of the present project since the mAb target human blood cells and because the therapy is used in humans. The *ex-vivo* human blood assay replaces *in vivo* animal toxicity assays and is therefore a more ethical method. Furthermore, the loop assay could be valuable for other biologics where the complement system is an important component.

Donors were verbally informed and signed a written informed consent form before participating in the study. Samples were coded with serial numbers and personal information was encrypted. At the end of the experiment all samples were destroyed. The study was voluntary, and the donors could at any time discontinue their cooperation.

6. Future perspectives

Cytokine release syndrome (CRS) is an issue for new mAbs and for the ones already on the market. More studies are needed to gain more knowledge of the pathophysiology of CRS. Further studies should include additional data on more donors healthy as supplement to the present study. A longer incubation period may be an option for further studies, however the impact on immune cells should be evaluated prior. The CD16 detection on B cells should also be further investigated to understand the mechanism behind it, what role CD16 has on B cells and what effect it has on ADCC as well as other immune reactions. Other rituximab variants should be studied in addition, for instance rituximab with enhanced affinity to C1q to compare with rituximab-GASDALIE to see which of ADCC and CDC is the more important immune mechanism for rituximab. Further studies would not only elucidate the study aim but also support and strengthen the presented results. Furthermore, the present study included healthy individuals although it is not the target population for the treatment. Therefore, all data should be interpreted as complementary data to future data from a replicated study with blood from patients where rituximab is used. This could further provide knowledge on the pathophysiology of the infusion reactions and, how to predict

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cytokine release and stratify patients accordingly.

7. Conclusions

Rituximab depleted B cells without inducing measurable cytokine release for healthy individuals. Among the studied treatments, Fc mutant rituximab seem to induce less B cell depletion. Rituximab-GASDALIE appear to elicit an enhanced NK cell activation. Moreover, the highly induced CD16 expression observed on B cells with rituximab-GASDALIE seem to be induced by trogocytosis. Further studies should include more donors as supplement and the results should be interpreted as complementary data to future data analyzed by performing the loop experiment using blood from patients.

8. Acknowledgements

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Populärvetenskaplig sammanfattning

Rituximab är en antikropp och används som ett biologiskt läkemedel för behandling av exempelvis leukemi. Läkemedlet binder till protein (CD20) på de immunceller som kallas B-celler och dödar dessa celler med hjälp av mekanismer tillhörande immunförsvaret; bland annat genom komplement-beroende cytotoxicitet (CDC) och antikroppsberoende cellmedierad cytotoxicitet (ADCC) som huvudsakligen aktiveras av kroppens naturliga mördarceller (natural killer cells eller NK celler). Rituximab tolereras ofta väl av patienter, men har rapporterats inducera frisättning av stora

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mängder cytokiner (proteiner) i blodet och således orsaka toxiska reaktioner i kroppen i samband med att läkemedlet ger en överaktiverad immunrespons. Syftet med detta arbete var att studera rituximab-inducerad B-celldöd och frisättning av cytokiner i blod från friska frivilliga samt om hur detta påverkades av modifierade versioner av antikroppen. Färskt blod från tre friska frivilliga förvarades i ett artificiellt blodloopssystem, som efterliknar hur blod cirkulerar i kroppen, i fyra timmar tillsammans med rituximab och tre olika modifierade versioner av rituximab. Studien genomfördes även med hjälp av immunologiska standardmetoder, såsom flödescytometri och enzymkopplad immunadsorberande analys (ELISA). Resultatet visade på att bland de studerade behandlingar så var rituximab mer effektiv på att döda B-celler än de modifierade rituximab-varianterna. I blodplasma upptäcktes ingen cytokinfrisättning, men däremot observerades en cytokinstimulering i NK celler från de friska individerna. Responsen från de olika behandlingarna i studien varierade och det berodde på huruvida antikroppen var modifierade eller inte. Dessutom spelade även modifieringstyperna en avgörande roll i responsen då det påverkade den funktionella aktiviteten hos antikroppen. Vidare studier borde utföras med fler friska frivilliga för att styrka all data och resultatet bör tolkas som kompletterande data till framtida data där blod ska analyseras från patienter.

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Appendices

Appendix A: Platelet stability analysis

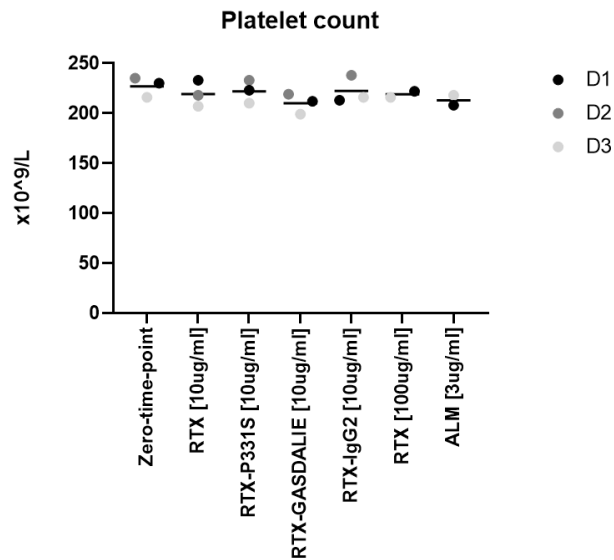


Figure 11. Platelet stability analysis. Fresh whole blood from healthy donors ($n=3$) was incubated for 4 hours with different rituximab treatments in blood loop system. Further treatment activation was stopped after 4 hours by diluting EDTA in samples. Platelet count was calculated with Sysmex XP-300 and plotted in $\times 10^9$ per liter. Final concentrations of all treatments are indicated in the figure. **RTX: rituximab, ALM: alemtuzumab, D1: donor 1, D2: donor 2, D3: donor 3.**

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Appendix B: B cell depletion and WBC count

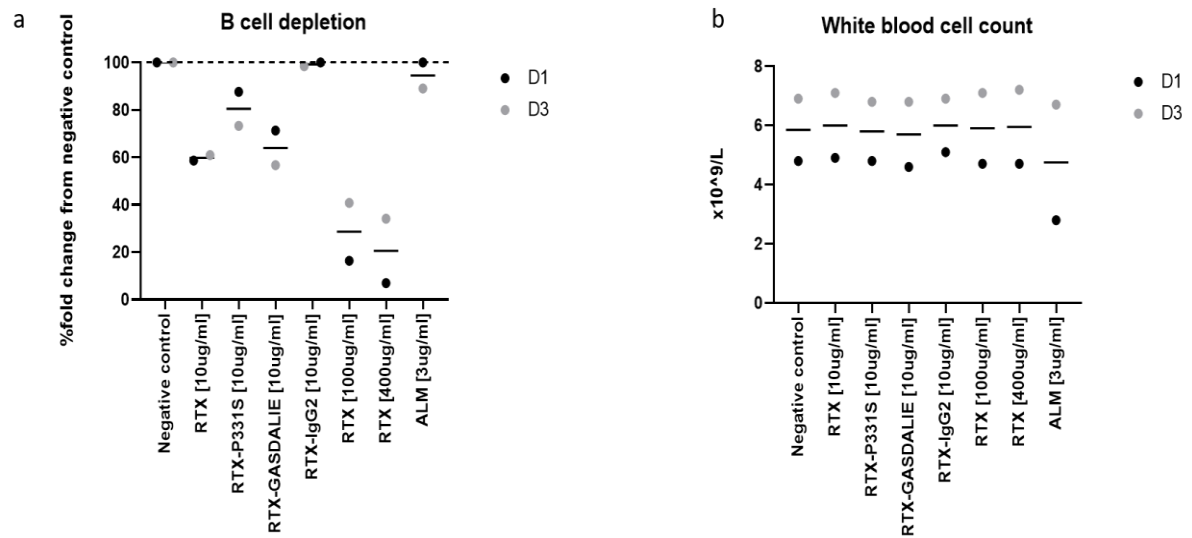


Figure 12. Rituximab-induced B cell depletion without brefeldin-A. Fresh blood from healthy donors ($n=2$) incubated with different rituximab treatments for 4 hours using blood loop system. **A.** B cell depletion was measured in flow cytometry as % viable CD19⁺ cells. Data was normalized as fold change of negative control. **B.** WBC count was calculated with Sysmex XP-300 hemocytometer and plotted in $\times 10^9$ per liter. Final concentrations of all treatments are indicated in the figure. **RTX:** rituximab, **ALM:** alemtuzumab, **D1:** donor 1, **D3:** donor 3

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Appendix C: CD16 expression on NK cells

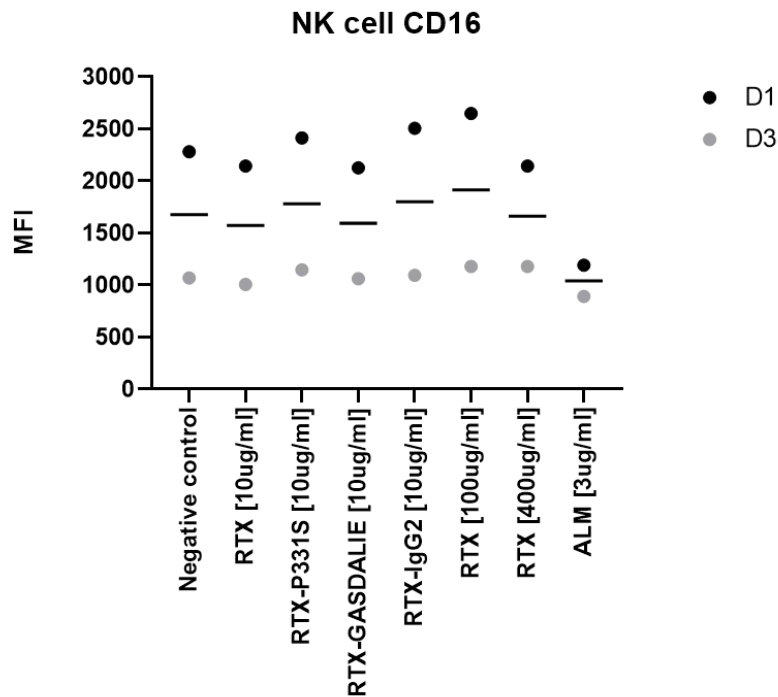


Figure 13. Extracellular NK cell CD16 expression. Fresh whole blood from healthy donors ($n=2$) was acquired after 4 hours incubation with different treatments using blood loop system. CD16 expression was measured in flow cytometry on NK cells (CD56+). Data was measured in mean fluorescence intensity (MFI). MFI describes the quantity of the expressed marker on every event. Final concentrations of all treatments are indicated in the figure. RTX: rituximab, ALM: alemtuzumab, MFI: mean fluorescence intensity, NK cell: natural killer cell, D1: donor 1, D3: donor 3.