Profiling of donor-specific immune effector signatures in response to rituximab in a human whole blood loop assay using blood from CLL patients

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ABSTRACT

Rituximab is widely used in the treatment of haematological malignancies, including chronic lymphocytic leukaemia (CLL), the most common leukaemia in adults. However, some patients, especially those with high tumour burden, develop cytokine release syndrome (CRS). It is likely that more patients will develop therapy-linked CRS in the future due to the implementation of other immunotherapies, such as CAR T-cell, for many malignancies. Current methods for CRS risk assessment are limited, hence there is a need to develop new methods. To better recapitulate an in vivo setting, we implemented a unique human whole blood “loop” system to study patient-specific immune responses to rituximab in blood derived from CLL patients. Upon rituximab infusion, both complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) profiles were evident in CLL patient blood, coincident with CLL cell depletion. Whereas B cell depletion is induced in healthy persons in the blood loop, only patients display B cell depletion coupled with CRS. With the exception of one donor who lacked NK cells, all other five patients displayed variable B cell depletion along with CRS profile. Additionally, inhibition of CDC or ADCC via either inhibitors or antibody Fc modification resulted in skewing of the immune killing mechanism consistent with published literature. Herein we have shown that the human whole blood loop model can be applied using blood from a specific indication to build a disease-specific CRS and immune activation profiling ex vivo system. Other therapeutic antibodies used for other indications may benefit from antibody characterization in a similar setting.

1. Introduction

Rituximab is a cornerstone therapy for many B-cell-malignancies, such as follicular lymphoma, diffuse large B cell lymphoma and chronic lymphocytic leukaemia (CLL), either as a monotherapy or in combination with chemotherapy [1]. Other indications include autoimmune diseases such as rheumatoid arthritis [2,3]. Although rituximab is generally well tolerated, some patients develop infusion reactions manifesting as cytokine release syndrome (CRS) commonly of grade 1–2. This type of reaction has been reported to occur early during rituximab treatment and has mainly been associated with the first infusion [4]. In CLL patients, high tumour burden is associated with an increased risk of CRS [4]. When CRS occurs, the patient can experience milder symptoms such as fever, nausea and vomiting. However, in severe cases,
involving dyspnoea and hypotension (grade 3–4), CRS can be life-threatening [5]. In parallel, a rise in biochemical markers such as TNF-α and IL-6 may be observed [6].

Responses to antibody-based CD20 targeted therapy in terms of the mode-of-action, adverse event (AE) profile and resistance mechanisms are likely both multifactorial and patient specific. Hence, it is difficult to identify a general and overarching rituximab specific mechanism for CRS development. For instance, some cytokine release assays (CRAs) using isolated peripheral blood mononuclear cells (PBMCs) from healthy donors can elicit cytokine release in response to rituximab [7]. However, the standard CRAs using whole blood from healthy donors in static plate assays do not elicit appropriate CRS readouts in response to rituximab stimulation [8]. This makes it challenging to predict susceptible patients at risk of severe AEs [9] given the variability in cytokine responses in the different assays.

The mode-of-action of the rituximab induced anti-tumour effect can be direct signalling induced apoptosis, complement-dependent cellular cytotoxicity (CDC) as well as antibody-dependent cellular cytotoxicity (ADCC) [10,11] and antibody-dependent cellular phagocyosis (ADCP), the latter possibly potentiated by complement activation [12]. Stimulated NK cells, which are the main cells involved in ADCC, can readily release large amounts of cytokines upon stimulation. Complement proteins are produced and released during activation of the complement cascade and through anaphylatoxins (C3a, C4a and C5a) can also contribute to the CRS pathogenesis [13].

Rapid and specific mode-of-action and AE profiling studies can be evaluated to some extent using in vitro assays [8]. These assays include effector cells from healthy individuals or cryopreserved cells from patients. However, all of these assays come with limitations in regards to the final readout as healthy cells do not stem from an immune-dysregulated environment and stored patient cells are commonly affected by the purification and freezing steps. In addition, most cell-based assays using human cells do not include an active complement system, either due to cell purification methods or, if whole blood is used, anticoagulants. Furthermore, the purification of certain cells for these types of in vitro assays strictly reduces the range of mechanisms and inter-relationships that can be investigated, reducing the physiological relevance. In a different way, murine models that are able to study responses to antibody-based therapies in vivo have limitations in their tumour kinetic outgrowth, as well as species-specific differences in immune responses to antibody-based therapeutics. These differences can, among other things, depend on target expression, genetic as well as the transcriptomic make-up of the antibody effector (FcgR and complement) repertoire [14–16], along with differences in gene expression patterns [17] as well as haematoepoiesis and immune cell distribution [18]. In contrast, whereas clinical trial data are very valuable, they cannot typically be used to test a hypothesis, but rather are hypothesis generating. Perhaps the best compromise is the use of whole human blood from patients which contains all of the main circulating components required to elicit an immune response, as well as tumour cells in many diseases, in the case of CLL. With this background, we set out to study the immune responses in human whole blood loop system [19,20], with intact complement, in blood from CLL patients. The loop assay has previously shown value when profiling known CRS inducing antibodies using blood from healthy volunteers [19]. However, as rituximab is not inducing cytokine release in healthy blood while causing problems in patients with tumours, we explored the nature of the adverse event in the loop model using blood from CLL patients. Disease-specific CRS and mode-of-action assays could also be explored when using therapeutic antibody strategies, for example in inflammatory conditions, if adverse event profiles may risk deviating from a non-inflammatory host.

2. Materials and methods

2.1. Materials and antibodies

Rituximab (MabThera®), alemtuzumab (Lemtrada®), and cetuximab (Erbitux®) were purchased from Sanofi (France), Roche (Switzerland) and Merck (Germany) respectively. Fc mutant rituximab P331S, rituximab-GASDALIE and rituximab-hlgG2 were produced in-house (University of Southampton, UK). C3 blocker (compsstatin) was kindly provided JD Lambris (University of Pennsylvania, School of Medicine, USA). CD16 blocking F(ab)2 (3G8) was purchased from Ancell (USA) and Heparin (Heparin LEO, Leo Pharma AB, Sweden) was purchased from Apoteket AB (Sweden). All fluorophore labelled antibodies were purchased from Bioplex (USA).

2.2. Study participants

The study population consisted of six CLL patients and five age-matched healthy volunteers. Inclusion criteria for CLL patients are a diagnosis according to the iwCLL-criteria who are newly diagnosed (no prior therapy) or previously diagnosed with CLL (history of prior therapy), and planned for further therapy. Patients who were receiving ongoing CLL therapy at the time of blood donation were excluded. The patients donated blood prior to receiving the planned therapy. Four CLL patients (D1, D2, D4 and D6) were included at the time of diagnosis prior to initiation of any specific CLL therapy, while two (D3 and D5) had received prior therapy for their CLL and were planned for treatment of relapsed disease. The patients were recruited at the Department of Haematology, Uppsala University Hospital in Sweden and had all had signed informed consent to participate after having been given oral and written information of the study. All CLL-patients were to receive treatment due to active CLL. None had overt signs of an ongoing infection (Table 1) displays the patients’ clinical characteristics.

2.3. Endpoints

The primary endpoint is to compare the development of cytokine release, complement activation and B cell depletion in the blood between healthy controls and CLL patients following a 4 h incubation with rituximab in the ex-vivo whole blood loop assay. The secondary endpoint is to investigate the mechanisms of the early rituximab induced B cell depletion (CDC vs ADCC) and the contribution of these mechanisms in CRS development.

2.4. Blood loop assay

The blood loop assay was conducted as described previously [19]. Briefly, 35–45 mL whole blood was collected from each of the study participants using open blood collection system. All surfaces in contact with blood were coated with insoluble heparin conjugates (Corline, Sweden). After acquiring the blood, soluble heparin (Heparin LEO) was added to the blood to a final concentration of 1 IU/ml. A volume of 2 mL of whole blood was subsequently added to surface heparinized PVC loops (Corline, Sweden) and a therapeutic antibody was added to each individual loop. For loops intended for intracellular cytokine staining, Brefeldin A (Sigma-Aldrich, USA) was added to the blood at a final concentration of 1 mg/mL. Blood loops were incubated with the treatment for 4 h at 37 °C and the loops were kept in motion by attaching them to a circulating wheel. Blood was sampled at zero-time-point (baseline analysis), after 15-min (complement analysis) and at 4 h (plasma and intracellular cytokines analysis). At sampling, blood was mixed with EDTA (Sigma-Aldrich) to a final concentration of 10 mM to prevent coagulation.
Table 1
Patient characteristics. Demographic and clinical characteristics of CLL patients. D = denotes donor number, D1-6 CLL patients. WBC = white blood cell count, ALC = absolute lymphocyte count, LDH = lactate dehydrogenase, CRS = cytokine release syndrome.

| ID  | Age | Diagnosis | Previous treatment | WBC (cells/L) | ALC (cells/L) | LDH (IU/L) | Treatment after loop ext. | CRS | Grade |
|-----|-----|-----------|-------------------|---------------|--------------|-----------|------------------------|-----|-------|
| D1  | 67  | CLL       | No                | 61.4          | 56           | 3.1       | Obinutuzumab iv         | Yes | 2     |
| D2  | 70  | CLL       | No                | 137.6         | 126.5        | 3.4       | BTK-inhibitor           | No  | NA    |
| D3  | 71  | CLL       | Yes               | 145.5         | 128.6        | 5.9       | Rituximab sc            | Yes | 1     |
| D4  | 81  | CLL       | No                | 185.9         | 182.1        | 2.8       | BTK-inhibitor           | No  | NA    |
| D5  | 70  | CLL       | Yes               | 122.9         | 107.2        | 7.1       | Rituximab iv            | Yes | 2     |
| D6  | 71  | CLL       | No                | 222.4         | 216.3        | 3.2       | BTK-inhibitor           | No  | NA    |

2.5. Cell counting

Red blood cell (RBC), white blood cell count (WBC) and platelet count were measured using haematology analyser XP-300 (Sysmex, Japan) before and after the incubation to monitor for changes in the counts caused by the experiment and to monitor for clotting of the blood.

2.6. Plasma cytokines, complement and proteomic analysis

EDTA plasma for cytokine, complement and proteomic analysis was isolated by centrifugation of whole blood at 2000g at 4°C for 20 min. For cytokines, plasma from 4 h blood was analysed by MSD Mesoscale V-plex kit for IFNγ or TNFα, IL-6 and IL-8 according to the manufacturer’s instructions. Complement split products C3a and C5a analysis was conducted on plasma isolated from blood collected at the 15-min time-point using Human C3 or C5 ELISA (RayBio, USA) according to the manufacturer’s instructions. Proteomic analysis was performed on 4 h plasma using the OLINK Proteomics (Uppsala, Sweden) multiplex platform. A panel consisting of 92 immuno-oncology related proteins was assayed.

2.7. Intracellular staining and flow cytometry

The following fluorochrome labelled antibodies were used for flow cytometry staining (antibody clone in parentheses): Anti-CD4 (OKT4), anti-CD8 (SK1), anti-CD3 (UVHT1), anti-CD19 (HIB19), anti-CD56 (NCAM), anti-CD14 (M5E2), anti-CD16 (3G8), anti-CD20 (2H7), anti-CD5 (L17F12) and anti-HLA-E (3D12). Anti-IFNγ (4S.B3) and anti-CD8 (SK1), anti-CD3 (UVHT1), anti-CD19 (HIB19), anti-CD56 (NCAM) antibodies. Cells were next washed with perm wash buffer and subsequently permeabilised by applying perm/wash buffer (BD, USA). Cells were incubated with 100 µl of whole blood for 30 min at 4°C before the red blood cells were lysed using RBC lysis buffer (BD, USA). Cells were washed twice with PBS before fixation and permeabilisation was performed by applying BD cytofix/cytoperm buffer (BD, USA) for 20 min at 4°C. Cells were then washed twice with PBS 780 were incubated with 100 µl of whole blood for 30 min at 4°C before the red blood cells were lysed using RBC lysis buffer (BD, USA). Cells were washed twice with PBS before fixation and permeabilisation was performed by applying BD cytofix/cytoperm buffer (BD, USA) for 20 min at 4°C. Cells were subsequently permeabilised by applying perm/wash buffer (BD, USA) before intracellular cytokine staining were stained for using anti-IFNγ and anti-TNFα antibodies. Cells were next washed with perm wash buffer and with PBS twice. Cells were analysed using Beckman Coulter CytoFLEX flow cytometer.

2.8. Ethical considerations

The study was approved by the Regional Ethical Committee in Uppsala (Dnr 2018/350) and performed according to the Helsinki declaration.

2.9. Data and statistical analysis

Flow cytometry data were analysed using FlowJo (LLC). Statistical analysis was performed in graphPad Prism 7.02 (GraphPad Software, Inc.) or R 4.0.0 (R Core Team, 2020). Multiple comparison analysis was performed using paired, non-parametric Friedman test with Dunn’s multiple comparison test. When indicated in the figure, outliers were identified and excluded using the ROUT method. *p < 0.05, **p < 0.01, ns = not significant. The ComBat function from the sva package (3.36.0) in R was used to remove the donor-dependent variation (batch effect) observed in the OLINK multiplex analysis and patients considered thereafter as independent. Treatments were interrogated with ANOVA and proteins with FDR < 0.05 presented in the heatmap.

3. Results

3.1. Rituximab induces a drop in white blood cell count and cytokine release in blood from CLL patients but not in blood from healthy donors

In order to investigate if a high white blood cell (WBC) count, i.e. high target cell number, is required for rituximab to induce cytokine release in the blood loop system, we compared responses in blood from six CLL patients with high WBC counts to blood from five age-matched healthy donors with normal WBC counts. WBC count was measured by a haematology analyser at sampling. CLL patient counts ranged between 51.8-223.1 × 10^9 (median 147.6 × 10^9) cells/L compared to 3.7-7.7 × 10^9 (median 3.83 × 10^9) cells/L in the healthy controls (Fig. 1A). After incubating rituximab with the blood for 4 h in the blood loop system, there was a drop in WBC count in the vehicle-treated blood from CLL patients. The WBC drop in vehicle-treated blood ranged from 6.7% to 55.3% (median 22.1%) from the zero time-point, while there was only a minor drop in the vehicle-treated blood from healthy donors, ranging between 0.3% and 8.9% (median 3.0%) from the zero time-point (Fig. 1B). Compared to the zero time-point, only rituximab induced a statistically significant drop in WBC count in CLL patient blood with a median of 31.0%, 37.3% and 44.5% drop in samples administered with 10 µg/ml, 100 µg/ml and 400 µg/ml rituximab, respectively (Fig. 1B). The greatest rituximab-induced drop of WBCs was seen in donors 5 and 6 (D5 and D6). In healthy donor blood, the WBC count drop was less pronounced by rituximab treatment and ranged between 0.9 and 11.3% of the zero time-point sample for all donors (Fig. 1C). The anti-EGFR hlgG1 antibody cetuximab was used as a negative control. In the absence of its target ligand, it did not impact WBC count or the B cell population in CLL patients or healthy blood donors and the drop in WBC count was comparable to the drop in the vehicle-treated blood. In contrast, incubation with a low dose of alemtuzumab, (used as a positive control) due to a broad target cell distribution (CD52+) cells in blood from both healthy and CLL, resulted in a drop in the WBC count both in both CLL and in some, but not all, healthy volunteers. In CLL patients, the median drop was 65.5% and in healthy donors the median drop was 9.5% compared to the zero time-point (Fig. 1B and C). To assess B cell as opposed to WBC depletion, we performed flow cytometry analysis. Despite that no drop in WBC count was apparent, rituximab depleted the B cell population (CD19+) in the loop of healthy donors blood with more prominent depletion at the highest concentration [rituximab 400 µg/ml] (Fig. 1D and E). This discrepancy is likely due to the fact that B cells constitute only a minor proportion of the total WBC count in healthy individuals and depletion do not impact WBC count.
3.2. Rituximab induces cytokine release from NK cells in the blood from CLL patients

In concordance with our previously published data [19], the level of cytokine release in blood from healthy donors was similar to the vehicle-treated group at all three concentrations of rituximab (Fig. 2A and Table 2). In contrast, rituximab induced clear cytokine release, reflective of potential CRS, in blood from CLL patients in the loop (Fig. 2A and Table 2). This increase in cytokines corresponded to a fold increase in the median cytokine release of (27–74)-fold, (4–6)-fold, (13–15)-fold and (2–4)-fold in IFNγ, TNFα, IL-8 and IL-6 levels, respectively compared to vehicle-treated CLL blood. However, these differences did not always reach statistical significance due to large interpatient variability in cytokine release. Interestingly, in one patient, donor 2 (D2), neither rituximab nor alemtuzumab gave rise to a cytokine release at any of the three concentrations tested (rituximab cytokine profile; IFNγ ≤ 11.4 pg/ml, TNFα ≤ 1.9 pg/ml, IL-8 ≤ 68.4 pg/ml and IL-6 ≤ 1.9 pg/ml) (Fig. 2A and B). Furthermore, the positive control for CRS, alemtuzumab, induced cytokine release in blood from both CLL patients and healthy donors (apart from D2). The cytokine levels in response to the negative control (cetuximab) was similar to the vehicle group in both CLL patients and healthy donors (Fig. 2 and Table 2). Of note, the donors D1, D3 and D5 that all displayed cytokine release in the loop, were treated with rituximab or obinutuzumab in the subsequent course of...
their treatment. In these donors, a grade 1–2 CRS profile was apparent during therapy, aligning the cytokine secretion data for these three donors with the CRS profile predicted by the whole blood loop model used herein (Table 1).

To identify the cellular source of rituximab-induced cytokine release in blood from CLL patients, brefeldin-A was added in another set of experiments, designed to capture cytokines intracellularly and enable flow cytometry analysis of intracellular cytokines. Intracellular IFNγ and TNFα were observed in NK cells (CD56+CD3−) (Fig. 2B) but not in B cells (CD19+), monocytes (CD14+) or T cells (CD3+) (Fig. 2C). Interestingly, D2 that did not show any sign of a CRS profile for any antibody used. D2 was also the only donor that lacked circulating NK cells (CD56+CD3−) (Fig. 2B), suggesting that NK cells are indeed the major source of IFNγ and TNFα release associated with rituximab and possibly also alemtuzumab treatment. For the other CLL patients, out of total gated NK cells, IFNγ+ NK cells had a median of 16.9%, 16.6% and 49.7% in rituximab 10 µg/ml, 100 µg/ml and 400 µg/ml treated blood. TNFα+ NK cells had a median of 10.3%, 10.3% and 34.9% in rituximab 10 µg/ml, 100 µg/ml and 400 µg/ml treated blood respectively (Fig. 2D and E).

In vehicle-treated blood from CLL patients, IFNγ+ and TNFα+ NK cells had respectively median of <0.2% and <0.8%. The cellular source of alemtuzumab-induced cytokines was also NK cells (data not shown) with no or low cytokine release observed in blood from D2 (Fig. 2D and E). Similar to plasma cytokine data, the percentage of IFNγ+ cells and TNFα+ NK cells were low (below 0.3% IFNγ and 0.5% TNFα) in healthy donors assessed for intracellular cytokine flow cytometry.

3.3. Rituximab induces complement activation in blood from CLL patients but not healthy donors

In concordance to our previous data [19], the levels of complement split products (C3a and C5a) in blood from healthy donors were

![Fig. 2. Cytokine release after 4 h in the blood loop system. Blood was acquired from CLL patients (n = 6) and age-matched healthy controls (n = 5) and incubated with antibodies (as displayed in the figure with concentrations in brackets [µg/ml]) in the blood loop system (as described in materials and methods). Aliquots of blood were sampled at time zero (before blood loop experiment) and after 4 h of incubation and the plasma levels of IFNγ, TNFα, IL-8 and IL-6 were analysed by MSD multiplex assay (A). Blood loops with brefeldin-A were included and analyse for intracellular IFNγ and TNFα levels in blood immune cells by flow cytometry. (B) Flow cytometry plots showing NK cell population in D2 (lacked circulating NK cells) and D3 (with circulating NK cells. (C) representative flow cytometry plots (D3) showing intracellular IFNγ, TNFα staining in CD56+, CD19+, CD14+ and CD3+ cells from rituximab treated blood or vehicle. Only NK cells from CLL patients expressed intracellular IFNγ and TNFα (D and E). Outliers are identified and excluded by the ROUT method (q = 0.001). Each dot represents a donor. The lines represent the mean. *p ≤ 0.05, **p ≤ 0.01, ns, not significant. Paired, non-parametric Friedman test with Dunn’s multiple comparisons test. D2 (appears in the figure) was excluded from the statistical analysis because the donor lacked circulation NK cells. D = denotes donor number, D1-6 CLL patients, D7-11 healthy donors.](image-url)
comparable in the vehicle-treated (median of 647.8 ng/ml C3a and 12.6 ng/ml C5a) and all three concentrations of rituximab (Fig. 3A, B and Table 3). In contrast, rituximab treatment increased the levels of C3a and C5a in blood from CLL patients compared to vehicle treatment with a 4.5–5.8 fold increase in the median C3 and 1.3–2.0 fold increase in the median C5 compared to vehicle (Fig. 3C, D and Table 3). The levels of
Complement split products were the highest in rituximab-treated blood plasma from CLL donors (mean of 1477.0 ng/ml C3a and 73.3 ng/ml C5a). However, only a slight increase was observed in healthy controls (mean of 844.0 ng/ml C3a and 20.4 ng/ml C5a) (Fig. 3 and Table 3).

3.4. Blockade of CDC or ADCC alters the killing activity and immune response profile of rituximab

Rituximab is a hlgG1 antibody that can kill target cells via both CDC and ADCC. In the loop system, both mechanisms are active. To investigate the killing mechanism in blood from CLL patients, different Fc-variants of rituximab as well as blocking agents of CDC or ADCC were examined. These included; rituximab with the P331S mutation (impaired for binding C1q), a hlgG2 rituximab (reduced C1q and FcgR binding) or wild type rituximab (hlgG1) in combination with either compstatin that blocks C3 of the complement cascade or anti-CD16 F(ab’)2 fragments to block NK-mediated ADCC. Rituximab (hlgG1), used as a single agent, resulted in an average 25.6% drop in WBC compared to vehicle control, while blocking complement by either using rituximab-P331S or addition of compstatin decreased rituximab induced WBC drop compared to the vehicle to 16.1% or 10.9%, respectively (Fig. 4A). Conversely, combining anti-CD16 F(ab’)2 fragments with rituximab (hlgG1) increased the WBC drop from a mean drop of 25.6% (rituximab alone) to 73.4% (Fig. 4A). The drop of WBC count with rituximab compared to vehicle varied between donors (Supplementary Fig. 1). However, the pattern was consistent in that by blocking ADCC, the B cell killing increased, whereas by blocking CDC (by compstatin or using rituximab-P331S) the killing capacity of rituximab was reduced, but not abolished. Although only assessed in a single donor (D11), the hlgG2 version of rituximab caused a relatively low WBC drop of 4.3% (Supplementary Fig. 1).

Whereas blocking of complement with either compstatin or rituximab-P331S resulted in decreased C3a and C5a compared to rituximab hlgG1 alone, the addition of CD16 F(ab’)2 fragments to rituximab hlgG1 increased complement activation in the system as compared to rituximab treatment alone (Fig. 4B and C). Complement proteins are suggested to hinder the NK cell mediated ADCC on rituximab coated B cells[23]. In the blood loop system, blocking complement either through compstatin or by using rituximab-P331S resulted in increased levels of IFNγ and TNFα secretion in plasma (Fig. 5A and B). However, blocking
CD16 via anti-CD16 F(ab)₂ increased the levels of TNFα, IL-6, IL-8 and to a relatively minor extent IFNγ in plasma compared to rituximab hIgG1 alone (Fig. 5A–D). Consistent to the plasma cytokines, rituximab-P331S induced more %IFNγ and %TNFα+ NK cells (mean of 18.4% IFNγ and 13.7% TNFα) than rituximab-hIgG1 (mean of 11.6% IFNγ and 8.2% TNFα) (Fig. 5E and F).

3.5. NK cell CD16 expression is downregulated upon rituximab treatment

Downregulation of activating receptors on NK cells has been reported upon NK cell activation [24–26]. We observed downregulation of the activating CD16 (FcγRIIIa) on the surface of NK cells upon treatment of CLL blood with rituximab in the blood loop system (Fig. 6A). Interestingly, CD16 downregulation was more prominent in rituximab-P331S as well as alemtuzumab treated blood compared to wild type rituximab treated blood. This may suggest a more pronounced NK cell activation and hence ADCC dependent B depletion pathway for these two treatments (Fig. 6A and Supplementary Fig. 2).

To further investigate CD16 downregulation upon NK activation by rituximab or rituximab with Fc mutations, we tested wild type rituximab (hIgG1), rituximab-P331S as well as rituximab-GASDALIE in whole blood from healthy donors in the blood loop system. Rituximab-GASDALIE, is a hIgG1 rituximab bearing the Fc mutations (Gly236Ala/Ser239Asp/Ala330Leu/Ile332Glu), leading to increased binding affinity to CD16 [27,28]. However, in healthy blood, we could not see changes in CD16 surface expression on NK cells upon treatment with rituximab or rituximab-GASDALIE (Supplementary Fig. 3). This was not surprising and could be attributed to the low or no NK cell activation in healthy blood with rituximab, due to relative scarcity of B cells in healthy blood compared to CLL blood where B cell numbers are extremely high. Interestingly, when we stained the blood for CD16 in flow cytometry, we observed an unexpected CD16 staining on the surface of B cells (gated CD19+CD3−CD56−) in the blood treated with rituximab-GASDALIE, but not with the other variants of rituximab or the other treatments (Fig. 6B). Notably, rituximab-GASDALIE treatment did not increase B cell depletion when compared to the wild type rituximab (hIgG1).

3.6. CD20, CD5 and HLA-E expression by B cells in blood from CLL patients

To investigate the correlation between surface marker expression on B cells and B cell depletion and CRS, we performed flow cytometry phenotyping on gated CD19+ cells from CLL patients and healthy donors to identify the baseline expression of CD20, CD5 and HLA-E. The cellular target for rituximab, CD20, has been demonstrated to be weakly expressed on CLL cells [29]. Accordingly, in our small cohort, the CLL cells expressed lower levels of CD20 compared to B cells

### Table 3

Plasma complement split products levels. Plasma C3a and C5a levels at zero time-point or 4 h after incubation of antibodies with blood from CLL patients (n = 6) or healthy donors (n = 5) in the blood loop system. Antibodies concentrations in µg/ml are shown between brackets. CLL = plasma from CLL donors, HC = plasma from healthy controls.

|          | C3a (ng/mL) |          | C5a (ng/mL) |
|----------|-------------|----------|-------------|
|          | Median | Mean | SEM | Median | Mean | SEM |
| CLL (n = 6) |        |        |     |        |        |     |
| Zero     | 223.9  | 230.2  | 35.3 | 9.1   | 12.3   | 4.0 |
| NaCl     | 501.7  | 568.1  | 115.2| 15.6  | 18.5   | 5.4 |
| Cetuximab [200] | 458.9  | 553.5  | 101.5| 15.6  | 18.9   | 5.8 |
| Rituximab [10] | 2273.0 | 1933.0 | 470.8| 20.8  | 28.8   | 7.8 |
| Rituximab [100] | 2870.0 | 2235.0 | 501.7| 30.7  | 47.6   | 18.4|
| Rituximab [400] | 2929.0 | 2388.0 | 499.5| 31.4  | 36.3   | 13.0|
| Alemtuzumab [3] | 1500.0 | 1477.0 | 508.6| 70.3  | 73.3   | 33.0|
| Zero     | 454.6  | 437.7  | 90.6 | 11.3  | 10.7   | 0.7 |
| NaCl     | 647.8  | 650.8  | 105.2| 12.6  | 13.1   | 1.3 |
| Cetuximab [200] | 743.8  | 709.2  | 133.0| 12.7  | 12.1   | 1.0 |
| Rituximab [10] | 670.8  | 646.7  | 102.2| 11.3  | 11.7   | 0.8 |
| Rituximab [100] | 752.1  | 729.8  | 112.2| 12.3  | 12.8   | 1.0 |
| Rituximab [400] | 693.5  | 708.1  | 110.0| 13.4  | 12.4   | 1.0 |
| Alemtuzumab [3] | 792.1  | 844.0  | 130.8| 18.6  | 20.4   | 3.6 |

### Fig. 4

Blocking of CDC/ADCC effects on rituximab-induced B cell depletion and complement activation. Blood was acquired from CLL patients and was incubated with antibodies (10 µg/ml) with/without CDC/ADCC blockers in the blood loop system (as described in Materials and Methods). The WBC count was automatically counted using a Sysmex XN-1-350 Haematology Analyser at time Zero (before blood loop experiment) and after 4 h of incubation, displayed as a percentage (%) of the vehicle as a mean value of all donors (A, n = 3–5). Complement split products C3a and C5a quantified in 15 min EDTA plasma samples using ELISA (n = 2–4) (B and C). WBC = white blood cell count.
and the plasma levels of IFN-γ of the 92 proteins assessed, 83 proteins were detectable in the EDTA plasma isolated after 4 h of treating blood with rituximab (or rituximab variants) from CLL patients expressed intracellular IFN-γ and TNFα in blood immune cells by flow cytometry. (E) representative flow cytometry plot of intracellular TNFα versus IFN-γ expressed by NK cells (gated CD56+ CD3–) from CLL patients expressed intracellular IFN-γ and TNFα and (F) pooled intracellular IFN-γ and TNFα from CLL patients (n = 3). Each dot represents a donor. The lines represent the mean. D = denotes donor number, D3-5 CLL patients.

However, no dose-dependent response of rituximab was observed and no difference between rituximab hIgG1 and the mutant versions of rituximab in relation to changes in the drug-induced release of plasma proteins was noted at this specific time-point.

4. Discussion

In order to investigate rituximab induced immune responses, researchers commonly apply rituximab to purified immune cells from healthy volunteers and stimulate these cell cultures for 24 h or more [8,11]. Many investigations concerning both mode-of-action or AE profiling of rituximab employ similar approaches and some commercial suppliers have applied rituximab as a positive control in CRA profiling assays [7,32–34]. However, using healthy donor blood/PBMCs, these CRAs often fail to induce a CRS signal in rituximab stimulated conditions [8,11]. This is likely not because CRS does not occur, but rather that it is related to the assay conditions and particularly target load on the cells present in the system [4]. In order to investigate the immediate infusion induced immune effector profiles in healthy volunteers and CLL patients, we made use of a unique human whole blood loop system. This system has been previously used to predict cytokine release by antibody-therapeutics from healthy blood [19,20] as well as to study the instant blood mediated immune reaction (IBMIR) related to when donor allogeneic transplant cells meet host blood [21]. The complexity and the donor-specific response to rituximab were apparent using this system.

Rituximab induced a clear drop in WBC in some, but not all CLL blood samples, but no drop in WBCs was noted in healthy volunteers. Although the drop in WBC in WBCs was not apparent in healthy volunteer blood, rituximab activity was observed as a dose-dependent B cell depletion in this cohort. Interestingly, in relation to this, profiling of immediate cytokine release in response to rituximab infusion resulted in elevated measurable cytokine release in blood from CLL patients but not from healthy volunteers. This observation supports the notion of tumour load dependent cytokine release in concordance with clinical observations [15]. Thus, to

3.7. Chemotactic and cell killing associated proteins are increased in the plasma of rituximab treated blood

In a further attempt to distinguish the profile of effector and regulatory mechanisms engaged by the monoclonal antibodies in the blood, we performed a multiplex analysis using a 92-feature proteomic panel with proximity extension assay (PEA) technology. This approach allows an unbiased assessment of protein changes in 92 different proteins in the plasma isolated after 4 h of treating blood with rituximab (or rituximab variants) or the isotype-controlled matched control cetuximab. The heatmap illustrates relative protein levels for the different treatments (Fig. 8). Out of the 92 proteins assessed, 83 proteins were detectable in the EDTA plasma of CLL patients. Based on the Log2-relative quantification values (NPX), 33 proteins showed a significant difference in blood treated with rituximab or the rituximab variant compared to cetuximab or vehicle-treated blood (FDR < 0.05) (Fig. 8). Markers that were upregulated included cytokines (IL-6, IL-7, IL-8, IFNγ, TNFα and TNFSF14), which is consistent with our plasma cytokine analysis performed by MSD multiplex assay (Fig. 2). In addition, chemotactic (CCL-3, CCL-4, CCL-17, CCL-20, CXCL-1, CXCL-5 CXCL-10, CXCL-11 and MCP-1, MCP-2, MCP-3, MCP-4) and cell killing related proteins (FASLG, GZMA-A, GZMB-B, GZMH- H) were also upregulated in rituximab treated samples, indicating more recruitment of immune cells and activation of killing activity. This also corresponds to our B cell depletion data in rituximab treated blood.

from healthy donors at the baseline (Fig. 7A). In addition, the T cell marker CD5, which is abnormally expressed on B cells of CLL patients [30] was upregulated on B cells in our CLL cohort compared to healthy B cells (Fig. 7B). However, no clear correlation between CD20 or CD5 expression levels and the degree of B cell depletion, complement activation or cytokine production was observed. Moreover, HLA-E is a class I MHC molecule that can interact with the inhibitory molecule NKG2A on NK cells to inhibit them [31]. The level of HLA-E expression on B cells was similar in the blood from CLL patients and healthy donors (Fig. 7 C).
only rely on blood/cells from healthy donors as a source to develop assays to predict cytokine release and mode-of-action of antibodies targeting blood-derived subpopulations may not be informative. Instead, patient-derived blood should be considered in these types of CRS assays.

The main source of IFNγ and TNFα was the circulating NK cells. However, other cytokines such as IL-8, indicated by the proteomic plasma analysis were not investigated by cell-specific staining. Hence, we cannot rule out that cytokines other than IFNγ and TNFα are produced by other immune cells as well as the malignant cells themselves. IL-8 is also known to be a down-stream mediator from anaphylatoxin stimulation and released by granulocytes [35,36] and elevated IL-8 is a likely result of antibody-induced CDC. Interestingly, D2 that lacked circulating NK cells did not exhibit rituximab induced cytokine release irrespective of whether it was NK cell mediated cytokine release (IFNγ and TNFα) or complement driven IL-8 secretion. Consistent with that, D2 had a relatively lower WBC drop upon rituximab treatment, which could be explained by the lack of NK cells to mediate ADCC as well as possible resistance to CDC in this patient. Resistance to CDC has previously been described to be associated with sialic acid content and recruitment of complement inhibitors [37].

Whether the blood loop assay could be used in routine clinical practice to predict response to rituximab (CDC and ADCC activity combined) remains to be investigated. However, if the loop can be used to identify patients that are both refractory to CDC and ADCC and thus are unlikely to respond to rituximab therapy, it would have value to assess such resistance mechanisms and for personalized therapeutic strategies.

When studying the NK cell response, no healthy volunteers displayed NK cell derived cytokine release (IFNγ and TNFα) to rituximab, whereas all rituximab doses assessed resulted in NK-cell mediated cytokine
release in all CLL patients apart from D2. For CDC responses, the results point towards a clear distinction between donor 3, 4, 5 and 6 whom all displayed CDC in response to rituximab, whereas donor 1 and 2 did not. However, as donor 1 had the highest measured cytokine release both in the plasma analysis and the intracellular staining (NK cells), this sample may exhibit compensation for the lack of CDC with an increased ADCC response. As donor 1 appeared to have a skewed response towards ADCC, and a reduced CDC profile, we decided to investigate how Fc mutants of rituximab or blockade of either CD16 or complement pathways would affect its ADCC/CDC profile. Two clinically used antibodies (obinutuzumab and ofatumumab) targeting CD20 have been developed that utilize either enhanced ADCC/P or improved CDC to overcome rituximab resistance [38,39]. These antibodies appear to have higher efficacy in treating CLL in the clinic but may be associated with an increased risk of AE [38,40,41] although results are not consistent and trials with different indications are difficult to compare head to head.

By impairing the complement pathways, there was a slight hampering of WBC killing, but the most drastic effect was noted when CD16 was blocked, resulting in enhanced CDC mediated killing. To understand if the diminished CDC, and white blood cell loss, with the C1q-impaired rituximab-P331S leads to improved NK cell activity, we performed intracellular NK cell IFNγ and TNFα staining. Our results indicated a tendency towards an increased cytokine release in NK cells when C1q binding is reduced. This effect might be more pronounced if the assay incubation was prolonged. Thus, it appears as if a reduction in C1q engagement slightly skew B cell killing towards NK mediated effector mechanisms (e.g. ADCC). However, hampering the rituximab/CD16 interaction appears to greatly facilitate WBC loss (presumably through increased CDC) during the initial blood/drug interface as previously described in the literature [42,43].

Interestingly, we also found an inverse correlation between loss of CD16 on NK cells and their activation profile with regards to cytokine production for the NK activating rituximab stimulations in CLL patient blood. When investigating healthy donor blood in the presence of rituximab, we saw an appearance of CD16 on B cells with rituximab-GASDALIE, which has an enhanced binding affinity for CD16. This could suggest that rituximab-GASDALIE leads to shaving (trogocytosis) of CD16 from the NK cell, resulting in uptake of the CD16 molecule onto the B cell plasma membrane. This "shaving" may lead to a regulation of NK cell activity over time and could dampen NK mediated ADCC, and as such ADCC potentiation from a block of CDC activity is less likely to occur. In clinical practice, it would be interesting to investigate CD16 shaving and B cell acquisition of CD16 with obinutuzumab, which also exhibits increased CD16 binding affinity. When studying expression markers on the B cell compartment, we noted that CD20 expression (based on flow cytometry) was decreased on B cells in CLL patients when compared to healthy donors, while CD5 expression appeared slightly upregulated. The regulatory molecule HLA-E that can interact with NKG2A on NK cells was upregulated in donor 3 compared to both CLL and healthy controls. NKG2A could regulate NK activation and cytotoxicity in these specific individuals and could explain the relatively lower rituximab induced cytotoxicity in this donor. A larger cohort study...
is however required to investigate this further.

The multiplex proteomic analysis displayed a strong rituximab signature; with increases in effector protein mediators in blood, linked to the cytotoxic response. Although too few donors were included to enable an analysis between the rituximab variants, there was a clear increase in the protein levels of almost all cytotoxic response genes tested in the group where CD16 was blocked. This is puzzling as granzyme release would normally be associated with an NK or T cell mediated toxicity. We propose that the source of the elevated cytotoxic and angiogenic proteomic profile induced by CD16 blockade is related to increase in complement activation and/or CD32A mediated effects and that it can either be due to platelet or polymorphonuclear neutrophils (PMNs) activation. PMNs are abundant in blood and store granzymes within their granules [44]. It is therefore plausible that CD16 blockade retargets killing to an ADCP-mediated pathway in blood, via complement ligation and interaction with complement receptor 3 (C3R) on granulocytes [12], or alternatively via a CD32A mediated pathway. The role of PMNs as mediators of the increase in granzyme and other immune-activating proteins is also supported by observed increases in VEGF, Hgf, TGFb, TNF and CXCL1, all of which can be related to PMN activation [45–49]. The alternative source for these mediators could be activated platelets. However, this is less likely as platelets normally activate the complement system, not the other way around, but reports indicate that complement deposition could drive platelet activation [50] in certain cases. Further investigations are required to examine the cellular source of the increased cytotoxic pattern during CD16 blockade. As VEGFA, PDGF and HFG are known pro-tumorigenic factors, their release could be donor/tumour related, or related to increased complement activity. However, these may counteract the effectiveness of an antibody with improved CDC profile and warrant further in-depth characterization of patient proteomic profile ahead.

Our data showed that the assay worked well in this setting to investigate rituximab infusion response in terms of effector mechanisms and cytokine release AE in CLL. The assay may be useful in the clinical setting to predict adverse events and response to treatment with rituximab or other haematology-relevant targeting antibodies. Thus, we propose the assay as a tool that enables extensive analysis of therapy efficacy and safety before administration. For that, a panel of plasma cytokines (TNFa, IFNy, IL-6 and IL-8) and plasma complement split product (C3a) can be feasible to implement widely before therapy initiation. Additionally, complete blood counts analysis pre- and post-treatment of the blood in the loop will give a valuable prediction of, at least, the initial response to therapies such as rituximab. However, our study and assay are not without limitations. The small sample size and the fact that not all patients participants received rituximab as a definitive therapy has hampered the correlation between the CRS clinical picture and CRS in the blood loop. Despite this, the three patients who received rituximab for their CLL, and have shown CRS clinically, have shown similar CRS indications in the blood loop. Additionally, the blood loop assay has a limited duration of incubation of therapy of around 4–6 h. This hampers the investigation of late reactions to a therapeutic beyond a 6 h timepoint.

In conclusion, our results show that the blood loop assay can add valuable information for patients treated with drugs known to cause CRS that exert their function via CDC and ADCP. The information that can be obtained using the system can be highly patient-specific and thus, this methodology may be an important tool to optimize tumour-directed antibody therapies within the haematological field ahead.

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CRediT authorship contribution statement

M. Eltahir: Data curation, Formal analysis, Investigation, Visualization, Writing - original draft, Writing - review & editing. E. Fletcher: Data curation, Formal analysis, Investigation, Visualization, Writing - original draft, Writing - review & editing. L. Dynesius: Data curation, Investigation, Writing - review & editing. J.L. Jarlborg: Conceptualization, Writing - review & editing. M. Lord: Formal analysis, Visualization, Writing - review & editing. I. Lauren: Data curation, Investigation, Writing - review & editing. M. Zekarias: Data curation, Investigation, Writing - review & editing. X. Yu: Resources, Writing - review & editing. M.S. Cragg: Resources, Writing - review & editing. C. Hammarström: Resources, Writing - review & editing. K.H. Levedahl: Resources, Writing - review & editing. M. Höglund: Conceptualization, Resources, Writing - review & editing. G. Ullenås: Writing - original draft, Writing - review & editing. M. Mattsson: Conceptualization, Resources, Writing - review & editing. S.M. Manghbo: Conceptualization, Funding acquisition, Data curation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. Supervision.

Declaration of Competing Interest

EF and SM are the founders of Immuneed AB and SM is the board member of Immuneed AB. LD is an employee of Immuneed AB and JLJ is a former employee of Immuneed AB. EF, SM, ME and JLJ are stockholders in Immuneed AB.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2020.107226.

References

[1] A. Carbone et al., Follicular lymphoma, Nat. Rev. Dis. Primers 5 (1) (2019) 83.
[2] J.C. Edwards et al., Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis, N. Engl. J. Med. 350 (25) (2004) 2572–2581.
[3] D. Huhn et al., Rituximab therapy of patients with B-cell chronic lymphocytic leukemia, Blood 98 (5) (2001) 1326–1331.
[4] M.D. Rombouts et al., Systematic review on infusion reactions to and infusion rate of monoclonal antibodies used in cancer treatment, Anticancer Res. 40 (3) (2020) 1201–1218.
[5] E. Yildizhan, L. Kaynar, Cytokine release syndrome, J. Oncol. Sci. 4 (3) (2018) 134–141.
[6] H. Murthy et al., Cytokine release syndrome: current perspectives, Immunotargets Ther. 8 (2019) 43–52.
[7] R. Stehbings et al., After TGN412: recent developments in cytokine release assays, J. Immunotoxicol. 10 (1) (2013) 75–82.
[8] S. Vessilier et al., Cytokine release assays for the prediction of therapeutic mAb safety in first-in man trials-Whole blood cytokine release assays are poorly predictive for TGN412 cytokine storm, J. Immunol. Methods 424 (2015) 43–52.
[9] M. Ghielmii et al., Single agent rituximab in patients with follicular or mantle cell lymphoma: clinical and biological factors that are predictive of response and event-free survival as well as the effect of rituximab on the immune system: a study of the Swiss Group for Clinical Cancer Research (SAKK), Ann. Oncol. 16 (10) (2005) 1675–1682.
[10] D.G. Maloney, B. Smith, A. Rose, Rituximab: mechanism of action and resistance, Semin. Oncol. 29 (3S2) (2002) 2–9.
[11] G.J. Weiner, Rituximab: mechanism of action, Semin. Hematol. 47 (2) (2010) 115–123.
[12] K.R. VanDerMeid et al., Cellular cytotoxicity of next-generation C202 monoclonal antibodies, Cancer Immunol. Res. 6 (10) (2018) 1150–1160.
[13] L.E. Van Der Kolk et al., Complement activation plays a key role in the side-effects of rituximab treatment, Br. J. Haematol. 115 (4) (2001) 807–811.
[14] P. Bruhns, Properties of mouse and human IgG receptors and their contribution to disease models, Blood 119 (24) (2012) 5640–5649.
[15] N.K. Banda et al., Mechanisms of complement activation by dextran-coated superparamagnetic iron oxide (SPION) nanosworns in mouse versus human serum, Part. Fibre Toxicol. 11 (2014), 64–64.
[16] N.K. Banda et al., Complement activation pathways in murine immune complex-induced arthritis and in C3a and C5a generation in vitro, Clin. Exp. Immunol. 159 (1) (2010) 100–108.
[17] T. Shay et al., Conservation and divergence in the transcriptional programs of the human and mouse immune systems, Proc. Natl. Acad. Sci. USA 110 (8) (2013) 2946–2951.
