Complement deficiencies limit CD20 monoclonal antibody treatment efficacy in CLL.

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Running Title: Complement exhaustion impairs immunotherapy action

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Conflict of Interest:

AB & CC are employed by GSK; ML, AMMcC, AMM and HW applied for and received funding from GSK to provide funding for this study. All other authors have no conflicts of interest to declare.

Funding:

This study was funded by GlaxoSmithKline, Kay Kendal Leukaemia Fund (KKL501) and a University of Glasgow-Paul O'Gorman PhD Studentship.

Abstract

Monoclonal antibodies (MAbs) form a central part of chronic lymphocytic leukaemia (CLL) treatment. We therefore evaluated whether complement defects in CLL patients reduced the induction of complement-dependent cytoxicity (CDC), using anti-CD20 MAbs rituximab (RTX) and ofatumumab (OFA). OFA elicited higher CDC levels than RTX in all CLL samples examined, particularly the poor prognosis cohorts (11q- and 17p-). Serum sample analyses revealed 38.1% of patients were deficient in one or more complement components, correlating with reduced CDC responses. While a proportion of patients with deficient complement levels initially induced high levels of CDC, on secondary challenge CDC activity in sera was significantly reduced, compared with normal human serum (NHS; $p<0.01$; $n=52$). Additionally, high CLL cell number contributed to rapid complement exhaustion. Supplementing CLL serum with NHS or individual complement components, particularly C2, restored CDC on secondary challenge to NHS levels ($p<0.0001$; $n=9$). *In vivo* studies revealed that complement components were exhausted in CLL patient sera post-RTX treatment, correlating with an inability to elicit CDC. Supplementing MAb treatment with fresh frozen plasma may therefore maintain CDC levels in CLL patients with a complement deficiency or high white blood cell count. This study has important implications for CLL patients receiving anti-CD20 MAb therapy.

Keywords: chronic lymphocytic leukemia, complement deficiencies, C2, complement-dependent cytotoxicity, monoclonal antibody, CD20.

Introduction

Monoclonal antibody (MAb) therapies form an integral part of the treatment regime for chronic lymphocytic leukaemia (CLL) patients. Current first line therapy involves administration of the purine analogue fludarabine (F) and the DNA alkylating agent cyclophosphamide (C), in combination with rituximab (RTX) a chimeric anti-CD20 MAb (FCR).[1, 2] The biological activity of CD20, a B-cell marker, is not fully elucidated, however it is thought to act as an ion channel and a store operated Ca^{2+} channel.[3, 4] As CD20 expression is restricted to the B-cell lineage, with no expression on stem cells or mature plasma cells, it makes an ideal therapeutic target for B cell malignancies. The inclusion of RTX to FC generated a radical improvement in progression free survival and response rates for CLL patients.[5] However despite these improvements relapse due to the re-emergence of minimal residual disease still poses a problem for CLL patients,[2, 6] especially those with adverse cytogenetics. Disease progression through acquisition of chromosomal deletions/mutations in 17p/p53 is associated with patients becoming refractory to fludarabine-based therapies, leaving few therapeutic options. These patients can be treated with alemtuzumab an anti-CD52 MAb, with variable levels of response, indicating that the clinical needs of this sub-set of CLL patients are still unmet by standard treatments. A new generation type I, human immunoglobulin (Ig) G_1k anti-CD20 MAb, ofatumumab (OFA), which binds to a novel epitope of CD20,[7] has been given FDA and EMEA approval for the treatment of double refractory patients, refractory to both F and alemtuzumab. OFA has shown promising results with >50% of CLL patients responding to treatment.[7-9]

MAbs exert anti-tumour activity by harnessing the body's own natural immune response especially antibody-dependent cellular cytotoxicity (ADCC) involving the recruitment of natural killer cells to cause phagocytosis and complement-dependent cytotoxicity (CDC) requiring the activation of the classical complement pathway, and/or apoptosis.[10] Type 1 MAbs, such as RTX and OFA, localise CD20 into lipid rafts enhancing C1q recruitment and activation of CDC.[11] CDC induction is critically dependent on the distance between MAb binding site and the plasma membrane, with closer binding associated with more efficient coating of active complement components onto the target cell. Previous studies have demonstrated that C1q binds more readily to OFA than RTX, with OFA also resulting in more effective deposition of C3b onto the surface of the membrane, due to the novel epitope binding site of OFA bringing the complex closer to the surface of the cell, thus increasing the amount of CDC.[11-14] This is of particular importance in CLL, as CD20 expression levels, which are relatively low

compared to B-cell lymphomas, linearly correlate with the lytic response of RTX.[15, 16] In addition, recent studies demonstrate that CLL cells can evade RTX, through sequestration of CD20-RTX complexes by phagocytic cells, resulting in trogocytosis. This enables CD20 depleted lymphocytes to remain in circulation, no longer responsive to RTX treatment.[17-19] These findings highlight the importance of generating a MAb capable of effectively inducing CDC.

To maximise the clinical effect of MAbs, it is important to consider whether the patient will be able to elicit CDC and ADCC responses to the drug. Early reports indicated that CLL patients harbour deficiencies in classical complement components C1 and C4 resulting in defective immune complex clearance. Deficiencies in classical complement components have also been linked to CLL patients being more susceptible to infections with organisms such as *Streptococcus pneumonia,* and exhibiting a propensity to develop autoimmune syndromes.[20-22] These findings raise two important questions, first how frequently do complement deficiencies occur in CLL and second how does this impact on the efficiency of MAb treatments? Here we demonstrate that 38.1% of our CLL patient cohort harbour deficiencies or reduced levels in one or more complement components, significantly impacting on their ability to elicit CDC response to OFA and RTX. Moreover, some patients that initially induced a high level of CDC, display a significantly reduced CDC activity upon secondary challenge *in vitro* and *in vivo*, indicating that rapid exhaustion of complement components occurs in CLL patient serum, compared to normal donors. A high circulating CLL cell burden also contributes to rapid complement exhaustion, in agreement with previously published work.[23] Importantly we establish that the reduction in CDC activity observed in CLL patient sera *in vitro* is overcome by supplementing CLL serum with individual complement components or normal healthy serum (NHS) as a source of complement. Collectively, our studies demonstrate that the majority of CLL patients have sub-optimal levels of complement proteins/activity, a finding that has important implications for CLL patients receiving MAb-based therapies.

Materials and methods

Patient samples, CLL cell isolation and serum collection

Peripheral blood samples were obtained after informed consent, from patients with confirmed CLL (Supplementary Table 1). Ethical approval for this study was obtained from the West of Scotland Research Ethics Committee, NHS Greater Glasgow and Clyde (United Kingdom). CLL cells were

isolated using RosetteSep^{TM} human B cell enrichment cocktail (StemCell Technologies, Vancouver, Canada) following the manufacturer's instructions. CLL cell purity was >95%, determined by flow cytometry (CLL: mean age 67.9 ± 6.78 years; range 48–88 years; percentage male 57.9%). Peripheral blood samples were also collected from healthy volunteers to isolate normal healthy serum (NHS; age range 25–50 years old) and age matched serum (AMS: mean age 64.8 ± 12.91 years; range 47–84 years; percentage male 41.7%; Supplementary Table 2). Serum was separated from freshly isolated blood collected using serum clot activator, by centrifugation at 3000*g* for 10 min. Sera was immediately frozen on dry ice and stored at -80^{o}C. Pooled NHS was prepared by mixing sera from 10 different healthy volunteers, prior to freezing.

Cell culture and cell line conditions

CLL primary cells and the CLL cell line HG3 were cultured in RPMI-1640 containing 10% FBS, 50 U/mL penicillin, 50 mg/mL streptomycin and 2 mM L-glutamine (complete medium; Invitrogen Ltd., Paisley, UK).

MAb treatment and assessment of cell death

Unless otherwise stated 2.5 x 10^6 cells/ml were pelleted by centrifuging for 5 min, 300*g* at room temperature (RT), and then resuspended in CDC buffer - Hanks Buffered Saline Solution (HBSS; PAA Laboratories, Austria), 10 mM Hepes, 1 mM sodium pyruvate and 10 μg/ml Gentamicin (Invitrogen Ltd.). 20 mg/ml OFA (GlaxoSmithKline, UK) and 10 mg/ml RTX (Roche, UK) were diluted in CDC buffer and added to cells at 20 μg/ml, for 30 min at RT. Untreated control cells were treated equivalently except no MAb was added to the CDC buffer. CLL cells were then pelleted and the supernatant removed. Thawed human serum was diluted in CDC buffer (1:1) and added to cells and mixed gently. CLL cells were then incubated with sera for 2 hr at 37^{o}C. Following MAb treatment, CLL cells were washed in CDC buffer, harvested and stained with 1 μg/ml propidium iodide (PI) (BD Biosciences, Oxford, UK). Following PI staining the percentage of CDC was determined by acquiring flow cytometry data using a FACSCantoII flow cytometer (BD Biosciences). PI^{-} cells were considered viable. When patient sera were supplemented, purified human complement components C2 and/or C4 (Complement Technology, Inc, Texas, USA) or NHS were added at the concentrations indicated.

Quantification of complement concentration and activity in sera

C1 and C2 levels in sera were determined by radial immunodiffusion (RID) (The Binding Site Group Ltd., Birmingham, UK) following the manufacturer's protocol. C3c and C4 were determined by

immunonephelometry in the clinical diagnostic immunology laboratory (Gartnavel General Hospital, NHS Greater Glasgow and Clyde). The activity of the classical complement cascade in serum was determined using total haemolytic complement kit, CH100 assay (The Binding Site Group Ltd.) following the manufacturer's instructions.

Statistical analysis and Software

All statistical analysis was performed using GraphPad Prism 4 software (GraphPad Software Inc., CA), P values were determined by students paired or unpaired *t*-test or mixed model Anova as indicated. Mean ± standard error of mean (SEM) is shown. Flow cytometry data were acquired using FACSDiva software (BD Biosciences) and analysed using FlowJo (Tree Star Inc., Ashland, OR) software.

Results

CLL patient sera display multiple deficiencies in components of the classical complement cascade.

Classical complement component levels (C1, C2, C3 and C4) were assessed in CLL patient sera and sera from AMS controls. Patients displaying single deficiencies/reduced levels were noted in all complement components screened (Figure 1A). In the AMS samples (n=12), complement deficiencies slightly below normal range for C1, C2 and C3/C4 were observed in a minority of donors. Of the CLL patients screened, 15.4% exhibited levels lower than the normal range of C1q in their sera (n=52) and approximately 20% of patients displayed deficiencies in the levels of C2 and C4 (17.9%, n=56 and 20.6% n=63 respectively). Analysis of C3 levels revealed that 11.1% of patient sera (n=63) were deficient in this component. Double deficiencies of C1/C4, C2/C4 and C3/C4 also occurred, at a frequency of 3.2%, 3.2% and 4.8% respectively. Triple deficiency of C2/C3/C4 was seen in 3.2% of CLL patients, and deficiency of C1q/C2/C3/C4 was observed in one patient within our cohort. In total 38.1% of CLL sera tested were deficient in at least one complement component (n=63). To determine how this impacted on the sequential activation of the classical complement cascade in the CLL sera we performed a CH100 assay. Surprisingly only 19.4% of CLL sera exhibited reduced complement activity levels (Figure 1B), despite the high proportion displaying at least one component deficiency. Complement deficiencies showed no correlation to prognostic markers, occurring at a similar frequency in the different Binet stage, cytogenetic and ZAP-70 status groups for our CLL patient cohort (Supplementary Figures 1-3).

OFA induces higher levels of CDC than RTX.

CDC optimisation experiments were carried out on CLL cell lines to determine the optimal concentration of RTX and OFA. Maximal cell kill occurred with 20 μg/ml OFA/RIT, and 50% sera (data not shown). Using these conditions we determined whether levels of CDC varied between poor prognosis patient cohorts. CDC was carried out on 15 patients, selected from three distinct cytogenetic subgroups; 11q-, 17p- and normal/13q. As previously reported OFA demonstrated much greater cell kill by CDC than RTX,[12, 13] with 20 μg/mL eliciting high levels of cell death in all cytogenetic groups (Figure 2A). Statistical analysis revealed that when compared to RTX, OFA induced significantly more CDC-mediated cell death in 11q- and 17p- cohorts, with borderline significant effect in normal/13q group (Figure 2B). As OFA induced high levels of cell kill it was therefore used in all subsequent *in vitro* experiments.

CLL sera readily exhaust of complement activity following MAb therapy.

Our findings indicated that low levels of individual complement components observed in CLL patient sera did not always directly impact on CH100 complement activity. We therefore hypothesised that CLL patient serum might exhaust the available complement components more readily after eliciting a CDC response. This is an important consideration given the repeated scheduling of MAbs in CLL. To validate complement exhaustion OFA-mediated CDC was performed on the CLL cell line HG3 using 50% CLL patient sera, to approximate physiological conditions, with a NHS control included for comparison. Following 2 hr incubation, cells were pelleted and CDC levels measured by PI staining. The sera removed from this experiment was then re-used to promote a second CDC response on OFA bound HG3 cells. Serum exhaustion was performed on 52 CLL patient sera and the difference in CDC cell kill from the first to the second round of serum use was then plotted (Figure 3A and Supplementary Figure 4), with representative data from three individual patient samples with poor (CLL08), medium (CLL32) and good (CLL85) CDC activity shown (Figure 3B). Our results clearly demonstrate that CLL patient sera show significantly more complement exhaustion compared to NHS controls, with 42.3% of CLL patient sera showing more than 30% reduction in CDC activity from primary to secondary use.

To determine whether CDC was affected by high tumour load, we carried out exhaustion assays using NHS exposed to increasing numbers of primary CLL cells. The efficiency of CDC in primary CLL cells did not decline significantly until 10000 cells/μl were present, at which point CDC activity

dropped by 48.1% in the NHS from the primary use (Figure 3C). Given that CLL sera is more readily depleted of complement activity than NHS, the CLL sera exhaustion assays carried out in Figure 3A & B and in subsequent experiments were performed using 2500 cells/µl. These findings indicate that tumour load will also have a significant negative impact on CDC activity.

CLL sera exhaustion can be reduced by the addition of a complement source.

To determine whether CDC could be improved in patients deficient in one or more complement component, individual components were added back to CLL patient sera. Similar to a report by Kennedy A.D., *et al.*, we identified C2 as being the limiting factor.[18] Upon addition of C2 to patient sera we were able to abrogate complement exhaustion in three individual patients (Figure 4A). In a larger patient cohort, supplementing the sera with C2 restored CDC cell kill from primary to secondary sera use to levels observed for the NHS control (Figure 4B). In addition, the C2 concentration displayed a significant negative correlation with sera exhaustion levels in CLL patients (Figure 4C; r^2= 0.5260, p <0.0001). The levels of C4 also displayed a significant negative correlation with sera exhaustion in CLL patients (Supplementary Figure 5), however only C2 alone or in combination with C4 was able to augment CDC in deficient sera, supporting previous findings that C2 is the limiting factor (Supplementary Figure 6). To determine whether we could protect against complement consumption in a clinically-relevant manner, we added back 10% and 20% NHS (equivalent to fresh frozen plasma (FFP) an available source of complement to CLL sera), prior to the first round of CDC. Our results clearly demonstrate that adding back 20% NHS not only gave significant improvement to the initial level of CDC but also decreased the amount of complement exhaustion on secondary use (Figure 4D).

CLL sera exhaustion is observed in vivo.

Next we screened the sera of CLL patients undergoing RTX immunotherapy to establish whether complement levels were severely diminished following RTX treatment *in vivo*. In addition *in vitro* CDC assays were carried out using OFA treated HG3 cells, using the RTX treated patient sera. OFA was chosen due to its ability to elicit higher levels of CDC *in vitro* than RTX, enabling a better range of CDC response to be measured. Analysis 24 hr following RTX infusion revealed that only one CLL patient serum sample (CLL108) demonstrated good CDC on OFA treated HG3 cells and no complement exhaustion following RTX treatment, with additional patients displaying complement levels falling below the expected normal range (Figure 5A), correlating with an inability to elicit a CDC

response (Figure 5B). For the majority of patients 28 days was sufficient for complement levels to be replenished following treatment, however this was not true for the patient already deficient in C2 (CLL106). Individual complement component levels also reflected how readily patient sera exhausted following subsequent CDC challenge on OFA treated HG3 cells, with CLL106 displaying a marked reduction in CDC cell kill *in vitro* prior to RTX infusion (Figure 5-Ci). This was more apparent post RTX infusion with the majority of patients showing complete ablation of complement activity *in vivo* (Figure 5-Cii). These findings have important implications for the future management of CLL patients receiving MAb therapy.

Discussion

MAbs are now firmly established in the treatment of CLL, both first-line and in the relapse setting. Type 1 anti-CD20 MAbs mediate their toxicity effects against CLL cells by using the patient's natural immune responses to induce cell death through ADCC and CDC. Previous reports have shown that CDC is a finite process with complement becoming exhausted following MAb induced cell lysis.[23] Earlier research indicated that around 50% of CLL patients are deficient in C1 and C4, key components of the classical complement cascade, and this low complement activity is associated with advanced disease and shorter survival time.[20, 21, 24, 25] Despite these early reports there has been little follow up to determine how these complement deficiencies influence the efficacy of MAb treatment, with drugs such as OFA and RTX that are potent inducers of CDC.

CLL patients have lower classical complement levels

Our comprehensive assessment of the levels of all complement components involved in the first stage of the classical cascade in CLL patient sera revealed that a high proportion of CLL patients harbour deficiencies, with 38.1% displaying a reduced level of either in C1, C2, C3 or C4. Patients exhibited a range of deficiencies from one to multiple complement components, with one individual demonstrating a deficiency in all classical complement components examined. Levels of C1 and C3 were often within the normal range, whereas subnormal levels of C2 (17.9% deficient) and C4 (20.6% deficient) were more prevalent in the CLL patient cohort examined. We were unable to define any significant differences in complement concentration within CLL patient clinical stage, poor prognostic cytogenetics or ZAP-70 status (Supplementary Figure 1-3). C2 and C4 are both cleaved into two fragments during classical complement activation, with C4b and C2a then forming a complex termed

C3-convertase. C3-convertase is essential for cleaving C3, which enables the C3b fragment to opsonise the target cell and act as a scaffold for the membrane attack complex to assemble so that the target cell can be lysed. C3b also functions to opsonise bacteria strains, for clearance by the classical pathway.[10] CLL patients are frequently unable to effectively coat bacterial pathogens with C3b, making them more susceptible to infection.[22] Therefore these deficiencies could increase the risk of life threatening infection, already a major concern in CLL patients.

CLL patient sera more readily exhaust complement, abrogating CDC activity

Although the high frequency of defects in complement levels did not correlate closely to overall classical complement activity *in vitro*, analysis of patient sera revealed that significantly more CLL patient sera samples underwent complement exhaustion on secondary challenge with bound MAb, compared to the NHS controls (42.3%; n=52). In addition tumour burden impacted on CDC efficiency, with high levels of sera exhaustion observed at medium-low tumour burden levels typically observed for CLL, 1 x 10^4 CLL cells/μl. These findings are corroborated by Beurskens F.J. *et al.,* who also observed an elevated complement consumption with high cell counts. Moreover they demonstrated that administering high concentrations of OFA did not substantially augment initial CDC induction, but did affect the ability to induce a second round of CDC due to exaggerated complement activation and consumption.[23] Therefore any MAb treatments requiring CDC for potency will be limited by tumour burden, and low complement component levels within CLL patient sera.

CDC activity can be restored in CLL patient sera with limited complement levels

Complement exhaustion has important implications for the dosing schedule of MAb treatment within CLL as frequent dosing, which in addition to limiting the efficacy of the drug, will lead to sustained complement exhaustion making patients more susceptible to infections. Having confirmed that C2 was depleted at a high frequency in CLL,[18] we established that impaired CDC could be restored to a normal NHS level by supplementing patient sera with C2. In addition, supplementing patient sera with 10 and 20% NHS was also sufficient to substantially improve OFA mediated CDC and enable protection against complement exhaustion. Other studies suggest that RTX is not as effective at depositing C3b onto the surface of cancer cells and is not as effective as OFA as a single agent in CLL, yet it still showed significant improvement when administered together with FFP in a clinical trial of fludarabine-refractory CLL patients, with minimal toxicity.[2, 11, 26, 27] OFA is currently licensed for the

treatment of double refractory patients and shows significant activity as a single agent, effectively targeting CLL cells previously treated with RTX.[7, 9] The improvement reported for the small cohort of patients treated with RTX in combination with FFP, lend support for further investigation of MAb therapy in combination with FFP infusions in clinical trials in the context of enhancing classical complement pathway activity. This would be of particular importance for MAbs like OFA, which are highly effective at using the complement system to induce cell death. Moreover, enhancement of CDC by supplementing MAb therapy with FFP may also reduce the level of trogocytosis thus preventing immune evasion of CLL cells. Indeed, our data highlight the importance of considering tumour burden and patient complement levels prior to MAb therapy, as these factors could negatively impact on the ability of the patient's natural immune system to clear the malignant cells, thus reducing clinical efficiency of the administered MAbs. Significantly, we demonstrate that complement exhaustion can occur following RTX therapy *in vivo*, reducing both complement activity as shown previously,[18] and the serum concentrations of complement components, particularly C2 and C4. Analysis of sera from CLL patients undergoing RTX immunotherapy, showed that 24 hr post RTX infusion, complement was rapidly consumed by OFA treated HG3 cells, leading to a substantial decrease in CDC activity. However in the majority of patients by day 28 CDC activity was restored, in line with complement levels. Interestingly the patient whose CDC activity remained low at day 28 was deficient in C2, which dropped even lower following RTX treatment and remained at low levels by day 28. We also observed that sera from this patient exhausted readily *in vitro*, as was observed *in vivo*. In contrast to RTX, OFA is typically administered as 8 weekly doses followed by 4 monthly treatments.[7, 9] In a previous study, patient complement levels were also reduced considerably following OFA infusion, especially after the first infusion when CD20 levels were initially high.[23] These findings have recently been confirmed by Baig et al., who demonstrate that there is a rapid decrease in serum complement levels following OFA treatment, which is sustained 24h post-treatment. In addition the CLL cells lose CD20 expression and become insensitive to *in vitro* OFA-mediated CDC, whilst retaining their ability to undergo alemtuzumab-mediated CDC when supplemented with 10% NHS.[28] The greater dose-intensity, coupled with the greater induction of CDC with OFA, suggests that sustained complement depletion is more likely to occur during OFA treatment, in susceptible patients. In patients with complement deficiencies, ineffective CDC may also result in non-lethal complement deposition on CLL cells, CD20 trogocytosis and subsequent resistance to OFA-mediated CDC.

Conclusion

This study highlights that complement deficiencies are an important clinical issue in CLL and have implications for the scheduling of MAb therapy, with repeated administration of MAb during treatment likely to reduce clinical efficacy in CLL patients due to complement consumption and depletion leading to an inability to elicit CDC. Indeed, our findings establish that physiological complement levels are fundamental to maintaining clinical efficacy of current MAb regimes. Provision of FFP as a source of complement in parallel with MAb therapy may provide a relatively simple and effective way to restore complement levels to a normal range, enhance the clinical efficacy of the MAb therapy and possibly reduce the risk of infection. This could ultimately result in improved response rates and progression free survival. A clinical study to assess the impact of complement replenishment using FFP in CLL patients receiving MAb therapy is clearly warranted.

Acknowledgements

The authors would like to thank the clinical diagnostic immunology laboratory at Gartnavel General Hospital, NHS Greater Glasgow and Clyde for carrying out the C3c and C4 analysis on the serum samples. In addition, we thank the CLL patients for kindly consenting for use of their cells and serum samples, without which this study would not have been possible.

Contributions of Authors

OM carried out the majority of the experimental work and drafted the manuscript; EC & ED carried out some experimental work, optimised the experimental conditions, assisted in data analysis and critically assessed the manuscript; AB & CC assisted in the conception and design of the study, enabled the provision of funding from GSK and critically assessed the manuscript; ML & AMMcC coordinated the CLL patient samples, assisted in developing the collaboration with GSK, applied for funding, assisted in the conception and design of the study and critically assessed the manuscript; AMM & HW designed the study, applied for funding, assisted in setting up the assays and the data analysis and wrote the manuscript.

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Figure legends

Figure 1. CLL patient sera exhibit deficiencies in key components of the classical complement cascade and reduced complement activity.

A. The concentrations of complement components of the classical cascade were assessed. Mean of individual patient samples and AMS controls is shown. NR: Normal Range. **i.** C1q levels were determined by RID. NR was established from the manufacturer's guidelines (AMS, n=12; CLL sera, n=52). **ii,** C2 levels were determined by RID. NR was established from our AMS controls (AMS, n=12; CLL sera, n=56). **iii & iv.** C3 & C4 concentrations respectively were determined by immunonephelometry performed by the clinical diagnostic laboratory (AMS, n=12; CLL sera, n=63). **B.** Activity of the classical complement cascade was measured using the CH100 assay in CLL sera (n=62) and AMS control (n=12). CH100 units were determined against a standard curve following lytic ring measurement produced by serum measured against a calibrator of known activity levels. An unpaired t test revealed no significant difference in the mean complement levels and complement activity when comparing AMS controls with CLL patient sera.

Figure 2. OFA induces greater cell kill through CDC than RTX.

A. CLL patient cells were treated with 20 μg/ml OFA or RTX and then incubated with 50% NHS and the level of CDC cell death measured by flow cytometry (% PI+ cells). The percentage of dead cells is expressed relative to untreated control, graphs represent the mean ± S.D (n=5 for each patient cohort). **B.** Statistical analysis using mixed model Anova following covariate adjustment of non-drug control for OFA and RTX populations.

Figure 3. CLL patient sera exhausts more readily than NHS.

HG3 cells were treated with 20 μg/ml OFA and then incubated with 50% CLL patient sera or NHS and the level of CDC cell death measured by flow cytometry (% PI+ cells). The percentage of dead cells is expressed relative to untreated control. **A.** CLL serum that caused ≥40% CDC in OFA treated HG3 cells in 1° use, was used again (2° use) and the percentage difference in CDC from 1° to 2° sera use enabled calculation of exhaustion levels (n=52 CLL patient sera vs. n=12 individual NHS. p values were determined by an unpaired t-test (** $p<0.01$). **B.** After induction of CDC from the first set of OFA-treated HG3 cells (1° use) sera were removed from the cells and used for a second time to determine the CDC activity induced on fresh HG3 cells treated with 20 μg/ml OFA (2° use). Representative data are shown from the sera of 3 CLL patients and NHS control. **C.** CLL primary cells (n=4 ± SEM) of

different cell densities were treated with 20 μg/ml OFA. Sera were removed and re-challenged with fresh CLL cells of matching cell density treated with 20 μg/ml OFA. CDC was measured at both stages. p values were determined by a paired t-test (*, $p<0.05$).

Figure 4. CDC activity in complement deficient serum can be restored with the addition of complement components.

C2 protects CLL patient sera against exhaustion. CLL sera (n=9) that exhibited high levels of serum exhaustion were supplemented with C2 (50 μg/ml), before being used to induce CDC in HG3 cells treated with 20 μg/ml OFA (1° use). After the incubation period sera was removed and re-challenged with HG3 cells treated with 20 μg/ml OFA (2° use). **A**. Representative results from 3 different CLL patients are shown. **B**. Analysis of the percentage change in CDC from the 1° to 2° use, p value was obtained from a paired t-test (*** $p<0.001$) **C.** C2 levels in CLL patient sera were compared against CLL patient sera exhaustion (n=47). Linear regression was applied to obtain values for r^2, 0.5260 and p value, <0.0001. **D.** 10 or 20% NHS was added to CLL sera (n=9 ± SEM) prior to CDC exhaustion, p value was obtained from paired t test. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). Percentage of dead cells is relative to untreated control.

Figure 5. Complement levels are exhausted *in vivo.*

Sera samples were collected from CLL patients undergoing RTX immunotherapy (concentration shown in Supplementary Table 1). Sera were collected prior to RTX therapy (pre – RTX dose 1), 24 hr after treatment (post – RTX dose 1) and prior to the second dose of RTX, 28 days after dose 1 (pre – RTX dose 2). **A.** The concentration of complement levels in CLL sera (n=6). **i.** C2 levels were determined by RID. **ii. & iii.** C3 & C4 concentrations were determined by immunonophelometry. p value was obtained from paired t test (* $p<0.05$; ** $p<0.01$). **B.** CDC was measured on HG3 cells treated with 20 μg/ml OFA in 50% CLL sera (n=6). p value was obtained from paired t test (* $p<0.05$; ** $p<0.01$). **C.** Exhaustion was measured in sera collected **i**, prior to RTX therapy and **ii**, post – RTX dose 1. After induction of CDC from the first set of OFA treated HG3 cells (1° use) sera were removed from the cells and re-challenged with HG3 cells treated with 20 μg/ml OFA and CDC measured (2° use). Percentage of dead cells is relative to untreated control.

Figure 1 - Middleton *et al.*

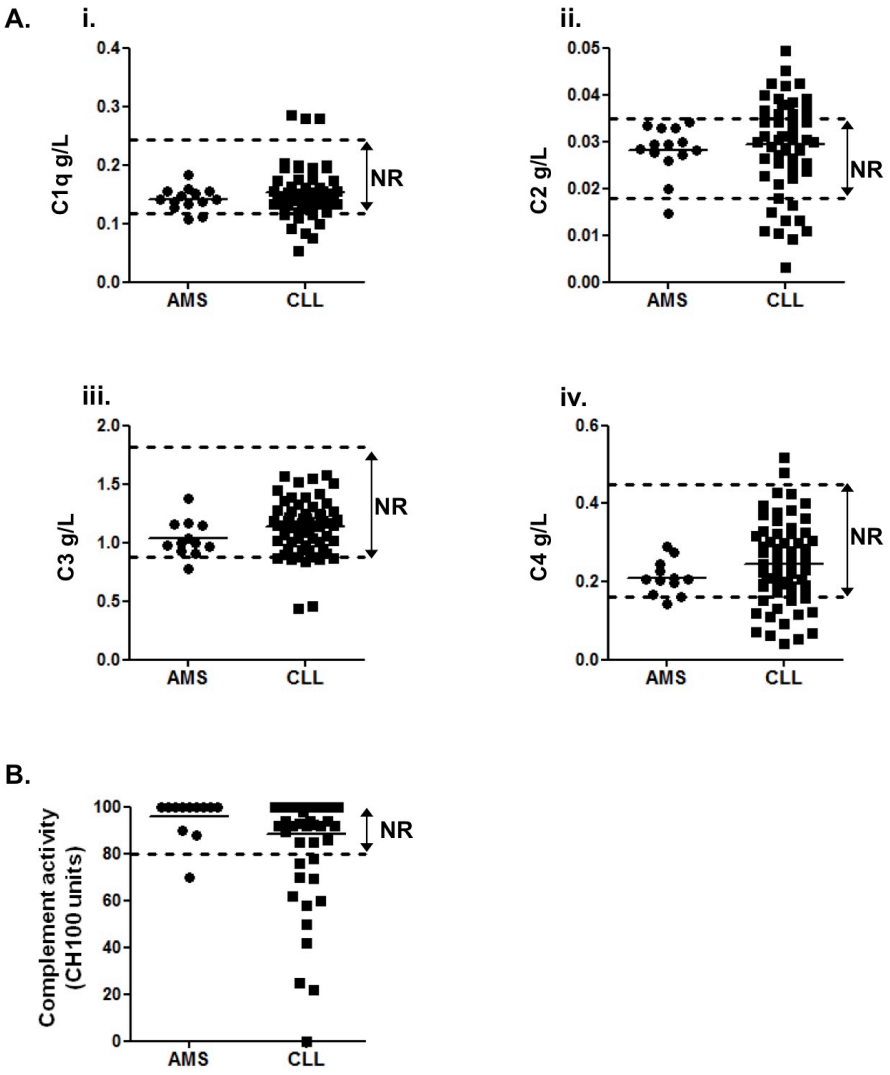
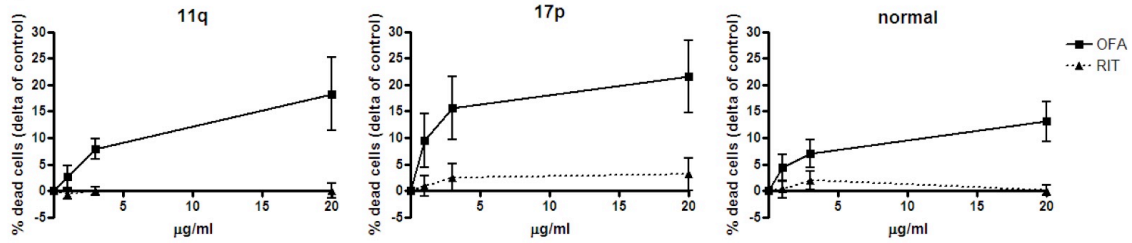


Figure 2 - Middleton *et al.*

A.



B.

Comparison	Difference	Standard Error	Lower 95% CI	Upper 95% CI	P value
OFA-RTX in 11q	18.4	6.53	4.6	32.3	0.0123
OFA-RTX in 17p	21.8	6.53	7.9	35.6	0.0042
OFA-RTX in normal	12.9	6.53	-0.9	26.7	0.0655

Figure 3 - Middleton *et al.*

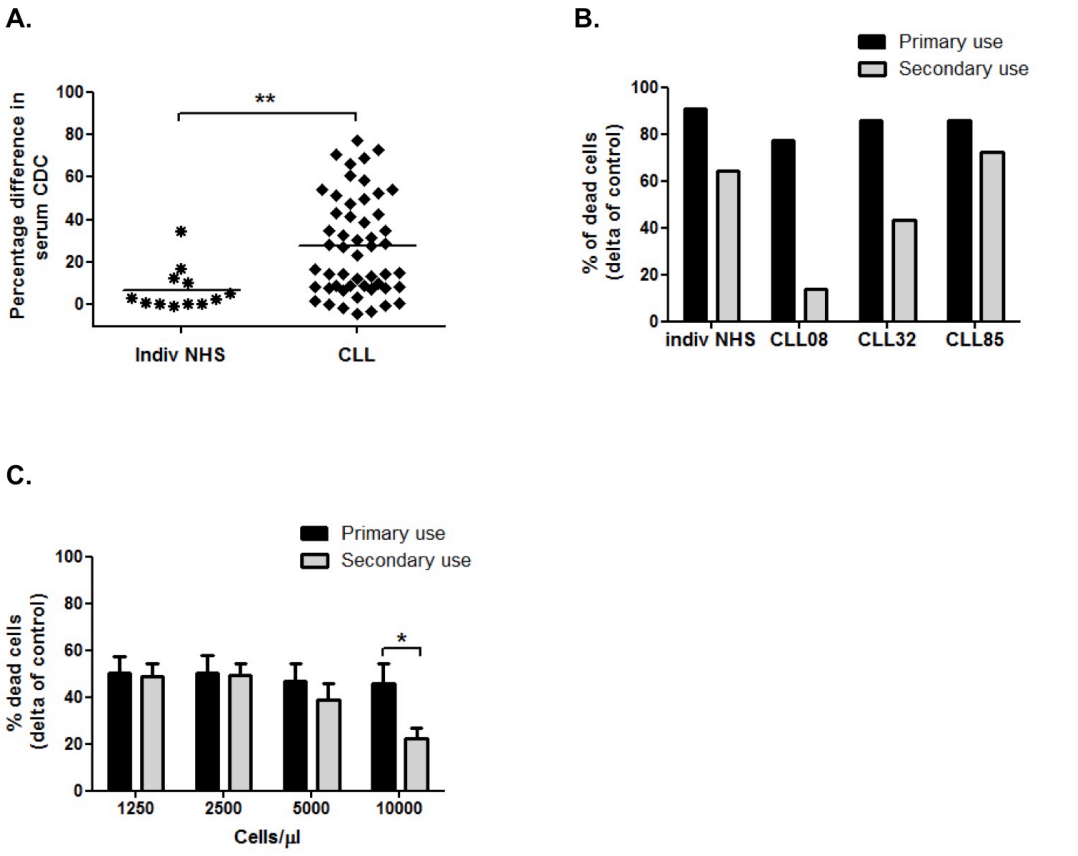


Figure 4 - Middleton *et al.*

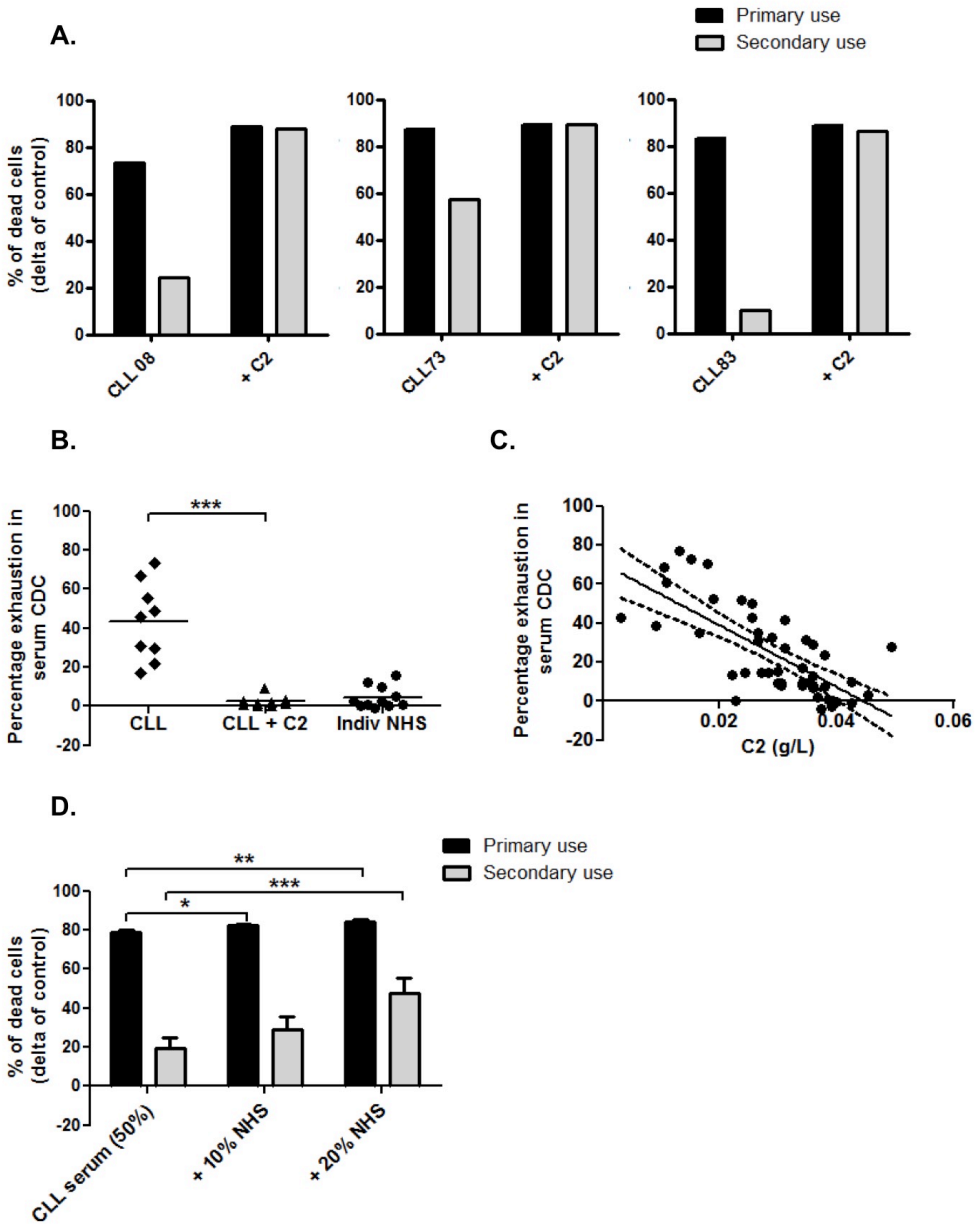


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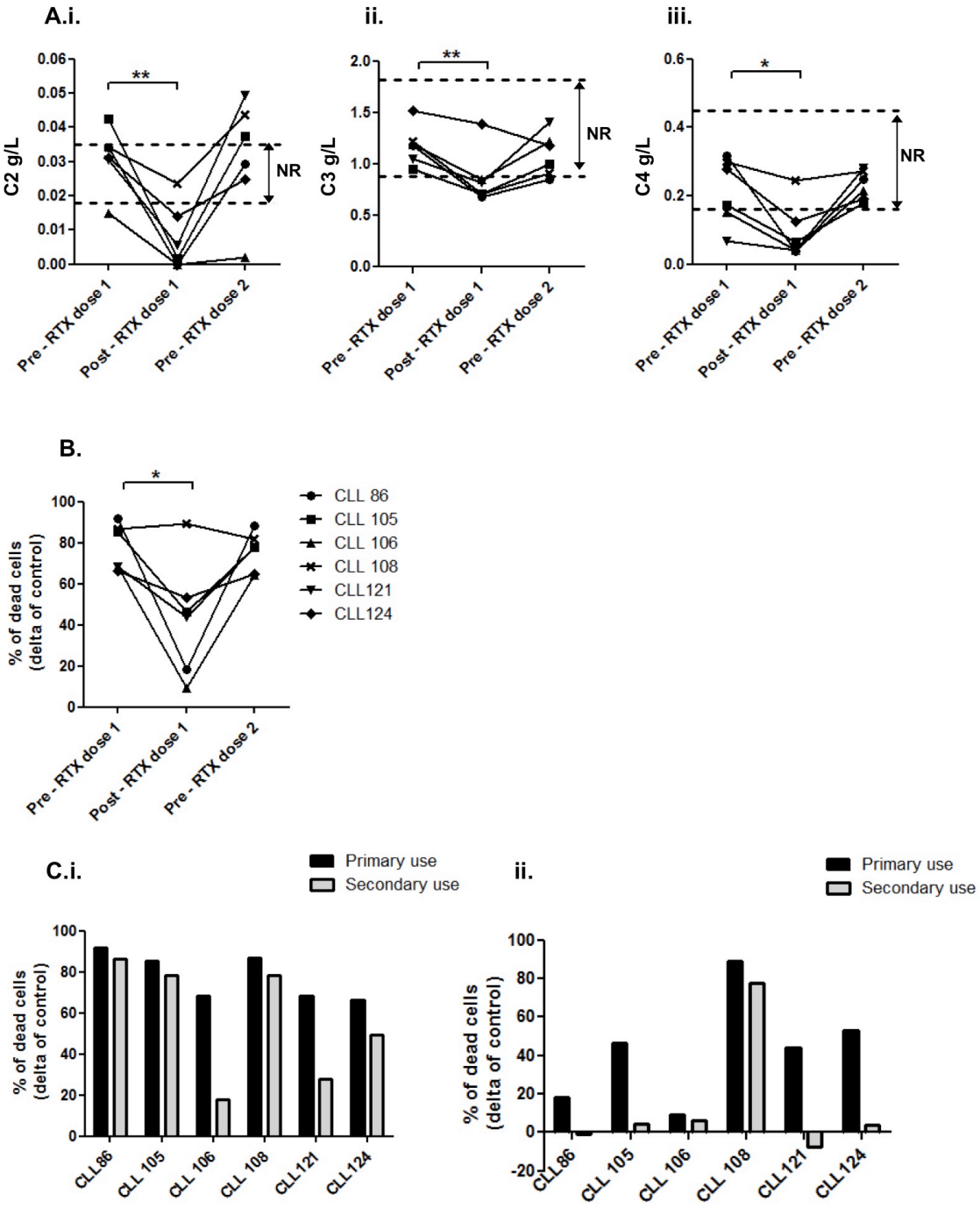
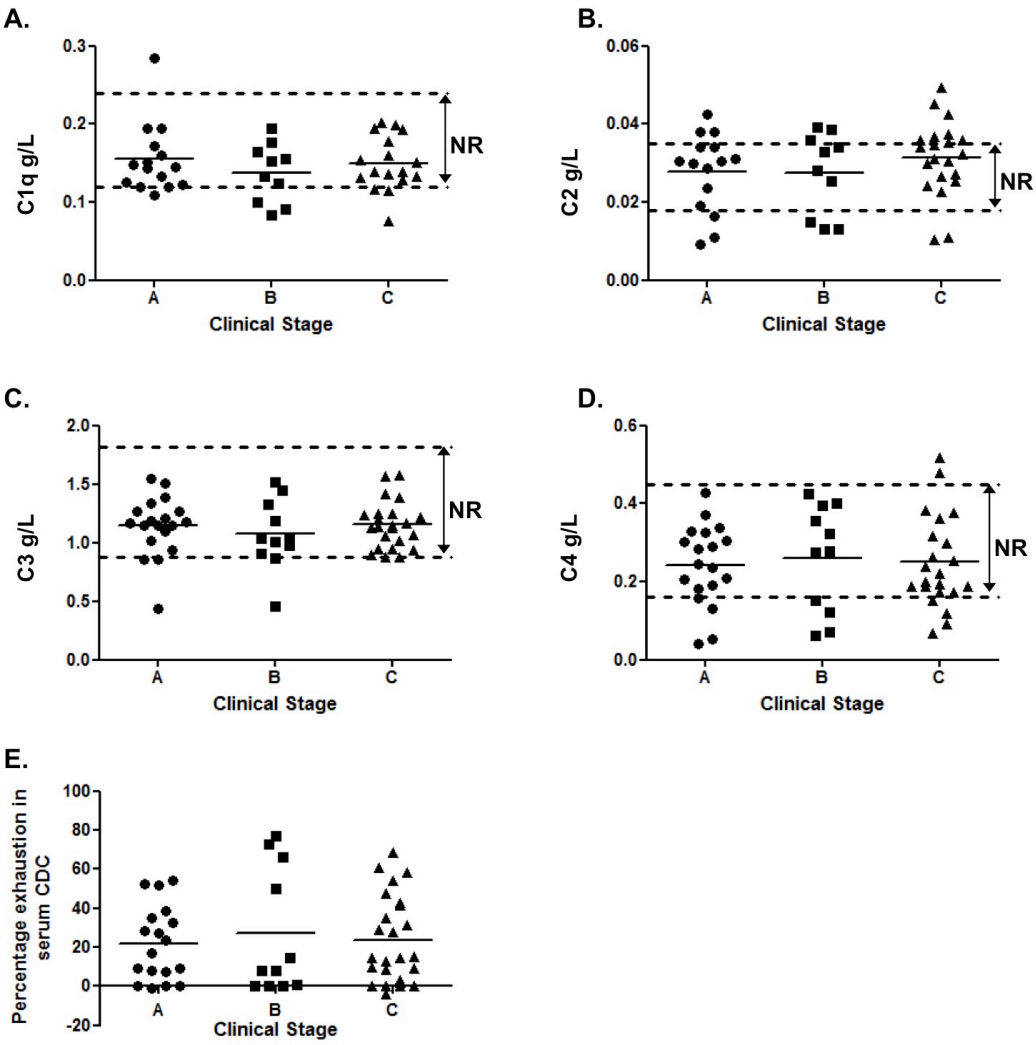
Figure 5 - Middleton *et al.*

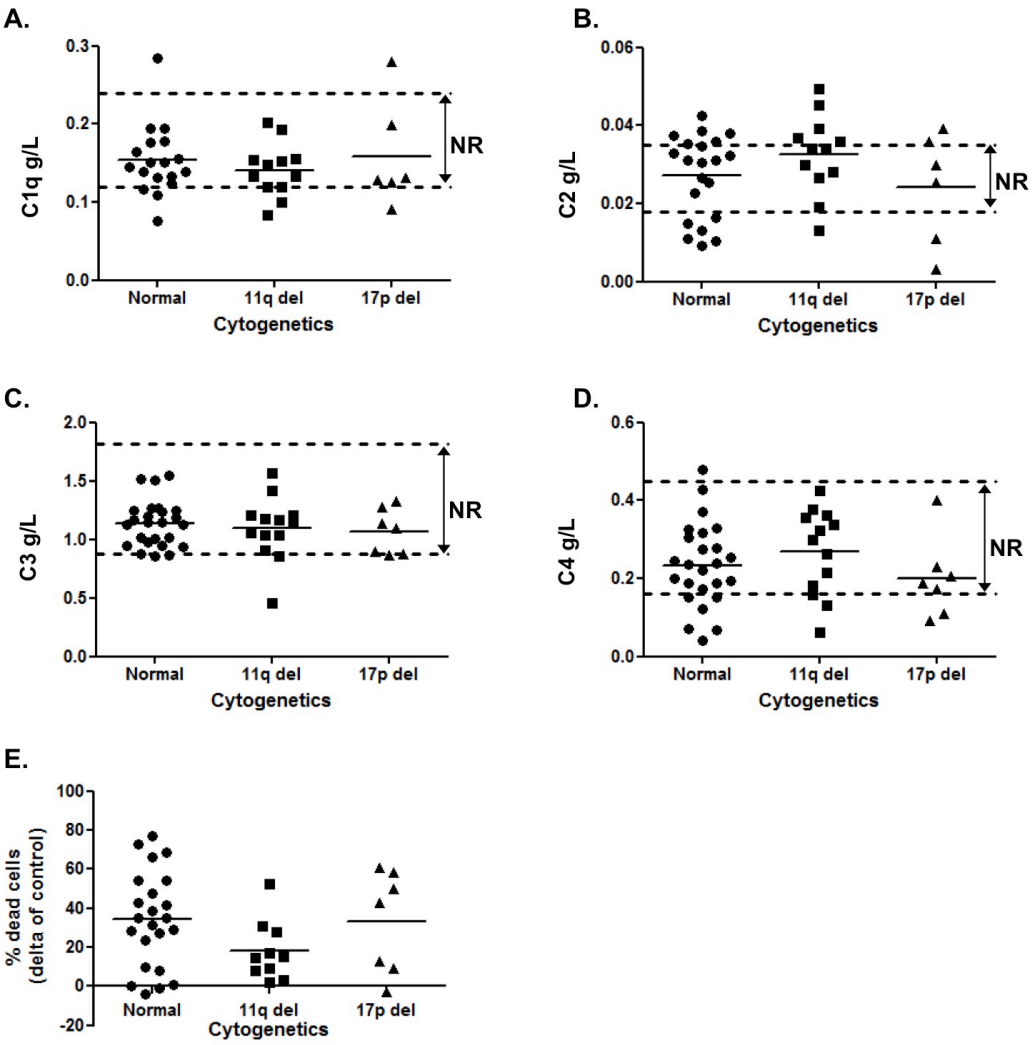


Supplementary Figure 1 - Middleton *et al.*



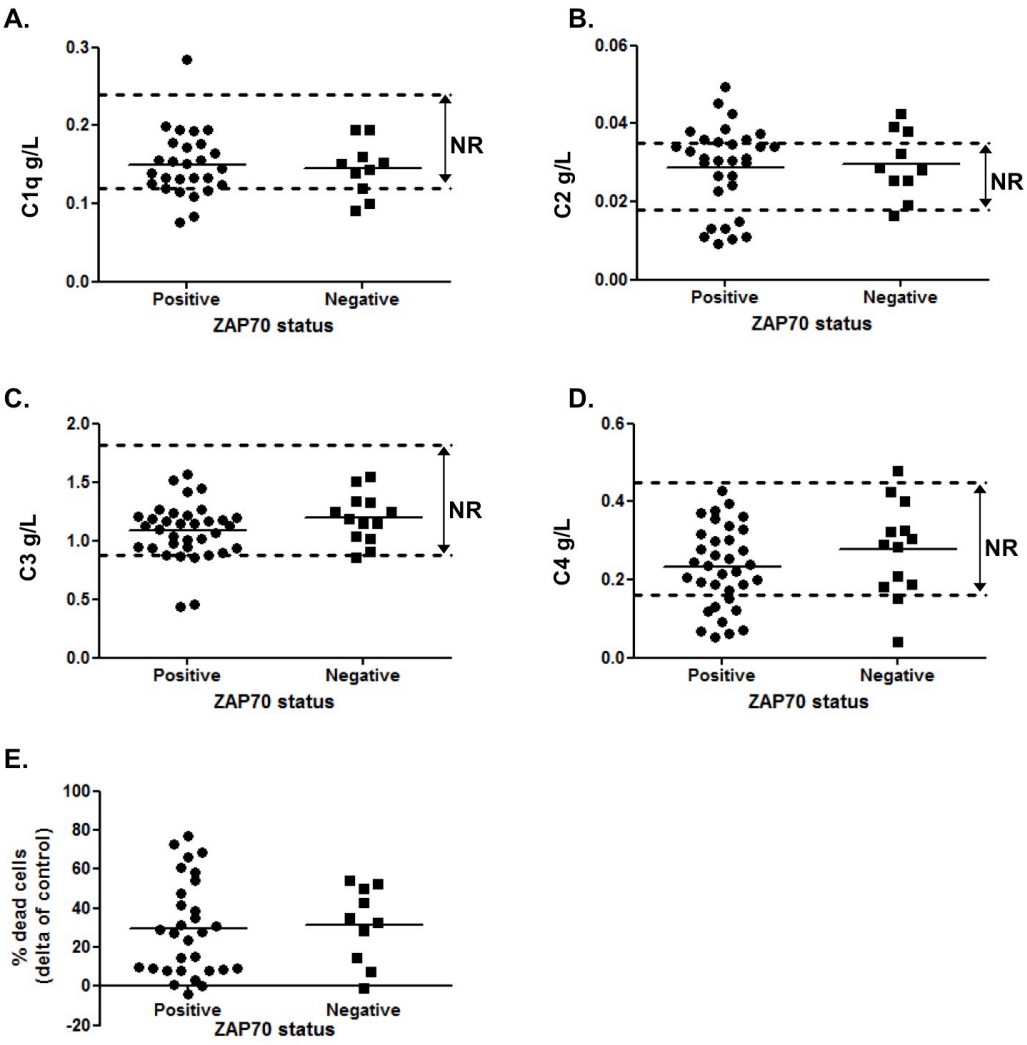
Supplementary Figure 1. Complement concentrations in CLL patient sera are not significantly affected by Binet stage.
The concentrations of complement components of the classical cascade and CLL patient sera exhaustion were compared against Binet stage of disease at the point of sample collection. Mean of individual patient samples is shown. **A.** C1q levels compared against clinical stage (n=42). **B.** C2 levels compared against clinical stage (n=46). **C.** C3 levels compared against clinical stage (n=53). **D.** C4 levels compared against clinical stage (n=53). **E.** CLL patient sera exhaustion was also compared against clinical stage (n=54). An unpaired t test was performed, with no significant difference observed between the mean complement levels or the level of CLL sera exhaustion and the different clinical stages of disease.

Supplementary Figure 2 - Middleton *et al.*



Supplementary Figure 2. Complement concentrations in CLL patient sera are not significantly affected by poor prognosis cytogenetics.
The concentrations of complement components of the classical cascade and CLL patient sera exhaustion were compared against the different CLL cytogenetic subgroups normal/13q, 11q del and 17p del. Mean of individual patient samples is shown. **A.** C1q levels compared against cytogenetic subgroups (n=36). **B.** C2 levels compared against cytogenetic subgroups (n=39). **C.** C3 levels compared against cytogenetic subgroups (n=45). **D.** C4 levels compared against cytogenetic subgroups (n=45). **E.** CLL patient sera exhaustion was also compared against cytogenetic subgroups (n=40). An unpaired t test was performed, with no significant difference observed between the mean complement levels or the level of CLL sera exhaustion and the different cytogenetic subgroups.

Supplementary Figure 3 - Middleton *et al.*



Supplementary Figure 3. Complement concentrations in CLL patient sera are not significantly affected by ZAP-70 status.
The concentrations of complement components of the classical cascade and CLL patient sera exhaustion were compared against the ZAP-70 status. Mean of individual patient samples is shown. **A.** C1q levels compared against ZAP-70 status (n=37). **B.** C2 levels compared against ZAP-70 status (n=41), 25% ZAP-70 positive CLL patients are deficient in C2 compared to 10% that are ZAP-70 negative. **C.** C3 levels compared against ZAP-70 status (n=48). **D.** C4 levels compared against ZAP-70 status (n=48). **E.** CLL patient sera exhaustion was also compared against ZAP-70 status (n=40). An unpaired t test was performed, with no significant difference observed between the mean complement levels or the level of CLL sera exhaustion and ZAP-70 status.

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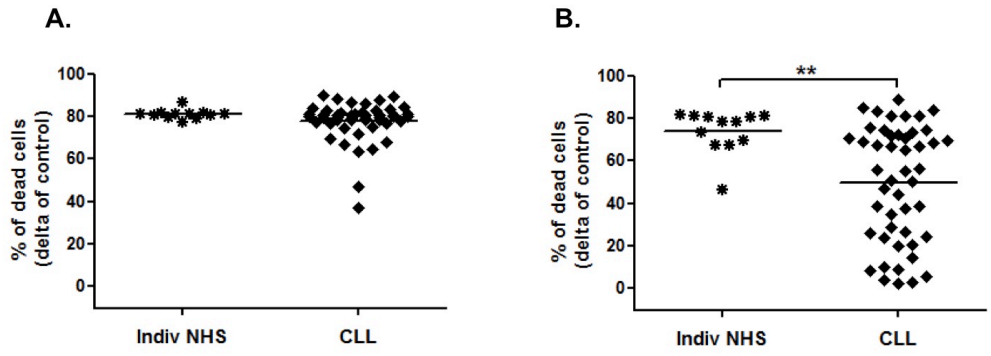
Supplementary Figure 4 - Middleton *et al.*



Supplementary Figure 4. CLL patient sera shows significantly reduced CDC activity when used for a second time.
HG3 cells were treated with 20 µg/ml OFA and then incubated with 50% CLL patient sera or NHS and the level of CDC cell death measured by flow cytometry (% PI+ cells). The percentage of dead cells is expressed relative to untreated control. **A.** Induction of CDC from CLL patient sera for the first set of OFA-treated HG3 cells was assessed by PI staining (NHS, n=12; CLL, n=48). Serum that caused ≥40% CDC was used for secondary CDC. **B.** Sera from the first set of OFA-treated HG3 cells were removed from the cells and used for a second time to determine the CDC activity induced on fresh HG3 cells treated with 20 µg/ml OFA (2° use) (NHS, n=12; CLL, n=48). p values were determined by an unpaired t-test (** $p<0.01$).

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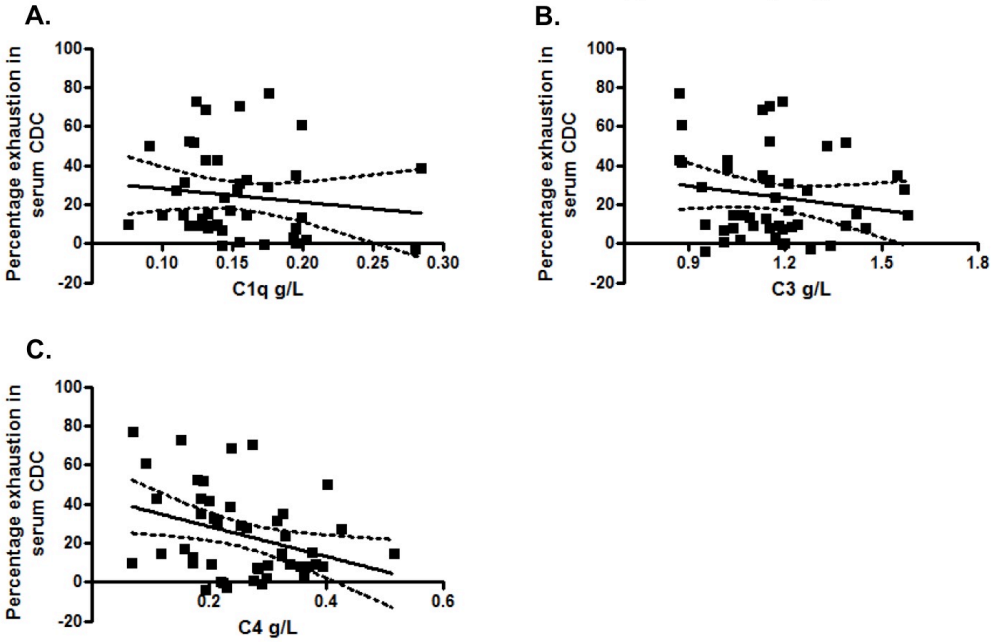
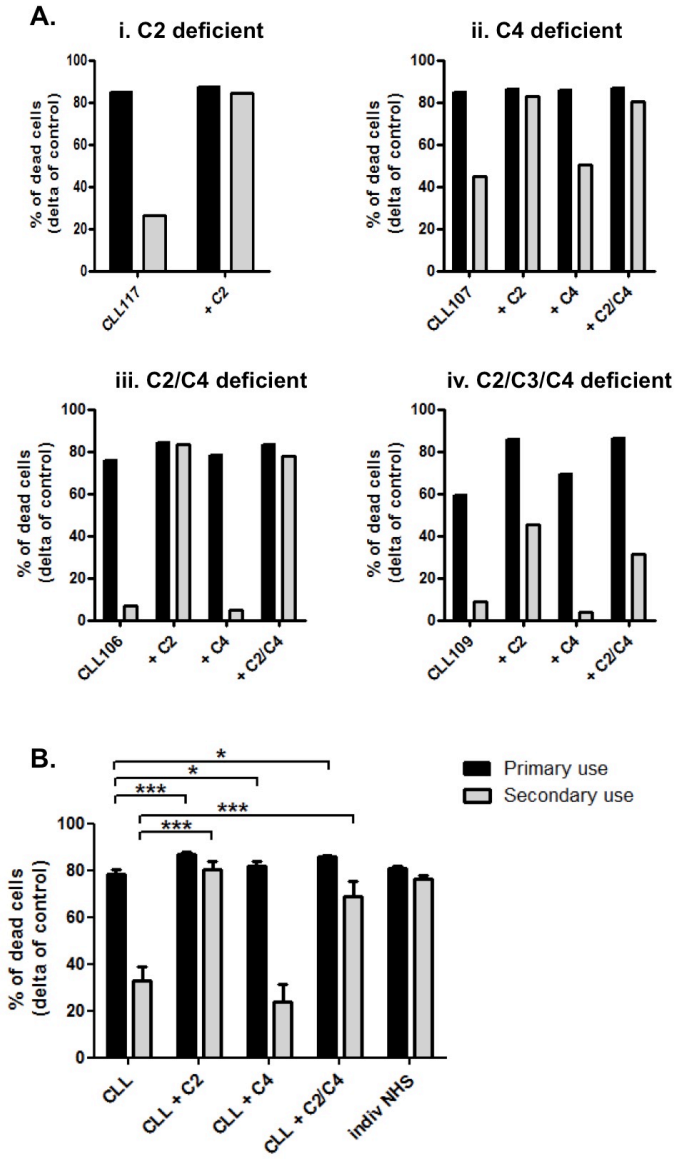
Supplementary Figure 5 - Middleton *et al.*



Supplementary Figure 5. CLL sera exhaustion also negatively correlates with C4 levels. Figure 4 demonstrates that complement component C2 concentration shows a significantly negative correlation with CLL patient sera exhaustion. Complement C4 concentration also displays a significant negative correlation with CLL sera exhaustion levels but to a lesser degree. **A.** C1q levels in CLL patient sera were compared against CLL patient sera exhaustion (n=43). Linear regression was applied to obtain values for r^2, 0.01681 and p value, 0.4073. **B.** C3 levels in CLL patient sera were compared against CLL patient sera exhaustion (n=47). Linear regression was applied to obtain values for r^2, 0.02727 and p value, 0.2673. **C.** C4 levels in CLL patient sera were compared against CLL patient sera exhaustion (n=43). Linear regression was applied to obtain values for r^2, 0.1559 and p value, 0.0192.

Supplementary Figure 6 - Middleton *et al.*



Supplementary Figure 6. CLL sera exhaustion in complement deficient serum can be restored with the addition of C2 alone.
CLL sera that had complement deficiencies in C2, C3 and/or C4 were supplemented with C2 (50 µg/ml), C4 (700 µg/ml) or both C2/C4 (25 µg/ml/350 µg/ml respectively), before being used to induce CDC in HG3 cells treated with 20 µg/ml OFA (1° use). After the incubation period, sera was removed and re-challenged with HG3 cells treated with 20 µg/ml OFA (2° use). Percentage of dead cells is relative to untreated control. **A. (i)** Representative CLL patient serum sample deficient in C2 alone. **(ii)** Representative CLL patient serum sample deficient in C4 alone. **(iii)** Representative CLL patient serum sample deficient in C2 and C4. **(iv)** Representative CLL patient serum sample deficient in C2, C3 and C4. **B.** CLL sera (n=16) that exhibited high levels of serum exhaustion were supplemented with C2 (n=14), C4 (n=9), C2 and C4 combined (n=8) Mean cell death $\pm$ SEM are shown. p value were obtained by paired t test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

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