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Original article

A single bout of vigorous intensity exercise enhances the efficacy of rituximab against autologous human chronic lymphocytic leukaemia B-cells *ex vivo.*

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Abstract

Chronic lymphocytic leukaemia (CLL) is characterised by the clonal proliferation and accumulation of mature B-cells and is often treated with rituximab, an anti-CD20 monoclonal antibody immunotherapy. Rituximab often fails to induce stringent disease eradication, due in part to failure of antibody-dependent cellular cytotoxicity which relies on natural killer (NK)-cells binding to rituximab-bound CD20 on B-cells. CLL cells are diffusely spread across

lymphoid and other bodily tissues, and ADCC resistance in survival niches may be due to several factors including low NK-cell frequency and a suppressive stromal environment that promotes CLL cell survival. It is well established that exercise bouts induce a transient relocation of NK-cells and B-cells into peripheral blood, which could be harnessed to enhance the efficacy of rituximab in CLL by relocating both target and effector cells together with rituximab in blood. In this pilot study, $n = 20$ patients with treatment-naïve CLL completed a bout of cycling 15% above their anaerobic threshold for ~30-minutes, with blood samples collected pre-, immediately post-, and 1-hour post-exercise. Flow cytometry revealed that exercise evoked a 254% increase in effector ($CD3^{-}CD56^{+}CD16^{+}$) NK-cells in blood, respectively, and a 67% increase in $CD5^{+}CD19^{+}CD20^{+}$ CLL cells in blood (all $p < 0.005$). NK-cells were isolated from blood samples pre-, and immediately post-exercise and incubated with primary isolated CLL cells with or without the presence of rituximab to determine specific lysis using a calcein-release assay. Rituximab-mediated cell lysis increased by 129% following exercise ($p < 0.001$). Direct NK-cell lysis of CLL cells – independent of rituximab – was unchanged following exercise ($p = 0.25$). We conclude that exercise improved the efficacy of rituximab-mediated antibody-dependent cellular cytotoxicity against autologous CLL cells *ex vivo* and propose that exercise should be explored as a means of enhancing clinical responses in patients receiving anti-CD20 immunotherapy.

Keywords: CLL_1, $Rituximab_2$, $Exercise_3$, $ADCC_4$, $NK\text{-}cells_5$, $B\text{-}cells_6$

1.1. Introduction

Chronic lymphocytic leukaemia (CLL) is the second most common adult blood cancer in the UK [1], with incidence peaking among people aged ≥65 years [2]. CLL is characterised by the clonal proliferation of mature B-cells in the blood, bone marrow, lymph nodes and spleen [3]. Often diagnosed incidentally, the clinical course of CLL is variable, ranging from indolent to aggressive. Patients typically present with asymptomatic, early-stage disease at diagnosis [4], and are monitored without therapy until progression to symptomatic CLL. These patients are referred to as treatment-naïve [3]. Once disease progression occurs, relatively more physically fit, younger patients have, for nearly two decades, typically received anti-CD20 monoclonal antibody (mAb) therapy (e.g. rituximab) combined with chemotherapy agents fludarabine and cyclophosphamide (FCR) [5–7]. Despite inducing favourable responses, rituximab with FC often fails to induce disease eradication. Several potential reasons have been described, related to both tumour cell intrinsic and extrinsic factors [8], and include the failure of antibody-dependent cellular cytotoxicity (ADCC).

Rituximab was one of the first mAb therapies approved for use in clinical practice and eliminates cancer cells via (i) antibody-dependent cellular cytotoxicity, (ii) complement-dependent cytotoxicity, (iii) antibody-dependent cellular phagocytosis, and (iv) direct apoptosis [9]. During an ADCC response, rituximab binds to CD20 on the CLL cell surface, allowing interactions between Fcγ receptors (such as CD16a) on NK-cells and monocytes, and the Fc portion of opsonised target cells, resulting in cell lysis via exocytosis of perforins and granzymes [10]. Rituximab with chemotherapy (e.g., FCR) achieves high complete remission rates, but, it does not induce disease eradication [7,11]. CLL cells are typically diffusely spread across lymphoid and other bodily tissues [12–14] and ADCC resistance in survival niches is thought to be driven by a variety of factors including a suppressive stromal environment that promotes CLL cell survival [15] and low $CD56^{dim}$ NK-cells – which express CD16 [16] – frequency in lymphoid tissue [17]. CLL cells that survive treatment – known as minimal residual disease (MRD) – exist below the detection threshold used for complete remission determination via flow cytometry (<1 CLL cell per 10^4 leukocytes) [3,18]. Therefore, disease

relapse is inevitable for most patients, and is often aggressive and treatment resistant [19,20]. Thus, novel approaches to tackle MRD in the setting of rituximab are warranted.

It is well established that during an individual bout of moderate to vigorous intensity aerobic exercise in healthy humans, immune cell frequency in peripheral blood increases profoundly. Driven by increased haemodynamic forces and catecholamine release in blood [21,22], the preferential, transient relocation of immune cell subtypes into the bloodstream is dependent on the cells expression of β_2-adrenergic receptors and their activation in response to increased adrenaline [22,23]. For example, NK-cells with a cytotoxic phenotype ($CD3^{-}CD56^{dim}$) are the most responsive, increasing by up to 1000% [24,25], followed by $CD8^{+}$ T-cells (+140%) [24], and $CD19^{+}$ B-cells (+130%) [26]. Given that $CD3^{-}CD56^{+}CD16^{+}$ NK-cells are required for rituximab-mediated ADCC, it may be that exercise is a potent means of enhancing the efficacy of rituximab against CLL cells in blood. Additionally, it has been shown in a study of $n = 16$ trained cyclists, that NK-cells expressing the terminal differentiation marker (CD57) increase in the blood by up to 500% following 30-minutes of cycling exercise (15% above lactate threshold) [25]. $CD57^{+}$ NK-cells contain large amounts of granzymes and perforins [16,27], and may be more sensitive to CD16 activation [27] than their immature counterparts and therefore, this phenotype of preferentially mobilised cells during exercise may enhance ADCC responses further. Importantly, cycling for 20-minutes at 80% maximal oxygen uptake ($\dot{V}O_{2MAX}$) has been shown to induce an 88% increase in blood $CD19^{+}$ B-cells in $n = 9$ healthy males [26]. Further, human B1 cells (CD19, CD27, CD43) which correspond to the phenotype of CLL cells [28], were mobilised by 84% in response to exercise in the same study [26]. It may therefore be the case that exercise can be harnessed to mobilise CLL cells into the blood, where they may be more susceptible to the mechanisms-of-action of rituximab. A rodent model of acute stress – which induces a similar adrenergic response to individual bouts of exercise – appeared to relocate B-cells from the bone marrow into the circulation [29] suggesting that B-cells mobilised in response to exercise may have lymphoid origins. The redistribution of CLL cells from the protection of the lymphoid tissues, into blood, may support efforts to delete more treatment resistant cells such as those responsible for MRD in CLL. Taken together, we hypothesise that an individual bout of cycling exercise will mobilise CLL cells from diffuse lymphoid tissues into the peripheral circulation, and will concomitantly mobilise cytotoxic NK-cells into blood, which in turn may enhance the efficacy of rituximab.

It is currently unknown how an individual bout of exercise influences circulating immune cell frequency in people with CLL. Therefore, the aim of this study was to characterise the frequency of immune cells and CLL cells in blood of people with treatment-naïve CLL in response to an individual bout of vigorous intensity cycling exercise, and determine if immune cell changes in blood correspond with improvements to rituximab efficacy *ex vivo* against autologous CLL cells.

1.2. Methods

1.2.1. Participants

Seventy-three patients diagnosed with CLL and aged ≥18-years were invited to participate in the study. Diagnosis of CLL was in-line with the 2018 International Workshop on CLL (iwCLL) guidelines, based on the presence of 5×10^3 B-cells/μL blood, sustained for ≥3-months and confirmed by blood smear, immunophenotyping and/or epigenetics [3].

Fifty-four treatment-naïve CLL patients were approached about the trial; treatment-naïve CLL was defined as asymptomatic, early-stage disease which is routinely monitored without

treatment. Of $n = 54$ treatment-naïve CLL patients approached, $n = 26$ declined to participate in the trial. Initial telephone screening involving verbal completion of the Physical Activity Readiness Questionnaire [30] resulted in $n = 2$ patients being excluded ($n = 1$ due to dyspnoea, $n = 1$ due to atrial fibrillation). A further $n = 2$ patients were excluded prior to the screening visit as the target sample size had been achieved. As such, $n = 24$ (44%) treatment-naïve CLL patients attended the screening visit (Figure 1). During the screening visit, patients were assessed for exclusion criteria: severe/uncontrolled cardiovascular, neurological, psychiatric, metabolic, and respiratory disease, musculoskeletal conditions that would impair the ability to cycle, metastatic/palliative malignancy, abnormal haematological results (e.g., haemoglobin <100g/dL), and an Eastern Cooperative Oncology Group [31] performance status >1. A 12-lead resting electrocardiogram (ECG) was recorded and reviewed by a cardiologist. Abnormal resting ECG resulted in $n = 2$ patients being excluded, meaning $n = 22$ patients with treatment-naïve CLL were recruited.

Our study was primarily focussed on patients with treatment-naïve CLL, and their results are discussed herein, unless otherwise stated. However, to explore whether cycling exercise could mobilise MRD CLL cells during remission, a subset of CLL patients in remission were also recruited. To be eligible, remission patients had achieved complete remission with incomplete marrow recovery or partial remission for ≥6-months following the completion of anti-CD20 treatment, as per iwCLL guidelines [3]. Inclusion/exclusion criteria were as described for treatment-naïve CLL. A total of $n = 19$ remission patients were approached. Two patients failed telephone screening ($n = 2$ due to abnormal haematological results) and nine patients declined to be involved in the trial. Screening of $n = 8$ remission patients resulted in $n = 4$ being excluded due to abnormal resting ECG, and $n = 4$ being recruited, with $n = 1$ withdrawing from the study before completion due to illness unrelated to the trial (Figure 1). All participants provided written informed consent prior to completing pre-experimental and experimental procedures. This study was approved by the National Health Service West of Scotland Research Ethics Committee 4 (20/WS/0049) and registered on ClinicalTrials.gov (NCT05093192).

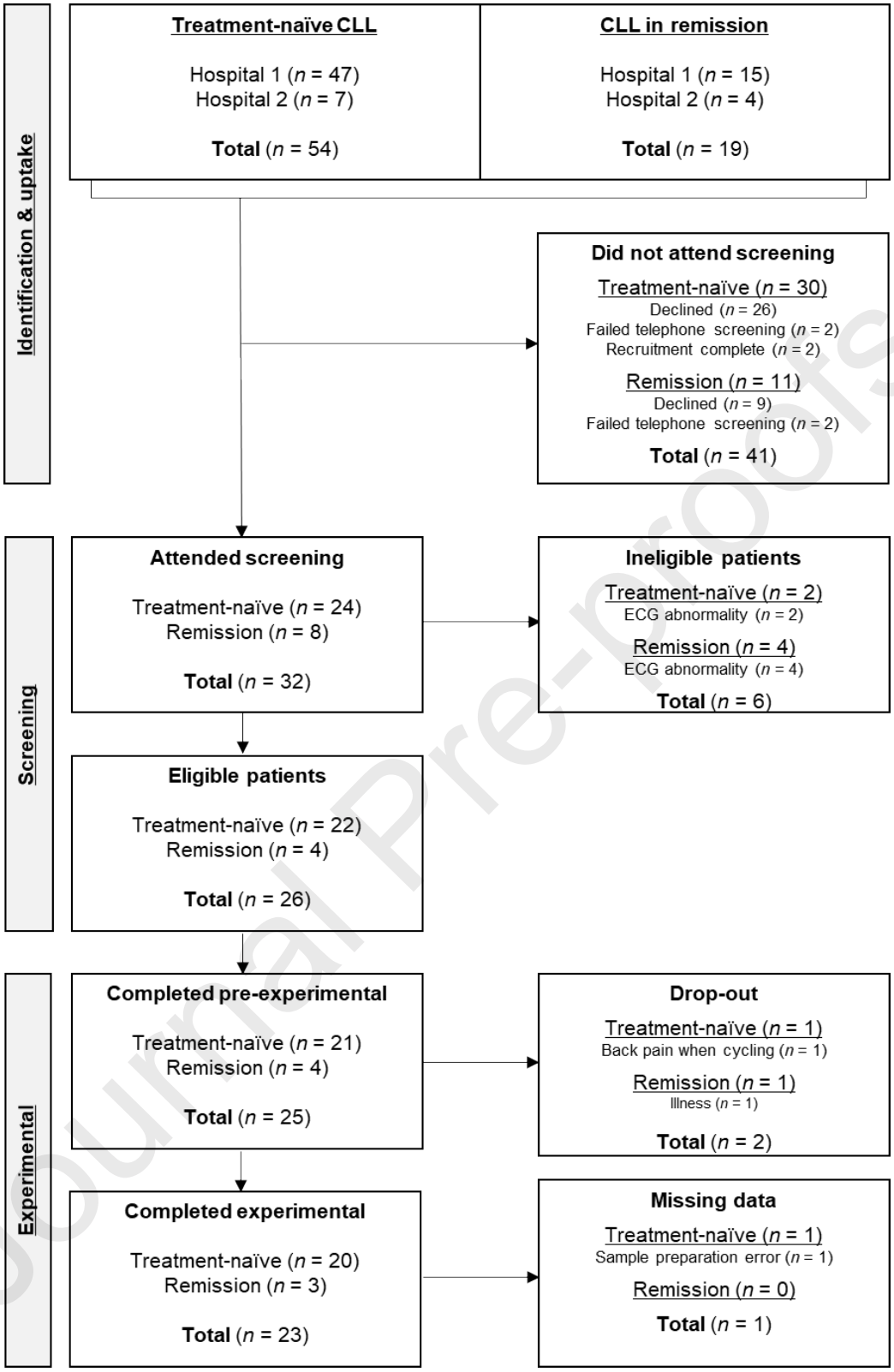

Figure 1. A CONSORT flow diagram of the recruitment, screening and experimental completion of the study.

1.2.2. Pre-experimental procedures

Participants were instructed to avoid strenuous exercise for 24-hours prior to the visit and arrived following a ≥4-hour fast. Height was assessed by a stadiometer (Seca, Birmingham, United Kingdom), body mass and fat percentage were determined by electronic scales and bioelectrical impedance analysis (Tanita InnerScan, Tokyo, Japan). Participants rested in a supine position for ~10-minutes prior to three blood pressure measurements using an automated blood pressure monitor (SunTech TangoM2, Wuxi, China). Anaerobic threshold was determined by a submaximal ramp incremental exercise test on a cycle ergometer (Lode Excalibur, Groningen, The Netherlands). Participants were instructed to maintain a cadence between 60-80 revolutions per minute throughout, and completed a 3-minute warm-up of unloaded cycling, followed by a 5-25 $W \cdot min^{-1}$ incremental phase up to a rating of perceived exertion (RPE) of ≥17 on a Borg 6-20 scale [32], or until the researchers were satisfied that anaerobic threshold was achieved (e.g. respiratory exchange ratio >1.0), and then an unloaded cool-down for 5-minutes. Breath-by-breath gas exchange/ventilation (Carefusion Vyntus CPX, CA, USA), heart rate via 12-lead ECG (Carefusion Vyntus ECG, CA, USA), and arterial oxygen saturation (SpO_2) via pulse oximetry (Nonin PureSAT, MN, USA) were recorded continuously during exercise, whilst RPE was recorded every 60-seconds and blood pressure was recorded pre- and immediately post-incremental exercise. Pulmonary oxygen uptake ($\dot{V}O_2$), carbon dioxide production ($\dot{V}CO_2$), and ventilatory equivalents of O_2 ($\dot{V}_E/\dot{V}O_2$) and CO_2 ($\dot{V}_E/\dot{V}CO_2$) data were interpolated to 15-second averages. The V-slope method [33] was used to determine anaerobic threshold and was further confirmed through visual inspection of $\dot{V}_E/\dot{V}O_2$ and $\dot{V}_E/\dot{V}CO_2$ measurements, independently by two researchers. Anaerobic threshold was reported in terms of $\dot{V}O_2$ ($mL \cdot kg^{-1} \cdot min^{-1}$), power output (W), and as a percentage of age predicted maximum heart rate, using the following equation:

% Age predicted heart rate max = (measured heart rate ÷ (220 – age in years)) × 100

1.2.3. Experimental procedures

At least 3-days after pre-experimental procedures, participants attended the experimental visit between 07:00-10:00 having avoided strenuous exercise and alcohol for 24-hours, and caffeine for ≥9-hours, and having fasted for ≥9-hours. Body mass was reassessed, and participants rested in a supine position for ~30-minutes prior to three blood pressure measurements and a 50 mL blood sample drawn from the antecubital fossa by venepuncture (baseline). The exercise intervention consisted of a 5-minute warm-up at 10% of the subsequent workload, and 20- to 30-minutes of cycling at a workload corresponding to 10-15% above individual anaerobic threshold, whilst maintaining a cadence of 60-80 revolutions per minute throughout. Breath-by-breath gas exchange/ventilation, heart rate, and SpO_2 data were recorded continuously during exercise and interpolated to 5-minute averages, whilst RPE was recorded every 5-minutes. Following exercise cessation, participants were immediately positioned in a supine position and a 50 mL blood sample was drawn within 3-minutes (post-exercise) and a final 20 mL blood sample was drawn after 1-hour (1-hr post-exercise).

1.2.4. Sample Processing

Blood was collected into sodium heparin (17 IU/mL), ethylenediaminetetraacetic acid (EDTA, 1.8 mg/mL), and silica act clot activator treated vacutainers (Becton & Dickinson, NJ, USA). Peripheral blood mononuclear cells (PBMCs) were isolated from sodium heparin-treated blood – diluted 1:1 with phosphate buffered saline (PBS; KCl 0.2 g/L, KH_2PO_4 0.2 g/L, NaCl 8.0 g/L, Na_2HPO_4 1.15 g/L; without $CaCl_2$ and $MgCl_2$ herein) (Sigma Aldrich, MI, USA)

containing 2% (v/v) heat-inactivated foetal calf serum (HI-FCS) (Gibco™, MA, USA) – in SepMate™ tubes (StemCell Technologies, Vancouver, Canada) that were pre-loaded with 15 mL Ficoll-Paque™ PLUS Media (Cytiva, MA, USA). SepMate™ tubes were centrifuged at 1,200 × g for 10-minutes at room-temperature and the isolated cells were transferred to a 50 mL conical tube (Falcon™, Thermo Fisher Scientific, Loughborough, UK). PBMCs were washed twice by centrifugation at 500 × g for 5-minutes at room-temperature and resuspended in 20-50 mL PBS (2% HI-FCS), depending on the density of the cell suspension. A small proportion of isolated PBMCs were cryopreserved at a concentration of 1×10^7 cells/mL in freezing medium, consisting of 10% (v/v) dimethyl sulfoxide (DMSO) (Invitrogen™, Thermo Fisher Scientific, Loughborough, UK) in HI-FCS, at −1°C/minute using a freezing container (Nalgene Mr Frosty, Thermo Fisher Scientific, Loughborough, UK), and subsequently transferred to −150°C freezer for long-term storage. The remaining PBMCs were used for ADCC assays.

EDTA-treated whole blood was analysed for blood lactate and blood glucose concentrations using rapid analysers (Lactate Plus Meter, Nova Biomedical, MA, USA and FreeStyle Optium Neo, Berkshire, UK, respectively). Subsequently, plasma was prepared from EDTA-treated blood by centrifugation at 2,000 × g for 15-minutes at 4°C within 4-hours of collection. Serum was prepared from silica-treated blood, which was allowed to clot for ≥60-minutes, prior to centrifugation at 1,300 × g for 10-minutes at 4°C. Both plasma and serum were cryopreserved at −80°C for long-term storage.

1.2.5. Immune cell analysis

1.2.5.1. Leukocyte differential

EDTA-treated whole blood was analysed in triplicate using an automated haematology analyser (Sysmex KX-21N, Kobe, Japan) for leukocyte and erythrocyte frequencies, haemoglobin, haematocrit, platelets, and proportions/numbers of lymphocytes, monocytes, neutrophils, eosinophils and basophils. In $n = 9$ treatment-naïve CLL patients, lymphocyte and monocyte proportions/number could not be determined by an automated haematology analyser. In these instances, monocyte proportions were determined by flow cytometry as HLA-DR$^+$ cells in the monocyte domain of a forward scatter-area × side scatter-area gate and lymphocyte proportions/numbers were calculated by negative deduction thereafter (Supplementary Figure 1).

1.2.5.2. Immunophenotyping leukocyte subsets

Sodium heparin-treated whole blood (1 mL from each time-point) was treated with 20 mL 1× FACS Lysing Solution (BD Biosciences, Wokingham, UK), and 217 ng/mL fixable viability stain 620 (FVS620 PE-CF594) (BD Biosciences, Wokingham, UK), for 15-minutes then centrifuged at 500 × g for 5-minutes at room temperature and washed twice with 5 mL PBS. Cells were resuspended in MACS buffer (PBS, 10% [v/v] HI-FCS, and 2 mM EDTA [Invitrogen™, Thermo Fisher Scientific, Loughborough, UK]) at a concentration of 1×10^7 cells/mL, and 1×10^6 cells were seeded in 5 mL round-bottom polystyrene test tubes (Falcon®, Corning, NY, USA). Cells from each time point were stained for 30-minutes at room-temperature with antibody-fluorochrome cocktails of two panels to investigate either CLL or NK-cells. The CLL panel compromised the following antibody-fluorochrome cocktails, including core markers recommended by the 2018 iwCLL for the monitoring of MRD [3]: anti-CD3 PE-Cy5.5 (SK7), anti-CD5 PE-Cy7 (L17F12), anti-CD19 APC-R700 (SJ2SC1), anti-CD20 APC-H7 (2H7), anti-CD38 BV421 (HIT2), anti-CD43 BV480 (1G10), anti-CD49d APC

(9F10), anti-CD79b PE (CB3-1), anti-CD81 FITC (JS-81), anti-sIgM BV605 (G20-127), and anti-CXCR4 BB700 (12G5). The NK-cell panel comprised the following antibody-fluorochrome cocktail: anti-CD3 APC-H7 (SK7), anti-CD14 PE-Cy5.5 (Tuk4), anti-CD16 BV480 (3G8), anti-CD32 PE (FLI8.26), anti-CD33 BV421 (P67.6), anti-CD56 PE-Cy7 (B159), anti-CD57 FITC (NK-1), anti-CD158a APC (HP-3E4), anti-NKG2A BB700 (131411), anti-HLA-DR AF700 (G46-6). Labelled cells were then centrifuged at 500 × g for 5-minutes at room temperature, resuspended in 500 µL MACS buffer and stored at 4°C until analysis. Samples were analysed using a FACS Aria III and FACS Diva software (BD Biosciences, Wokingham, UK) within six hours of preparation.

All antibodies were pre-titrated to ensure optimal fluorescent staining was achieved (data not shown). Both unstained cells from each time-point, and single stained tubes containing anti-mouse positive (Igκ) and negative control compensation particles (BD Biosciences, Wokingham, UK) were used in each assay to account for spectral overlap. Data were analysed in FlowJo (Version 10.9, BD Biosciences, Wokingham, UK) according to the representative gating strategies reported in Figures 2 and 3.

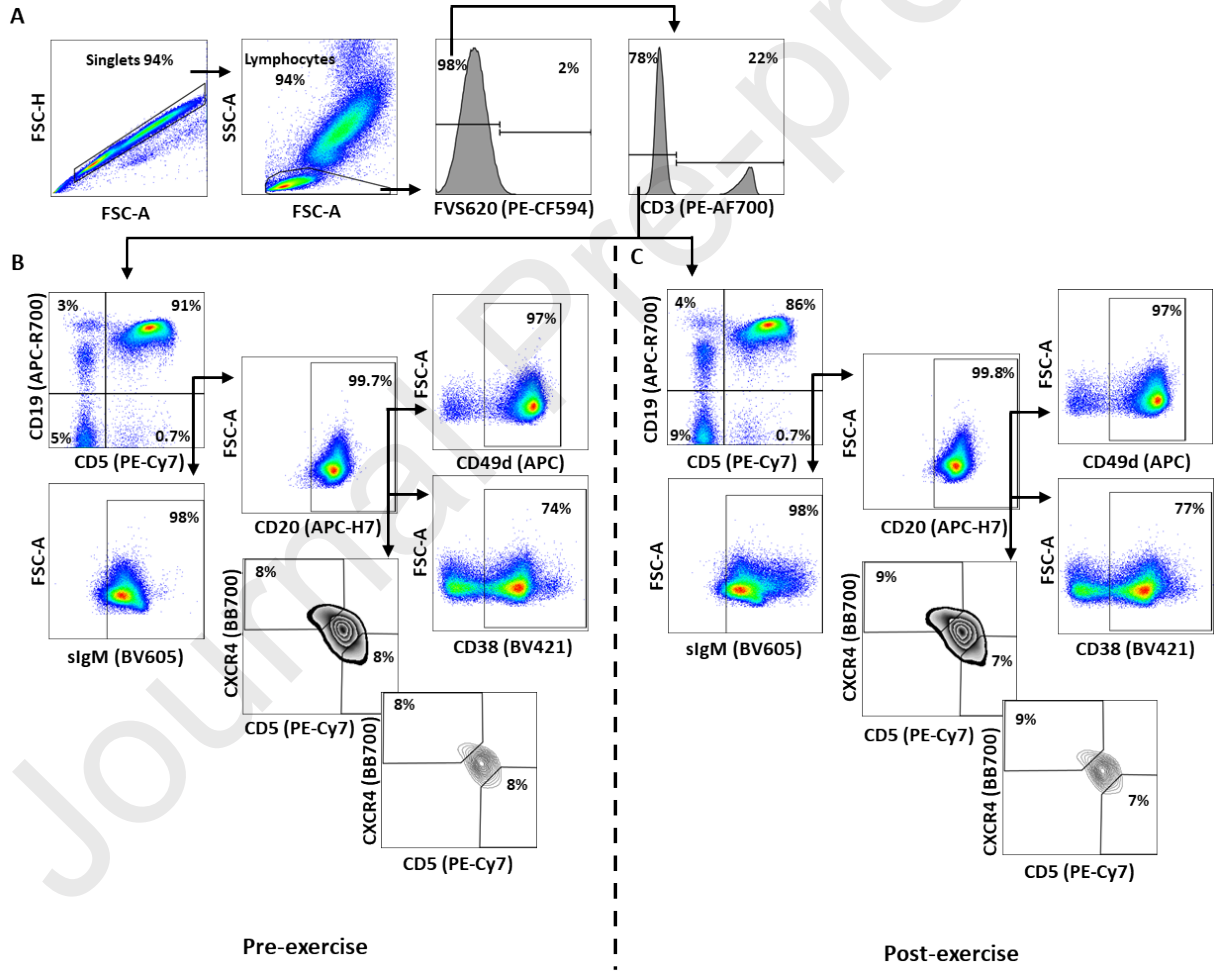

Figure 2. Representative gating strategy for CLL cells from one participant with treatment-naïve CLL. **A)** Doublets and debris were removed by plotting FSC-H against FSC-A, lymphocytes were then gated from singlets by plotting SSC-A against FSC-A. Using a fixable viability stain (FVS) viable cells were gated as FVS620^{-} using a histogram. CD3 was then gated in viable cells to remove any T cells from further analysis using a histogram. **B)** In a pre-exercise sample, following the exclusion of T cells, CD19 was plotted against CD5 to identify

total CLL cells ($CD5^+CD19^+$). In the CLL cell population, FSC-A was plotted against sIgM to identify B-cell receptor engagement, and FSC-A was plotted against CD20 to identify CLL cells susceptible to rituximab. CLL cells susceptible to rituximab ($CD5^+CD19^+CD20^+$) were then gated to identify subsets with prognostic relevance (FSC-A against CD49d and FSC-A against CD38), a phenotype consistent with a propensity to migrate ($CD5^{dim}CXCR4^{bright}$), and with a phenotype consistent with recent egress from lymphoid tissue ($CD5^{bright}CXCR4^{dim}$). **C)** Represents the same gating strategy as in '**B)**' but from a post-exercise sample. CLL, chronic lymphocytic leukaemia; FSC-H, forward scatter-height; FSC-A, forward scatter-area; SSC-A, side scatter-area.

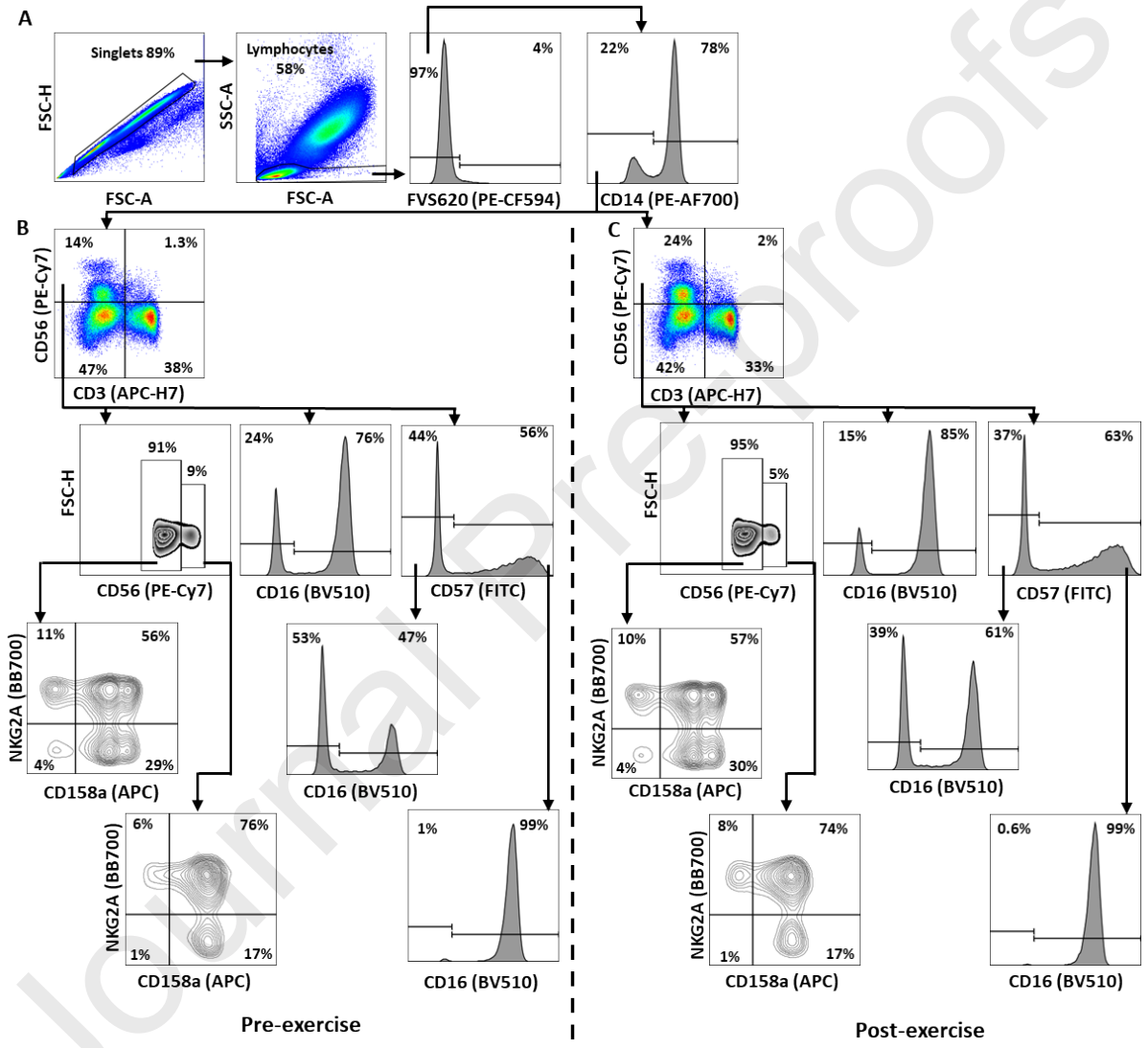

Figure 3. Representative gating strategy for NK-cells from one treatment-naïve CLL participant. **A)** Doublets and debris were removed by plotting FSC-H against FSC-A, lymphocytes were then gated from singlets by plotting SSC-A against FSC-A. Using a fixable viability stain (FVS) viable cells were gated as $FVS620^-$ using a histogram. CD14 was then gated in viable cells to remove any monocytes from further analysis using a histogram. **B)** In a pre-exercise sample, following the exclusion of monocytes, CD56 was plotted against CD3 to isolate total NK-cells ($CD3^-CD56^+$). In the $CD3^-CD56^+$ population, $CD56^{dim}$ and $CD56^{bright}$ NK-cells were gated by plotting FSC-H against CD56, $CD16^-$ and $CD16^+$ NK-cells were gated using a histogram, and $CD57^-$ and $CD56^+$ NK-cells were gated using a histogram. In both the

$CD56^{dim}$ and $CD56^{bright}$ populations, NKG2A was plotted against CD158a to determine NK-cell differentiation status including, low-differentiated ($CD158a^{-}NKG2A^{+}$), medium-differentiated ($CD158^{+}NKG2A^{+}$) and highly differentiated ($CD158a^{+}NKG2A^{-}$). **C)** Represents the same gating strategy as '**B)**' but from a post-exercise sample. CLL, chronic lymphocytic leukaemia; FSC-H, forward scatter-height; FSC-A, forward scatter-area; SSC-A, side scatter-area.

1.2.6. Antibody-dependent cellular cytotoxicity (ADCC) assay

CLL cells were isolated from 1 mL of sodium heparin-treated whole blood collected pre-exercise by EasySep™ direct immuno-magnetic negative selection, as per manufacturer's instruction (StemCell Technologies, Vancouver, Canada), achieving a mean purity of 87 ± 15% viable $CD19^{+}CD56^{-}$ cells (Supplementary Figure 2). Enriched CLL cells were resuspended in 2 mL PBS and labelled with 10 µg/mL calcein acetoxymethyl ester (calcein-AM) (Invitrogen™, Thermo Fisher Scientific, Loughborough, UK). Non-fluorescent calcein-AM passively diffuses across the membrane of live cells, where it is converted to green, fluorescent calcein following acetoxymethyl ester hydrolysis by intracellular esterases, which renders it membrane-impermeable. Following removal of excess calcein-AM through washing, the relative fluorescence intensity in the supernatant is proportional to cell lysis.

Labelled CLL cells were washed thrice by centrifugation at 500 × g for 5-minutes at room-temperature, and resuspended in 15 mL PBS, followed by a final resuspension at 2×10^5 cells/mL in PBS. CLL cells were seeded at a concentration of 5×10^3/well in U-shaped, non-tissue culture treated 96-well plates (Falcon®, Corning, NY, USA) and experimental wells were immediately treated, for 30-minutes at 37°C and 5% CO_2, with 10 µg/mL of either anti-CD20 rituximab (ADCC) (Selleckchem, TX, USA) or anti-HER2 herceptin (antibody-independent cellular cytotoxicity [AICC]) (isotype control; Selleckchem, TX, USA). CLL cells were also seeded in control wells, which were cultured in PBS (10% [v/v] HI-FCS) to determine spontaneous lysis (negative control) and maximum lysis (positive control).

NK-cells from each time-point were isolated by EasySep™ immuno-magnetic negative selection from PBMCs, as per manufacturer's instruction (StemCell Technologies, Vancouver, Canada), achieving a mean purity of 68 ± 30% $CD19^{-}CD56^{+}$ cells pre-exercise, and 80 ± 24% $CD19^{-}CD56^{+}$ cells post-exercise (Supplementary Figure 2). Enriched NK-cells were suspended in PBS at 2× concentration of the blood they were isolated from and, subsequently, diluted 1:1 in the 96-well plate to establish an NK-cell concentration representative of blood. To investigate whether CLL cell lysis was specific to the interaction of target cell bound rituximab with CD16 on NK-cells (i.e., ADCC), a fraction of NK-cells were treated with 50 µg/mL anti-CD16 mAb (B73.1) (ADCC + anti-CD16) (Invitrogen™, Thermo Fisher Scientific, Loughborough, UK) for 1-hour at room-temperature, prior to being added to the 96-well plate. It should be noted here that two isoforms of CD16 exist (CD16a, CD16b) with CD16a considered the primary receptor involving NK-cell mediated ADCC. Nevertheless the CD16 on NK-cells is typically the CD16a isoform, with CD16b restricted neutrophils [34]. Culture was performed in 10% (v/v) HI-FCS. In a subset of patients, the influence of human plasma on ADCC was explored, where culture of NK-cells isolated pre-exercise and immediately post-exercise was performed in 10% (v/v) plasma collected pre-exercise, or 10% (v/v) plasma collected post-exercise, respectively. In all conditions, PBS was added to each well to achieve a final volume of 200 µL after addition of CLL cells, NK-cells, mAb, and HI-FCS or human plasma. The plate was incubated for 2-hours at 37°C and 5% CO_2.

Following the 2-hour incubation, maximum lysis wells were treated with 100 µL/well of 4% (v/v) Triton X-100 (Invitrogen™, Thermo Fisher Scientific, Loughborough, UK) and the plate was centrifuged at 100 × g for 2-minutes at room temperature. Subsequently, 75 µL/well of acellular supernatant was transferred to a 96-well flat-bottom black plate (Corning™, Thermo Fischer Scientific, Loughborough, UK). Fluorescence (485 nm, 530 nm) was measured using a Pherostar plate reader (BMG Labtech, Ortenberg, Germany) with the gain (based on positive controls) and read height optimised for each plate. Arbitrary relative fluorescence was converted to the percentage of specific lysis using the following equation:

% Specific Lysis = ((Sample – Spontaneous) ÷ (Triton X-100 – Spontaneous)) x 100

Spontaneous and maximum lysis conditions were evaluated over six replicates, whilst experimental wells were seeded in at least triplicate. All ADCC assay procedures were conducted in aseptic conditions. Assay procedures for remissive CLL patients are described in Supplementary Methods.

1.2.7. Statistical analysis

Statistical analyses were performed using SPSS (IBM SPSS Statistics Version 28, IL, USA). Data are presented as mean ± standard deviation (SD) unless otherwise stated. Paired sample t-tests or Wilcoxon signed-rank tests (if non-parametric distribution was observed following a Shapiro-Wilk test) were used to compare differences pre- to post-exercise for ADCC, AICC, and ADCC + anti-CD16. Statistical analysis was one-way repeated measures analysis of variance (ANOVA) for immune cell populations and physiological responses to exercise. Where significant effects were observed, *post hoc* Bonferroni comparisons were performed as reported in Figure/Table legends. Effect sizes from t-tests were reported as Cohen's *d*, with effects determined small ($d = 0.2$), medium ($d = 0.5$), or large ($d = 0.8$), and from ANOVA as partial eta squared (ηp^2), with effects determined small ($\eta p^2 = 0.01$), medium ($\eta p^2 = 0.06$), or large ($\eta p^2 = 0.14$) [35]. Additionally, Spearman's correlations were performed to assess the relationship between $CD16^+$ NK-cells and ADCC responses, and statistical significance was achieved if $p \leq 0.05$.

1.3. Results

1.3.1. Treatment-naïve CLL

Of $n = 22$ treatment-naïve CLL patients that provided informed consent for the trial, $n = 20$ participants completed all experimental procedures, and their baseline characteristics are reported in Table 1.

Table 1. Participant Characteristics.

Variable	Treatment-naïve	Remission
Total participants (*n*)	20	3
Male:female participants (*n*:*n*)	15:5	2:1

Age (years)	62 ± 10	63 ± 9
Height (cm)	174.0 ± 7.5	174.3 ± 6.9
Body mass (kg)	83.3 ± 16.8	81.8 ± 12.2
Body mass index ($kg.m^{-2}$)	27.4 ± 5.0	26.9 ± 3.2
Body fat (%)	31.7 ± 9.8	29.4 ± 10.8
Systolic blood pressure (mmHg)	134 ± 18	144 ± 14
Diastolic blood pressure (mmHg)	82 ± 12	90 ± 7
Erythrocytes ($\times 10^{12}$/L)	4.35 ± 0.37	4.64 ± 0.45
Haemoglobin (g/L)	132 ± 10	143 ± 13
Haematocrit (L/L)	0.396 ± 0.025	0.402 ± 0.040
Leukocytes ($\times 10^{9}$/L)	30.70 ± 22.21	3.68 ± 0.49
Lymphocytes ($\times 10^{9}$/L)	26.58 ± 22.03	0.82 ± 0.41
Neutrophils ($\times 10^{9}$/L)	3.29 ± 1.34	2.41 ± 0.59
Monocytes ($\times 10^{9}$/L)	0.51 ± 0.28	0.35 ± 0.09
Anaerobic threshold ($mL \cdot kg^{-1} \cdot min^{-1}$)	14.1 ± 2.9	15.8 ± 5.6
Anaerobic threshold (W)	71 ± 30	90 ± 49
Anaerobic threshold (% age predicted heart rate max)	65 ± 13	70 ± 9

1.3.1.1. Safety

One adverse event (syncope) occurred following pre-experimental exercise procedures. No other adverse events or serious adverse events occurred.

1.3.1.2. Characteristics of cycling exercise

All participants completed a single bout of cycling at a workload corresponding to 10-15% above anaerobic threshold, and $n = 14$ (70%) completed 30-minutes of cycling. The remaining $n = 6$ (30%) participants ceased cycling after 21-minutes and 36-seconds ± 3-minutes and 18-seconds (range: 20-minutes and 0-seconds to 28-minutes and 30-seconds) due to volitional exhaustion. Importantly, the $n = 6$ who did not complete the prescribed 30-minutes exercise duration exhibited physiological responses – such as RPE and blood lactate – in line with the rest of the cohort (Supplementary Tables 2 and 3) and therefore were included in subsequent within group analyses. Physiological responses including, $\dot{V}O_2$ ($mL \cdot kg^{-1} \cdot min^{-1}$), $\dot{V}O_2$ relative to anaerobic threshold (%), $\dot{V}CO_2$ ($mL \cdot kg^{-1} \cdot min^{-1}$), $\dot{V}E$ ($L \cdot min^{-1}$), respiratory exchange ratio, heart rate (bpm), heart rate as a percentage of age-predicted maximum (%), and RPE (6-20 Borg scale) [32] were averaged into two, 15-minute segments and are displayed in Table 2. Participants cycled above their anaerobic threshold throughout the cycling trial, with a significant increase in $\dot{V}O_2$ relative to anaerobic threshold from warm-up to 15- and 30-minutes (61 ± 18% vs 116 ± 30%, $p < 0.001$ and 127 ± 31%, $p < 0.001$) in addition to a significant increase from 15-minutes to 30-minutes ($p < 0.001$). Blood lactate increased by 145% ($p < 0.001$) from pre- to post-exercise (Table 3). The effect of the acute bout of cycling on leukocyte, lymphocyte, monocyte, neutrophil, and erythrocyte frequency are displayed in Table 3.

Table 2. Characteristics of vigorous intensity cycling exercise in treatment-naïve CLL, with main effect from repeated measures ANOVA. Data are mean ± SD, $n = 20$.

Variable	Warm-up	15-minutes	30-minutes	Main effect of time
$\dot{V}O_2$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	8.3 ± 1.8	16.0 ± 4.0***	17.4 ± 4.1***†††	$F_{(1.05,20.03)} = 119.89$, $p < 0.001$, $\eta p^2 = 0.86$
$\dot{V}O_2$ (% anaerobic threshold)	61 ± 18	116 ± 30***	127 ± 31***†††	$F_{(1.08,20.48)} = 143.11$, $p < 0.001$, $\eta p^2 = 0.88$
$\dot{V}CO_2$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	6.5 ± 1.5	14.7 ± 3.9***	15.8 ± 3.9***†††	$F_{(1.05,19.94)} = 126.26$, $p < 0.001$, $\eta p^2 = 0.87$
$\dot{V}E$ ($L \cdot min^{-1}$)	19.8 ± 5.3	39.9 ± 11.6***	44.7 ± 13.4***†††	$F_{(1.06,20.13)} = 82.00$, $p < 0.001$, $\eta p^2 = 0.81$
Respiratory exchange ratio	0.79 ± 0.05	0.91 ± 0.04***	0.91 ± 0.03***	$F_{(1.29,24.54)} = 149.34$, $p < 0.001$, $\eta p^2 = 0.89$
Heart rate (bpm)	77 ± 10	107 ± 14***	119 ± 15***†††	$F_{(1.25,23.79)} = 194.41$, $p < 0.001$, $\eta p^2 = 0.91$
Heart rate (% age predicted max)$_a$	49 ± 6	68 ± 8***	75 ± 9***†††	$F_{(1.32,25.12)} = 198.72$, $p < 0.001$, $\eta p^2 = 0.91$
Rating of perceived exertion	9 ± 2	12 ± 2***	15 ± 2***†††	$F_{(1.46,27.70)} = 85.34$, $p < 0.001$, $\eta p^2 = 0.82$

***indicates a significant difference from 'warm-up' at $p < 0.001$, †††indicates a significant difference from '15-minutes' at $p < 0.001$, following *post hoc* Bonferroni comparisons. CLL, chronic lymphocytic leukaemia; ANOVA, analysis of variance.

Table 3. Haemodynamic variables pre-exercise, immediately post-exercise, and 1-hr post-exercise with percentage change (%Δ) pre- to post-exercise and main effect from one-way repeated measures ANOVA in treatment-naïve CLL. Data are mean ± SD, $n = 20$.

	Pre-exercise	Post-exercise	1-hr post-exercise	%Δ Pre-Post	Main effect of time
Leukocytes ($\times10^9$/L)	30.70 ± 22.21	49.17 ± 38.04***	29.67 ± 21.02†††	60 ± 37	$F_{(1.03,19.51)} = 20.64$, $p < 0.001$, $\eta p^2 = 0.52$
Lymphocytes ($\times10^9$/L)	26.58 ± 22.03	43.23 ± 37.67**	25.27 ± 20.86††	65 ± 48	$F_{(1.03,19.56)} = 17.94$, $p < 0.001$, $\eta p^2 = 0.49$
Monocytes ($\times10^9$/L)	0.51 ± 0.28	0.67 ± 0.34**	0.52 ± 0.33†	40 ± 32	$F_{(2,38)} = 6.46$, $p = 0.004$, $\eta p^2 = 0.25$
Neutrophils ($\times10^9$/L)	3.29 ± 1.34	4.84 ± 2.09***	3.59 ± 1.37**†††	49 ± 31	$F_{(1.23,23.35)} = 38.52$, $p < 0.001$, $\eta p^2 = 0.67$
Erythrocytes ($\times10^{12}$/L)	4.35 ± 0.37	4.74 ± 0.38***	4.36 ± 0.37†††	9 ± 5	$F_{(2,38)} = 69.07$, $p < 0.001$, $\eta p^2 = 0.78$
Blood lactate (mmol/L)	1.5 ± 1.4	3.0 ± 1.7***	1.3 ± 1.3†††	145 ± 113	$F_{(1.26,23.86)} = 35.60$, $p < 0.001$, $\eta p^2 = 0.65$
Blood glucose (mmol/L)	5.4 ± 1.6	5.0 ± 1.5	5.2 ± 1.5	−36 ± 78	$F_{(1.49,28.27)} = 2.84$, $p = 0.089$, $\eta p^2 = 0.13$

**indicates a significant difference from pre-exercise at $p < 0.01$, ***indicates a significant difference from pre-exercise at $p < 0.001$, †indicates a significant difference from post-exercise at $p < 0.05$, ††indicates a significant difference from post-exercise at $p < 0.01$, †††indicates a significant difference from post-exercise at $p < 0.001$ following *post hoc* Bonferroni comparisons. ANOVA, analysis of variance; CLL, chronic lymphocytic leukaemia.

1.3.1.3. ADCC changes in response to cycling exercise

There was a significant 129% increase observed in ADCC pre- to post-exercise ($t_{(17)} = 5.04$, $p < 0.001$, $d = 1.19$) with no differences pre- to post-exercise in AICC ($Z = 1.61$, $p = 0.11$) or ADCC + anti-CD16 ($Z = 0.94$, $p = 0.35$) on CLL cell lysis (Figure 4A). We note that a significant 12% difference was observed between the purity of NK-cells isolated pre-exercise and post-exercise ($Z = 2.36$, $p = 0.018$). As a result, we determined whether this difference in NK-cell purity accounted for the change in ADCC responses observed, but it did not alter the main effects observed (see Supplementary Results).

In a subset of treatment-naïve participants ($n = 9$), ADCC against autologous CLL cells cultured with autologous time-point matched plasma ($ADCC_{Plasma}$) was compared to ADCC against autologous CLL cells cultured with HI-FCS ($ADCC_{HI\text{-}FCS}$) (Figure 4B). This additional experiment was conducted to investigate whether time-point matched plasma affected ADCC responses. There was a significant increase pre- to post-exercise for $ADCC_{Plasma}$ (+99%) and $ADCC_{HI\text{-}FCS}$ (+92%) ($t_{(8)} = 2.49$, $p = 0.038$, $d = 0.83$ and $t_{(8)} = 3.71$, $p = 0.006$, $d = 1.24$, respectively) on CLL cell lysis. Additionally, there was no difference between $ADCC_{Plasma}$ and $ADCC_{HI\text{-}FCS}$ pre-exercise ($t_{(8)} = 1.82$, $p = 0.11$, $d = 0.61$), or post-exercise ($t_{(8)} = 2.10$, $p = 0.069$, $d = 0.70$).

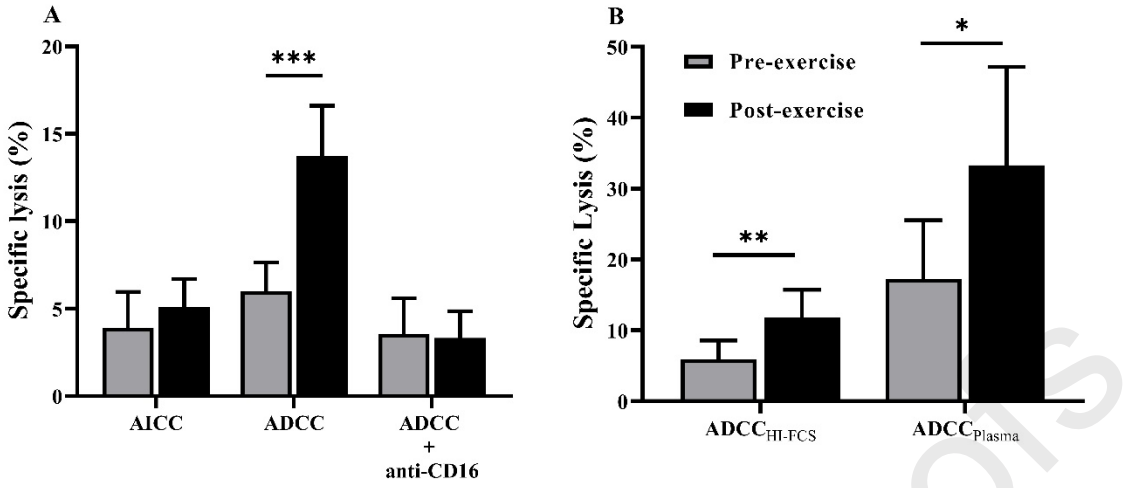

Figure 4. Specific lysis of treatment-naïve autologous CLL cells pre-exercise (grey bars) and post-exercise (black bars). **A)** Specific lysis independent of rituximab (AICC), mediated by rituximab (ADCC), and mediated by rituximab in the presence of a CD16 blocking antibody (ADCC + anti-CD16), $n = 18$. **B)** Specific lysis mediated by rituximab in the presence of HI-FCS ($ADCC_{HI\text{-}FCS}$) and time-point matched autologous plasma ($ADCC_{Plasma}$), $n = 9$. *indicates a significant difference at $p < 0.05$, **indicates a significant difference at $p < 0.01$, ***indicates a significant difference at $p < 0.001$. CLL, chronic lymphocytic leukaemia; AICC, antibody-independent cellular cytotoxicity; ADCC, antibody-dependent cellular cytotoxicity; HI-FCS, heat inactivated foetal calf serum. Data are mean ± SEM.

1.3.1.4. NK-cell mobilisation during cycling exercise

There was a significant effect of time on the number of $CD3^{-}CD56^{+}$ NK-cells ($F_{(1.05,18.96)} = 27.89$, $p < 0.001$, $\eta p^2 = 0.61$), $CD3^{-}CD56^{dim}$ ($F_{(1.07,19.17)} = 26.52$, $p < 0.001$, $\eta p^2 = 0.60$) and $CD3^{-}CD56^{bright}$ ($F_{(1.09,19.55)} = 13.58$, $p = 0.001$, $\eta p^2 = 0.43$) subsets in blood, increasing by 156 ± 101% ($p < 0.001$), 161 ± 113% ($p < 0.001$), and 68 ± 53% ($p = 0.004$) pre- to post-exercise, respectively (Table 4).

As ADCC by NK-cells is mediated by CD16a, the effect of time on NK-cells expressing CD16 was explored. There was a significant effect of time on $CD3^{-}CD56^{+}CD16^{+}$ ($F_{(1.03,18.60)} = 24.23$, $p < 0.001$, $\eta p^2 = 0.57$), mature $CD3^{-}CD56^{+}CD57^{+}CD16^{+}$ ($F_{(1.04,18.69)} = 22.64$, $p < 0.001$, $\eta p^2 = 0.56$) and immature $CD3^{-}CD56^{+}CD57^{-}CD16^{+}$ ($F_{(1.04,18.72)} = 22.99$, $p < 0.001$, $\eta p^2 = 0.56$) NK-cells (Table 4). From pre- to post-exercise, there was a significant, 254 ± 145% ($p < 0.001$) increase in $CD3^{-}CD56^{+}CD16^{+}$ NK-cells, a 323 ± 224% ($p < 0.001$) increase in $CD3^{-}CD56^{+}CD57^{+}CD16^{+}$ NK-cells, and a 190 ± 96% ($p < 0.001$) increase in $CD3^{-}CD56^{+}CD57^{-}CD16^{+}$ NK-cells in blood.

Spearman's correlations were performed to assess the relationship between ADCC and $CD16^{+}$ NK-cells pre- and post-exercise, revealing significantly positive correlations between ADCC and $CD3^{-}CD56^{+}CD16^{+}$ NK-cells ($\rho_{(34)} = 0.351$, $p = 0.036$), $CD3^{-}CD56^{+}CD57^{-}CD16^{+}$ NK-cells ($\rho_{(34)} = 0.368$, $p = 0.027$) and $CD3^{-}CD56^{+}$ $CD57^{+}CD16^{+}$ NK-cells ($\rho_{(34)} = 0.330$, $p = 0.050$).

Within the $CD3^{-}CD56^{dim}$ subset, there was a significant effect of time on low-differentiated ($F_{(1.02,18.37)} = 13.17$, $p = 0.002$, $\eta p^2 = 0.42$), medium-differentiated ($F_{(1.03,18.58)} = 21.30$, $p <$

0.001, $\eta p^2 = 0.54$), and highly-differentiated ($F_{(1.08,19.39)} = 29.84$, $p < 0.001$, $\eta p^2 = 0.62$) NK-cells, increasing significantly pre- to post-exercise by 121 ± 123% ($p = 0.004$), 167 ± 108% ($p < 0.001$), and 158 ± 127% ($p < 0.001$), respectively (Table 4). Similarly, there was an effect of time on low-differentiated ($F_{(1.09,19.65)} = 7.09$, $p = 0.013$, $\eta p^2 = 0.28$), medium-differentiated ($F_{(1.10,19.79)} = 13.03$, $p = 0.001$, $\eta p^2 = 0.42$), and highly-differentiated ($F_{(2,36)} = 10.34$, $p < 0.001$, $\eta p^2 = 0.37$) $CD3^{-}CD56^{bright}$ NK-cells, with a significant pre- to post-exercise increase of 71 ± 58% ($p = 0.005$) in medium-differentiated $CD3^{-}CD56^{bright}$ NK-cells, and a 67 ± 65% ($p = 0.014$), in $CD3^{-}CD56^{bright}$ NK-cells (Table 4).

Table 4. Treatment-naïve CLL NK-cell subsets pre-exercise, immediately post-exercise, and 1-hr post-exercise with percentage change (%Δ) pre- to post-exercise, and main effect from repeated measures ANOVA. Data are mean ± SD, $n = 19$.

Lymphocyte (cells/μL)	Phenotype	Pre-exercise	Post-exercise	1-hr post-exercise	%Δ Pre-Post	Main effect of time
NK-cells	$CD3^{-}CD56^{+}$	185 ± 120	468 ± 321***	143 ± 83*†††	156 ± 101	$F_{(1.05,18.96)} = 27.89$, $p < 0.001$, $\eta p^2 = 0.61$
	$CD3^{-}CD56^{+}CD16^{+}$	102 ± 77	352 ± 270***	80 ± 56†††	254 ± 145	$F_{(1.03,18.60)} = 24.23$, $p < 0.001$, $\eta p^2 = 0.57$
	$CD3^{-}CD56^{dim}$	172 ± 112	438 ± 308***	127 ± 75*†††	161 ± 113	$F_{(1.07,19.17)} = 26.52$, $p < 0.001$, $\eta p^2 = 0.60$
	$CD3^{-}CD56^{bright}$	17 ± 15	30 ± 28**	15 ± 15††	68 ± 52	$F_{(1.09,19.55)} = 13.58$, $p = 0.001$, $\eta p^2 = 0.43$
	$CD3^{-}CD56^{+}CD57^{+}CD16^{+}$	65 ± 45	253 ± 201***	49 ± 37†††	323 ± 224	$F_{(1.04,18.69)} = 22.64$, $p < 0.001$, $\eta p^2 = 0.56$
	$CD3^{-}CD56^{+}CD57^{-}CD16^{+}$	37 ± 36	102 ± 88***	29 ± 25†††	190 ± 96	$F_{(1.04,18.72)} = 22.99$, $p < 0.001$, $\eta p^2 = 0.56$
$CD3^{-}CD56^{dim}$ NK-cells	$CD158^{-}NKG2A^{+}$	21 ± 17	44 ± 41**	14 ± 10††	121 ± 123	$F_{(1.02,18.37)} = 13.17$, $p = 0.002$, $\eta p^2 = 0.42$

	$CD158^{+}NKG2A^{+}$	115 ± 81	299 ± 226***	85 ± 52†††	167 ± 108	$F_{(1.03,18.58)} = 21.30$, $p < 0.001$, $\eta p^2 = 0.54$
	$CD158^{+}NKG2A^{-}$	31 ± 19	78 ± 48***	24 ± 17†††	158 ± 127	$F_{(1.08,19.39)} = 29.84$, $p < 0.001$, $\eta p^2 = 0.62$
$CD3^{-}CD56^{bright}$ NK-cells	$CD158^{-}NKG2A^{+}$	1.8 ± 1.8	3.5 ± 4.3	1.8 ± 2.1†	151 ± 341	$F_{(1.09,19.65)} = 7.09$, $p = 0.013$, $\eta p^2 = 0.28$
	$CD158^{+}NKG2A^{+}$	13.3 ± 12.6	23.6 ± 23.1**	12.2 ± 12.3††	71 ± 58	$F_{(1.10,19.79)} = 13.03$, $p = 0.001$, $\eta p^2 = 0.42$
	$CD158^{+}NKG2A^{-}$	1.6 ± 1.0	2.6 ± 2.0*	1.3 ± 1.2††	67 ± 65	$F_{(2,36)} = 10.34$, $p < 0.001$, $\eta p^2 = 0.37$

*indicates a significant difference from pre-exercise at $p < 0.05$, **indicates a significant difference from pre-exercise at $p < 0.01$, ***indicates a significant difference from pre-exercise at $p < 0.001$, †indicates a significant difference from post-exercise at $p < 0.05$, ††indicates a significant difference from post-exercise at $p < 0.01$, †††indicates a significant difference from post-exercise at $p < 0.001$ following *post hoc* Bonferroni comparisons. ANOVA, analysis of variance.

1.3.1.5. CLL cell mobilisation during cycling exercise

The effect of exercise on CLL cell frequencies is presented in Table 5. Briefly, there was a significant effect of time on total $CD3^{-}CD5^{+}CD19^{+}$ CLL cells ($F_{(1.05,19.92)} = 15.31$, $p < 0.001$, $\eta p^2 = 0.45$) and $CD3^{-}CD5^{+}CD19^{+}CD20^{+}$ CLL cells ($F_{(1.05,19.92)} = 15.25$, $p < 0.001$, $\eta p^2 = 0.45$), with a 67 ± 47% ($p = 0.002$) increase in blood pre- to post-exercise for both populations before returning to baseline 1-hour post-exercise.

Within the $CD20^{+}$ CLL cell population – i.e., CLL cells susceptible to the actions of rituximab – there was a significant effect of time on $CD5^{bright}CXCR4^{dim}$ CLL cells (i.e., CLL cells recently egressed from lymphoid tissue [36,37] ($F_{(1.15,21.76)} = 12.60$, $p = 0.001$, $\eta p^2 = 0.40$) and $CD5^{dim}CXCR4^{bright}$ CLL cells (i.e., CLL cells with a propensity to migrate to tissues) [36,37] ($F_{(1.54,29.17)} = 8.47$, $p = 0.003$, $\eta p^2 = 0.31$) increasing by 74 ± 51% ($p = 0.005$) and 70 ± 62% ($p = 0.002$) pre- to post-exercise, respectively. Additionally, a significant effect of exercise was observed on $CD20^{+}$ CLL cells expressing the prognostic markers CD49d ($F_{(1.01,19.16)} = 7.99$, $p = 0.011$, $\eta p^2 = 0.30$) and CD38 [38] ($F_{(1.04,19.69)} = 14.99$, $p < 0.001$, $\eta p^2 = 0.44$), which increased by 72 ± 47% ($p = 0.022$) and 66 ± 47% ($p = 0.002$) pre- to post-exercise, respectively.

A further effect of time was observed on the expression of CD20 ($F_{(2,38)} = 3.36$, $p = 0.045$, $\eta p^2 = 0.15$) on $CD5^{+}CD19^{+}CD20^{+}$ CLL cells, however inspection of follow-up comparisons revealed no significant changes ($p > 0.05$) to CD20 expression. No effects of exercise were observed on sIgM expression on $CD3^{-}CD5^{+}CD19^{+}$ CLL cells ($F_{(1.54,29.25)} = 0.58$, $p = 0.53$, $\eta p^2 = 0.03$), as measured by median fluorescence intensity.

We also explored whether an individual bout of vigorous intensity exercise could mobilise CLL cells into the blood in a subset of CLL patients in remission. All remission patients included ($n = 3$) were MRD negative in blood – defined as <1 CLL cell/10^4 leukocytes – with no change observed in the number of total $CD5^{+}CD19^{+}$ CLL cells pre- to post-exercise ($F_{(2,4)} = 1.23$, $p = 0.38$, $\eta p^2 = 0.38$) (Supplementary Table 5).

Table 5. Treatment-naïve CLL B-cell subsets pre-exercise, immediately post-exercise, and 1-hr post-exercise, with percentage change (%Δ) pre- to post-exercise and main effect from repeated measures ANOVA. Data are mean ± SD, $n = 20$.

$CD19^+$ B-cells (cells/μL)	Pre-exercise	Post-exercise	1-hr post-exercise	%Δ Pre-Post	Main effect of time
$CD5^+$	20,028 ± 17,707	33,508 ± 31,584**	19,624 ± 17,635††	67 ± 47	$F_{(1.05,19.92)} = 15.31$, $p < 0.001$, $\eta p^2 = 0.45$
$CD5^+CD20^+$	19,890 ± 17,635	33,358 ± 31,550**	19,421 ± 17,070††	67 ± 47	$F_{(1.05,19.92)} = 15.25$, $p < 0.001$, $\eta p^2 = 0.45$
$CD20^+CD49d^+$	9,716 ± 13,397	16,489 ± 23,302*	9,411 ± 11,962†	72 ± 47	$F_{(1.01,19.16)} = 7.99$, $p = 0.011$, $\eta p^2 = 0.30$
$CD20^+CD38^+$	15,522 ± 14,993	25,393 ± 25,120**	14,880 ± 13,603††	66 ± 47	$F_{(1.04,19.69)} = 14.99$, $p < 0.001$, $\eta p^2 = 0.44$
$CD20^+CD5^{bright}CXCR4^{dim}$	2,810 ± 3,261	4,566 ± 5,239**	2,985 ± 3,342††	74 ± 51	$F_{(1.15,21.76)} = 12.60$, $p = 0.001$, $\eta p^2 = 0.40$
$CD20^+CD5^{bright}CXCR4^{dim}CD49d^+$	1,139 ± 1,263	1,896 ± 2,143**	1,266 ± 1,378††	76 ± 52	$F_{(1.15,21.92)} = 12.23$, $p = 0.001$, $\eta p^2 = 0.39$
$CD20^+CD5^{bright}CXCR4^{dim}CD38^+$	2,312 ± 3,063	3,706 ± 4,814*	2,407 ± 2,994†	72 ± 50	$F_{(1.10,20.91)} = 10.18$, $p = 0.004$, $\eta p^2 = 0.35$
$CD20^+CD5^{dim}CXCR4^{bright}$	1,440 ± 1,101	2,487 ± 2,130**	1,558 ± 1,312†	70 ± 62	$F_{(1.54,29.17)} = 8.47$, $p = 0.003$, $\eta p^2 = 0.31$
$CD20^+CD5^{dim}CXCR4^{bright}CD49d^+$	623 ± 678	1,081 ± 1,205**	595 ± 678††	73 ± 58	$F_{(1.10,20.91)} = 13.34$, $p = 0.001$, $\eta p^2 = 0.41$

$CD20^{+}CD5^{dim}CXCR4^{bright}CD38^{+}$	1,136 ± 921	1,931 ± 1,643**	1,054 ± 887†††	69 ± 63	$F_{(1.19,22.52)} = 18.20$, $p < 0.001$, $\eta p^2 = 0.49$
$CD5^{-}$	296 ± 246	538 ± 426**	289 ± 252††	103 ± 89	$F_{(1.15,21.78)} = 14.79$, $p < 0.001$, $\eta p^2 = 0.44$
$CD5^{-}CD20^{+}$	268 ± 221	510 ± 416**	270 ± 230††	99 ± 80	$F_{(1.10,20.96)} = 15.45$, $p < 0.001$, $\eta p^2 = 0.45$

*indicates a significant difference from pre-exercise at $p < 0.05$, **indicates a significant difference from pre-exercise at $p < 0.01$, †indicates a significant difference from post-exercise at $p < 0.05$, ††indicates a significant difference from post-exercise at $p < 0.01$, †††indicates a significant difference from post-exercise at $p < 0.001$ following *post hoc* Bonferroni comparisons. CLL, chronic lymphocytic leukaemia; ANOVA, analysis of variance.

1.3.1.6. Monocyte mobilisation during cycling exercise

Given that monocytes can express CD16 and CD32 – both of which can initiate mAb-mediated ADCC [39] – the effect of vigorous intensity cycling was explored on monocyte subsets. A significant effect of time was observed for non-classical monocytes (HLA-DR$^+$CD14^{dim}CD16$^+$) ($F_{(1.20,21.60)}$ = 39.97, p < 0.001, ηp^2 = 0.69), intermediate monocytes (HLA-DR$^+$CD14$^+$CD16$^+$) ($F_{(1.52,27.33)}$ = 12.66, p < 0.001, ηp^2 = 0.41), classical monocytes (HLA-DR$^+$CD14$^+$CD16$^-$) ($F_{(1.09,19.5949)}$ = 27.50, p < 0.001, ηp^2 = 0.60), and effector (HLA-DR$^+$CD14$^+$CD32$^+$) monocytes ($F_{(1.12,20.10)}$ = 28.55, p < 0.001, ηp^2 = 0.61), with an increase of 147 ± 150% (p < 0.001), 100 ± 114% (p = 0.004), 47 ± 28% (p < 0.001), and 59 ± 33% (p < 0.001) pre- to post-exercise, respectively (Supplementary Table 4).

1.3.1.7. Myeloid-derived suppressor cell mobilisation during cycling exercise

A significant effect of time was also observed for HLA-DR$^-$CD33$^+$ cells which may represent myeloid-derived suppressor cells (MDSC) [40,41] ($F_{(1.13,20.34)}$ = 28.54, p < 0.001, ηp^2 = 0.61), monocytic (M)-MDSCs (HLA-DR$^-$CD33$^+$CD14$^+$) ($F_{(1.05,18.92)}$ = 6.77, p = 0.017, ηp^2 = 0.27), and polymorphonuclear (PMN)-MDSCs (HLA-DR$^-$CD33$^+$CD14$^-$) ($F_{(1.16,20.89)}$ = 34.80, p < 0.001, ηp^2 = 0.66), which increased by 70 ± 60% (p < 0.001), 84 ± 89% (p = 0.045), and 65 ± 53% (p < 0.001) pre- to post-exercise, respectively (Supplementary Table 4).

1.4. Discussion

In this study, we analysed the effects of an individual bout of vigorous intensity cycling on the frequency of immune cells and CLL cells in the blood of people with treatment-naïve CLL, and investigated whether immune cell changes in blood correspond to improvements in rituximab-mediated ADCC against autologous CLL cells *ex vivo*. NK-cells and their subsets increased immediately post-exercise. ADCC increased post-exercise when autologous NK-cells and CLL cells were incubated in the presence of rituximab. Additionally, CLL cells also increased during exercise, including those with a recently egressed from lymphoid tissue phenotype.

This study is the first to show that a single bout of vigorous intensity cycling exercise improves rituximab-mediated ADCC against autologous CLL cells *ex vivo*. The effects of ADCC were blunted when NK-cells expressing CD16 – which make up approximately 55-75% of total CD3$^-$CD56$^+$ NK-cells herein – were blocked with an anti-CD16 antibody. Additionally, exercise had no effect on AICC against autologous CLL cells, which is unsurprising given that CLL cells in treatment-naïve CLL patients are poorly immunogenic to NK-cells, and solely mobilising immune cells via exercise is unlikely to elicit anti-tumour effects if those cells lack immunogenicity [42]. Taken together these findings indicate that the increase in ADCC was independent of a change in AICC and suggests that the increase was dependent on the preferential mobilisation of NK-cells, specifically the mobilisation of CD3$^-$CD56$^+$CD16$^+$ NK-cells (+254%) – supported by the positive correlation between CD3$^-$CD56$^+$CD16$^+$ NK-cells and ADCC responses. It is important to note that the purity of isolated NK-cells used in ADCC assays was 12% greater in samples collected immediately post-exercise, likely due to NK-cells increasing in proportion (of total lymphocytes) post-exercise (1.6%) compared to pre-exercise (1.0%). Nevertheless, controlling for purity as a confounding variable did not nullify the ADCC responses observed. As NK-cells differentiate, their cytotoxic potential increases [43]. Terminally differentiated NK-cells (CD57$^+$) contain granzymes and perforins [16,27] – important for ADCC mediated elimination of target cells [10] – and are more sensitive to CD16 activation compared to their immature counterparts [27]. We also showed that cycling exercise

induced a preferential mobilisation of mature, cytotoxic $CD3^{-}CD56^{+}CD57^{+}CD16^{+}$ NK-cells (+323%), which also positively correlated with ADCC responses. The mobilisation of mature, cytotoxic NK-cells is consistent with previous findings showing an increase of ~500% in $CD3^{-}CD56^{+}CD57^{+}$ NK-cells following 30-minutes of cycling 15% above lactate threshold (analogous to anaerobic threshold) [25]. Indeed, the mobilisation of mature, cytotoxic NK-cells herein was ~180% less than previously reported. However, the previous study did not include CD16 in their NK-cell phenotyping and recruited healthy, trained cyclists resulting in a greater relative exercise intensity (age predicted heart rate max = 88 ± 4%) [25] compared to our study (age predicted heart rate max = 75 ± 9%).

In a subset of participants, we also assessed rituximab-mediated ADCC when target cells were cultured with autologous time-point matched (i.e. pre- and post-exercise) plasma. We did this to explore a more physiologically relevant milieu containing autologous NK-cells, B-cells and plasma. ADCC with plasma increased by 92% pre- to post-exercise, and a medium effect was observed for greater ADCC with plasma compared to $ADCC_{HI\text{-}FCS}$ pre- and post-exercise (d = 0.61 and d = 0.70, respectively), with a specific trend towards a difference post-exercise (p = 0.069). This medium effect size indicates a meaningful difference, with the non-significant change likely attributed to the smaller sample size (n = 9). The trend towards a difference between ADCC with plasma and $ADCC_{HI\text{-}FCS}$ may be explained by the presence of hormones and cytokines (e.g., cortisol and IFN-γ) following vigorous cycling exercise [44]. Indeed, it is likely that incubating purified NK-cells with serum collected 1-hour post-exercise could enhance ADCC activity further due to reduced cortisol and elevated IFN-γ serum concentrations resulting in enhanced NK-cell activity per cell, as previously reported [44].

Our ADDC assay used NK-cells as effector cells, however, *in vivo*, other immune cells play a role in rituximab's mechanisms-of-action, for example, monocytes and macrophages [45]. Non-classical ($HLA\text{-}DR^{+}CD14^{dim}CD16^{+}$) monocytes and $HLA\text{-}DR^{+}CD14^{+}CD32^{+}$ monocytes were elevated by 147% and 59% post-exercise, respectively, and these cells can initiate ADCC [46]. Thus, the mobilisation of effector monocytes may further enhance rituximab's efficacy *in vivo*. It is important to note here that immunosuppressive MDSCs, were also elevated post-exercise (+70%), and these cells have been shown to inhibit NK-cell mediated ADCC against solid tumour cell lines *in vitro*, attributed to MDSC secretion of nitric oxide [47]. The calcein-release assay used herein allows for only a small number of target cells needed to observe the impact of effector cells. As such, in our assay, the number of target cells was fixed at 5×10^{4} cells/well and were isolated from blood sampled pre-exercise. We posited that the above arguments would have greater, clinically relevant implications if a concomitant increase in CLL cells was observed following cycling exercise, as discussed below.

The mobilisation of $CD5^{+}CD19^{+}$ CLL cells and their subsets in response to cycling exercise in this study is a novel finding not previously described. Indeed, CLL cells possess a similar phenotype to that of healthy B-cells (e.g., CD19) and these cells may increase up to 100% following a bout of vigorous cycling in healthy humans [24]. Specifically, CLL cells increased by 67% herein and these cells are broadly consistent with the 'B1' cell phenotype [18] which increase by 84% in response to vigorous cycling exercise in healthy humans [24] The difference in magnitude of change could be the result of the different relative exercise intensity in the present study (age predicted heart rate max = 75 ± 9%) compared to that reported previously (age predicted heart rate max = 94 ± 4%) [26] as exercise-induced lymphocytosis is intensity-dependent [24,25]. We also assessed whether CLL cells mobilised by cycling exercise were susceptible to rituximab-mediated ADCC, with a 67% increase to $CD5^{+}CD19^{+}CD20^{+}$ CLL cells and no change to the expression of surface CD20. Additionally,

cycling exercise appeared to relocate CLL cells from their protective stromal niches in lymphoid tissue into the blood – typified as $CD20^{+}CD5^{bright}CXCR4^{dim}$ [36,37] – and increased the frequency of CLL cells with prognostic relevance ($CD20^{+}CD49d^{+}$, +72% and $CD20^{+}CD38^{+}$, +66%). In a subset of remissive CLL patients we explored whether a single bout of vigorous intensity cycling could mobilise CLL cells into the blood. All remissive patients included in the study were MRD negative in blood – defined as <1 CLL cell/10,000 leukocytes – with no change in the number of CLL cells observed during vigorous intensity cycling (Supplementary Table 5).

The findings of our study have potentially important clinical implications. For instance, rituximab is effective in achieving complete remission rates in many patients, but it does not induce disease eradication [7,11]. This is because CLL cells are typically diffusely spread across lymphoid and other bodily tissues [12–14] and ADCC resistance in survival niches is thought to be driven by a variety of factors including a suppressive stromal environment that promotes CLL cell survival [15] and an inadequate frequency of $CD56^{dim}$ NK-cells expressing CD16 [16] in lymphoid tissue [17]. Thus, relocating CLL cells from their protective stromal niches into the blood may render these cells more susceptible to the mechanisms-of-action of rituximab. This relocation also suggests that individual bouts of vigorous intensity exercise may have the potential to be employed alongside Bruton's Tyrosine Kinase (BTK) inhibitors, which have been shown to transiently increase lymphocyte frequency, peaking in the blood approximately 4-weeks (+~300%) after therapy in people with CLL [48,49]. The mobilisation of CLL cells also has important implications for patients in CLL remission, specifically, individual bouts of cycling exercise may aid in identifying MRD positivity and may improve the responses to rituximab in a relapse setting. However, preliminary evidence from herein (n = 3) suggests that this may not occur in patients who are MRD negative in blood. Furthermore, the concomitant increase in $CD16^{+}$ NK-cells and our demonstration of enhanced rituximab mediated ADCC provides a compelling rationale for future research to investigate the effects of individual bouts of exercise alongside rituximab therapy *in vivo*. Given that rituximab has a half-life of ~28-days, which is typically the time when an additional treatment cycle commences [50], it is not necessary for exercise bouts to be performed during rituximab infusions, but rather, exercise bouts can be performed by patients away from the clinic in the days and weeks after infusion [51]. Additionally, a subset of treatment-naïve patients herein did not complete the 30-minute prescribed cycling bout and yet were observed to exhibit increased immune cell frequency in blood and enhanced rituximab-mediated ADCC. For example, ADCC pre- and immediately post-exercise was 2.8 ± 8.2% and 11.9 ± 16.7%, respectively, and $CD3^{-}CD56^{+}CD16^{+}$ NK-cell frequency pre- and immediately post-exercise was 94 ± 71 cells/μL and 444 ± 439 cells/μL, respectively (Supplementary Table 3), suggesting that vigorous intensity exercise for ~20-minutes may be sufficient to induce relevant immunomodulation. Collectively, the results of our study suggest that other mAbs used to treat CLL and other haematological cancers – particularly those with neoplastic fractions residing in treatment-resistant niches – may also benefit from individual bouts of vigorous intensity exercise, as recently described [51].

A strength of this study, but a point that could also be considered a limitation, was the inclusion of treatment-naïve participants. This population enabled the examination of the effects of an individual bout of cycling exercise without confounding variables such as anti-CLL therapies – which not only would have interfered in our assays, but may cause leukocytopenia, and anaemia [7] – or disease associated comorbidities, which together may result in greater contra-indications to exercise, such as, cardiac arrhythmias [52]. However, this may be considered a limitation as the effects of exercise on rituximab efficacy was not tested in patients receiving

rituximab who may present with greater immune dysregulation such as reduced cytotoxic potential and altered phenotype of NK-cells [53]. Therefore, it is important for future research to assess the safety, and acceptability of moderate to vigorous intensity exercise in patients undergoing anti-CLL therapy, particularly given that chemo-immunotherapy may result in immunosuppression, which may nullify the immunomodulatory changes that arise in response to individual bouts of exercise.

To summarise, our findings show that an individual bout of vigorous intensity cycling temporarily increased the frequency of $CD3^{-}CD56^{+}CD16^{+}$ NK-cells and $CD5^{+}CD19^{+}CD20^{+}$ CLL cells in the blood of patients with treatment-naïve CLL. Our *ex vivo* investigations demonstrated enhanced rituximab mediated ADCC because of elevated $CD16^{+}$ NK-cells in blood. Thus, exercise could be explored as a means of improving clinical responses in patients receiving rituximab, and/or other anti-CD20 monoclonal antibodies such as, obinutuzumab.

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1.6. Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

1.7. Appendix A. Supplementary materials

Supplementary materials include: (i) supplementary methods for the antibody-dependent cellular cytotoxicity (ADCC) assay procedures for patients with remissive CLL; (ii) supplementary results for ADCC assay changes in treatment-naïve CLL adjusting for covariance; (iii) supplementary results for the characteristics of exercise and ADCC changes in response to exercise in patients with remissive CLL; (iv) supplementary figures; and (v) supplementary tables.

1.8. References

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A single bout of vigorous intensity exercise enhances the efficacy of rituximab against autologous human chronic lymphocytic leukaemia B-cells *ex vivo*

Highlights

- Vigorous intensity exercise increases the frequency of NK-cells and CLL cells in the peripheral blood of patients with treatment-naïve CLL.
- Vigorous intensity exercise improves the efficacy of rituximab against autologous CLL cells in treatment-naïve CLL *ex vivo*.