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COVID-19 vaccines elicit strong cellular immunity and robust clinical protection in CLL

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B cell chronic lymphocytic leukaemia (CLL) is the most common subtype of adult leukaemia and is associated with profound secondary immunodeficiency. SARS-CoV-2 infection has been a significant cause of morbidity and mortality (Mato et al., 2020; Pagano et al., 2021) and immunological responses against SARS-CoV-2 vaccines are impaired (Fendler et al., 2021) in patients with CLL. In particular, reduced rates of seroconversion and antibody titre have been reported (Parry et al., 2021; Greenberger et al., 2021; Herishanu et al., 2022) and associate with reduced serum immunoglobulin level or use of medication such as Bruton tyrosine kinase inhibitors or anti-CD20 antibodies (Parry et al., 2021; Herishanu et al., 2022). However, questions regarding optimal immune protection remain unresolved including the potential for additional vaccine doses to increase seroconversion rate, potential humoral and cellular immune protection against Omicron, and the impact of vaccine delivery on breakthrough infection rate and clinical outcome.

We determined antibody and cellular immune responses after 3rd and 4th vaccine dose in participants of the CLL-VR study together with age-matched healthy donor controls (n=93). Blood samples were taken from 404 patients at a median time of 20 days following 3rd dose, of which 161 (40%) had received the BNT162b2 vaccine (Pfizer/BioNTech) as primary series and 243 (60%) had received the ChAdOx1 vaccine (Oxford/AstraZeneca). Almost all patients (393/404) received an mRNA vaccine for 3rd dose (375: BNT162b2; 18: mRNA-1273). Samples were also collected from 186 patients following the 4th vaccine dose (Table S1). Patients with clinical or serological evidence of prior natural SARS-CoV-2 infection were excluded from analysis.

Spike-specific antibody responses have previously been reported to develop in 66% (322/486) of patients within the CLL-VR study following the first 2 vaccine doses compared to 100% of controls (Parry et al., 2021). This response rate improved to 80% following the 3rd vaccine dose (298/374) ($p<0.0001$) (Figure S1A). Analysis of vaccine subtype received during the first 2 doses showed no difference in seroconversion rate following a heterologous or homologous 3rd dose (ChAdOx1/mRNA response rate 81% [187/230] vs. BNT162b2/mRNA response rate 77% [111/144], $p=0.28$).

However, the seroconversion rate was not increased further after a 4th vaccine (77%; 132/171) indicating that the proportion of patients who develop a spike-specific antibody response following COVID-19 vaccination plateaus after the 3rd vaccine (Figure S1A). Three seronegative patients became available for study following a breakthrough infection and

natural infection also failed to generate spike-specific antibodies, indicating that seronegative patient subgroup are broadly refractory to seroconversion. Regardless of vaccine dose number, a low serum IgM, current BTKi therapy or imminent planned treatment were independent predictors of poor response with an 81% (p=0.003), 90% (p=0.021) and 96% (p=0.027) reduction in probability of response respectively after the 4th dose.

In those patients with a positive antibody response following vaccination, titers increased by 4.5-fold after the 3rd vaccine dose (Geometric mean [GM] 404 AU/ml [95% CI 311-526] vs.1820 AU/ml [95% CI 1340-2480], p<0.0001) and became comparable to values seen within healthy controls following primary series dual vaccination (GM 2317 [95% CI 1191-4508] AU/ml) (Figure S1B). No difference in antibody titer was observed following heterologous or homologous vaccination (ChAdOx1/mRNA GM 2580 [95% CI 1150-5780] vs BNT162b2/mRNA: 1830 [95% CI 526-6340], p=0.72).

Cellular responses were initially assessed by IFNγ-QuantiFERON after second (n=19) and third vaccine dose (n=70). These were robust and comparable with values seen in control donors after two vaccine doses (CLL: 2 doses: 0.25 [IQR 0.08-0.46] IU/ml; 3 doses: 0.15 [IQR 0.03-0.3] IU/ml vs. controls: 2 doses: 0.14 [IQR 0.06-0.36] IU/ml) (Figure S1C). Response was found to be markedly higher after 3rd dose in patients who had a heterologous vaccine course (ChAdOx1/mRNA 0.22 [IQR 0.06- 0.55] IU/ml vs mRNA/mRNA 0.04 [IQR 0.02-0.25] IU/ml; p=0.009).

We next assessed the quality of humoral and cellular vaccine-induced immunity against the Omicron variant that has become globally dominant since its original description in November 2021. Neutralising antibody titers after 3rd vaccine dose were markedly reduced against Omicron compared to ancestral variant but were equivalent in patients and controls (ancestral: CLL GM 1780 [95% CI 969-3280] U/ml vs controls 2600 [95% CI 1423- 4738] U/ml; Omicron: CLL 122 [95% CI 88-170] vs controls 215 U/ml [95% CI 99-465]) (Figure S1D). In contrast, ELISpot analysis of peptide specific pools for ancestral and Omicron showed no difference in the magnitude of the cellular responses amongst vaccinated CLL patients, with a median 246 SFC/10^6 PBMC (IQR 85-679) against ancestral peptides compared to 238 SFC/10^6 against Omicron peptides (IQR 71-725; p=0.33) (Figure S1E). As such, vaccine-induced cellular responses in patients with CLL may provide strong cross-protection against the Omicron variant.

Clinical data on breakthrough infection were collected from the whole cohort on 21 February, 2022. At that point, 491 participants remained in study (7 participants had withdrawn and 2 participants had non-COVID-related mortality). Data were obtained on 486 participants (99%) and the remaining 5 were confirmed to be alive. 79/486 (16%) reported a confirmed COVID-19 infection at least once since vaccination. A further 8 donors were found to be nucleocapsid-specific antibody positive without a history of infection and together 18% (87/486) of participants suffered SARS-CoV-2 breakthrough infection.

We next obtained information on breakthrough infection to assess if correlates of protection might be observed within the CLL-VR cohort. 66 of 486 patients (14%) reported a COVID-19 infection during the 14 months since the first vaccine dose was administered, of which 3 also had a reinfection. 5 infections (7.6%) occurred between January-June 2021 when the Alpha variant was dominant, 22 (33%) between July-December 2021 during the Delta wave and 39 cases (59%) in the last 3 months during Omicron transmission. The proportion of patients requiring hospitalisation during these three phases was 20% (1/5), 32% (7/22) and 7.7% (3/39), respectively (Figure S1F).

Somewhat unexpectedly, patients who were seropositive after the second dose showed a 79% increase in infection rate (n=471; HR 1.79 [95% CI 1.0-3.1]; p=0.046) during median follow up time of 46 (IQR 43-54) weeks. Younger age (p=0.001) and low total serum IgM (p=0.03) were independent predictors for breakthrough infection by multivariate analysis.

These findings reveal the utility and limitations of current COVID-19 vaccines in patients with CLL. Although 3 vaccine doses increase the rate of seroconversion to 80%, it represents a plateau that is not overcome by further vaccine doses or natural infection. As a result, 20% of patients continue to lack any detectable anti-spike response, reflecting the inherent immunodeficiency of CLL and the immunosuppressive impact of CLL-directed therapy. Indeed, hypogammaglobulinaemia and BTKi therapy were also associated with failure of seroconversion in patients following breakthrough infection. This patient group appear resistant to improvement in humoural immunity and require alternative approaches for immune protection, such as prophylactic monoclonal antibody treatment.

However, there were also encouraging findings. Antibody titres after 3 vaccine doses in those patients who did develop an antibody response were comparable with those seen in healthy donors after primary series vaccination. Furthermore, cellular immune responses

were also comparable. Homologous and heterologous vaccination protocols elicited comparable humoral responses although cellular immunity was stronger following ChAdOx1 primary series. A similar finding has been reported in healthy donors and patients with other hematologic malignancies, and suggests that adenoviral-based vaccines may be particularly effective in generating cellular immunity in patients with immune suppression (Collier et al., 2021; Lim et al., 2022). Furthermore, we found neutralization of Omicron was low, although values were broadly equivalent in both CLL participants and controls following a third vaccine dose, whereas cellular responses against Omicron were equivalent to those seen against the ancestral strain amongst vaccinated CLL patients.

The most important consideration in SARS-CoV-2 vaccination is clinical efficacy. Vaccine breakthrough infection occurred in 14% of patients but encouragingly there were no COVID-19-related deaths in this cohort that were recruited at the time of the vaccination roll out. The observed increased risk of infection in seropositive patients is thought likely to reflect differences in social behaviour and population mixing as this group was younger than the group that remained seronegative. However, this observation emphasizes that definition of an immune correlate of protection will be challenging in patients with immune suppression and indicates a need for caution in predicting individual infection risk on the basis of antibody status in the clinic. Hospitalisation rates were high, at 32% for the pre-Omicron variants although falling to 7.7% during the Omicron wave. Monoclonal antibody therapy became available in the community in December 2021 and may have contributed to the reduced rate of hospitalisation, although only 36% of those testing positive during the same period received therapy.

In conclusion, SARS-CoV-2 vaccines are currently providing robust clinical protection for patients with CLL but approximately 20% of patients are refractory to seroconversion and are at increased risk of infection. In contrast, cellular responses after vaccination are comparable with healthy donors and may be critical for preventing severe disease.

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RB, PM designed the study. HP, GM, RB, TR, SH, UA, SD, SS, GP, SP recruited participants. PM, HP wrote the manuscript and HP, GM, JZ, SD, BW, DB verified and analysed the data. TL, GT, PS, AD, DB, HP, AR, NL, SS, SA, AJ, SF JZ, CS, KV, TR performed the experiments. All authors had full access to the data and approved submission of this report.

Declaration of Interests

The authors declare no conflicts of interest

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Supplemental Figure S1. COVID-19vaccinees elicit robust cellular immunity and clinical protection in CLL.

A. Proportion of CLL participants who developed a positive antibody response following 1st (n=267), 2nd (n=486), 3rd (n=374) and 4th (n=171) vaccine dose compared to healthy donors following 1st (n=93), 2nd (n=93) and 3rd dose (n=9). Participants with evidence of natural infection were excluded from analysis. B. Spike-specific antibody titer in participants with a positive antibody response following 2nd dose (n=257) compared to controls (n=85) ($p<0.0001$), and in CLL participants following 3rd dose (n=54) ($p<0.0001$) (Post hoc Dunn analysis). Cut-off for positive response is indicated by dotted line (GM and 95% CI shown). C. IFNγ concentration following whole blood overnight stimulation with SARS-CoV-2 peptide pools is shown in CLL participants after 2nd (n=19) and 3rd vaccine doses (n=70) and controls (n=61) following 2nd dose (median and IQR shown). D. ND50 neutralising antibody titers against viral pseudotypes bearing ancestral, Delta and Omicron spike glycoproteins following 3rd vaccine dose from age-matched controls (n=9) and participants with CLL (n=37) (post hoc Dunn for Delta $p= 0.03$) (GM and 95% CI shown). E. IFN-γ ELISpot assay following 3rd vaccine dose in infection-naïve CLL patients following peptide stimulation with either ancestral or Omicron peptide pools ($p=0.33$) (Wilcoxon) (Median and IQR shown) (n=14). F. Bar chart to show the number and timing of breakthrough infections since first vaccine dose (n=66). Colors indicate dominant viral variants over the sample period. The proportion of infected participants who required hospitalisation is shown by the black line.

Supplementary Table S1. Patient demographics by vaccine dose

		post 2nd vaccine	Post 3rd vaccine	post 4th vaccine
Number of patients		500	404	186
Age (years)	Median	67	67	68
	IQR	60 to 72	62 to 73	63 to 72
	Range	39 to 89	40 to 89	45 to 84
Sex	Men	267 (53%)	216 (54%)	102 (55%)
	Women	233 (47%)	188 (46%)	84 (45%)
Vaccine received (first two doses)	BNT162b2	204 (41%)	161 (40%)	71 (38%)
	ChAdOx1	296 (59%)	243 (60%)	115 (62%)
Vaccine received (third dose)	BNT162b2		375 (93%)	
	mRNA1273		18 (5%)	
	NVX-Co2373		10 (3%)	
	ChAdOx1		1 (0.2%)	
Vaccine received (fourth dose)	BNT162b2			150 (81%)
	mRNA1273			36 (19%)
Time from vaccine dose to blood test (days)	Median	20	20	21
	IQR	17 to 29	17 to 27	17 to 29
	Range	4 to 133	0 to 163	0 to 125
Time since CLL diagnosis (months)	Median	73	77	75
	IQR	34 to 133	36 to 135	37 to 124
	Range	1 to 408	1 to 408	1 to 373
CLL stage at diagnosis	A	429 (86%)	348 (86%)	158 (85%)
	B	30 (6%)	21 (5%)	10 (5%)
	C	41 (8%)	35 (9%)	18 (10%)
Previous treatment	Watch and Wait	279 (56%)	225 (56%)	102 (55%)
	Treatment planned	13 (3%)	10 (2%)	4 (2%)
	1 line	128 (26%)	104 (26%)	46 (25%)
	2 lines	48 (10%)	40 (10%)	23 (12%)
	3+ lines	32 (6%)	25 (6%)	11 (6%)
On BTKi		99 (20%)	82 (20%)	38 (20%)
On venetoclax		21 (4%)	18 (5%)	11 (6%)
Previous chemotherapy		143 (29%)	117 (29%)	56 (30%)
Previous anti-CD20		153 (31%)	125 (31%)	61 (33%)
History of infection	Frequent infections	145 (29%)	114 (28%)	59 (32%)
	Hospitalisation with infection	95 (19%)	72 (18%)	35 (17%)
Prophylactic antibiotics		37 (7%)	31 (8%)	15 (8%)
IVIG		41 (8%)	33 (8%)	23 (12%)
Immunoglobulin deficiency	Number	471	381	174
	IgG (<6g/L)	236 (50%)	188 (49%)	97 (56%)
	IgA (<0.8g/L)	232 (49%)	189 (50%)	88 (51%)
	IgM (<0.5g/L)	177 (38%)	136 (36%)	65 (37%)
*Patients on a delayed vaccine interval for first and second doses.				

Supplementary methods

Study design and participants

Patients with a diagnosis of CLL or small lymphocytic leukaemia (SLL) were recruited to study with no additional exclusion criteria. Informed consent was obtained by remote consultation and work performed under the CIA UPH IRAS approval (REC 20\NW\0240) from North-West and Preston ethics committee and conducted according to the Declaration of Helsinki. The dates and type of SARS-CoV-2 vaccination were obtained with self-reported information on stage and date of CLL diagnosis, CLL treatment and infection history as previously described. Participant demographics can be found in Table S1.

Samples were obtained 2-3 weeks following the second, third and fourth dose of vaccination. Local participants undertook phlebotomy whilst those more distant donated a dried blood spot sample (DBS). 93 healthy donor controls were recruited from local primary care networks (median age 73; (IQR 68-74.5); 56 were female (60%) and 59 received ChAdOx1 primary course and 34 received BNT162b2) .

Roche Elecsys® electrochemiluminescence immunoassay (ECLIA)

Using ECLIA, qualitative IgG/A/M Anti-nucleocapsid protein (NP) antibodies specific to SARS-CoV-2 were detected (COV2, Product code: 09203079190); cut-off index value ≥1.0 considered positive for anti-nucleocapsid antibodies. Using the quantitative ECLIA assay, anti-spike (S) receptor binding domain antibodies were detected (COV2 S, Product code 09289275190) with values ≥0.8 U/ml considered positive.

Dried blood spot ELISA analysis

Dried blood spot (DBS) analysis was carried out as previously described to ascertain the sero-positive rate amongst donors (Cook et al., 2021). IgG, IgA and IgM antibody isotypes against stabilised trimeric SARS-CoV-2 spike glycoprotein are reported with a positive result classed as a ratio of 1 or more.

Serum Immunoglobulin concentration

Quantification of IgG, IgA and IgM was evaluated using the COBAS 6000 (Roche) at the University of Birmingham Clinical Immunology Service as previously described (Parry *et al.*, 2021).

Neutralization assays

HEK293, HEK293T and 293-ACE2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 200mM L-glutamine, 100µg/ml streptomycin and 100 IU/ml penicillin. HEK293T cells were transfected with the appropriate SARS-CoV-2 spike gene expression vector in conjunction with lentiviral vectors p8.91 and pCSFLW using polyethylenimine (PEI, Polysciences, Warrington, USA). HIV (SARS-CoV-2) pseudotype-containing supernatants were harvested 48 hours post-transfection, aliquoted and frozen at -80^{o}C prior to use. The SARS-CoV-2 spike glycoprotein expression constructs for ancestral Hu-1, B.1.617.2 and Omicron have been described previously (Willett et al., 2022). The delta construct bore the following mutations relative to the ancestral Hu-1 sequence (GenBank: MN908947): T19R, G142D, E156del, F157del, R158G, L452R, T478K, D614G, P681R, D950N. 293-ACE2 target cells were maintained in complete DMEM supplemented with 2µg/ml puromycin.

Neutralising activity in each sample was measured by a serial dilution approach. Each sample was serially diluted in triplicate from 1:50 to 1:36,450 in complete DMEM prior to incubation with approximately 1x10^{6} CPS per well of HIV (SARS-CoV-2) pseudotypes, incubated for 1 hour, and plated onto 239-ACE2 target cells. Luciferase activity was quantified after 48-72 hours by the addition of Steadylite Plus chemiluminescence substrate and analysis on a Perkin Elmer EnSight multimode plate reader (Perkin Elmer, Beaconsfield, UK). Antibody titer was then estimated by interpolating the point at which infectivity had been reduced to 50% of the value for the 'no serum' control samples.

QuantiFERON assay

T cell responses were measured by QuantiFERON assay, using the QuantiFERON SARS-CoV-2 assay (Catalogue 626715, QuantiFERON SARS-CoV-2 RUO, Qiagen). 1ml of whole blood was added to the test tubes, including QuantiFERON Nil (negative control), QuantiFERON Mitogen BCTs (positive control) and a QFN SARS CoV-2 Ag2 tube containing epitopes from whole spike that simulate both CD4+ and CD8+ T cells. After 18hr incubation, plasma was retrieved from each tube and an ELISA for IFN-γ release performed (Catalogue number 626410 QuanitFERON ELISA, Qiagen). The concentration of IFN-γ in IU/ml was confirmed after deduction of the QFN-SARS-CoV-2 Nil concentration.

ELISpot assay

250,000 PBMC were incubated overnight with peptide pools containing 15-mer peptides overlapping by 10aa from SARS-CoV-2 spike S1 or S2 domains for either the ancestral

strain or the Omicron variant (PepMix™ SARS-CoV-2 (Spike B.1.1.529 / Omicron) Product Code: PM-SARS2-SMUT08-1
PepMix™ SARS-CoV-2 (Spike Glycoprotein) Product Code: PM-WCPV-S-2
JPT Peptide Technologies, Germany). T cell responses were determined using a Human IFNγ ELISpot PRO kit (3420-3PT Mabtech, Sweden) and plates read using the Bioreader5000 (Bio-Sys, Germany)

Statistical analysis

For comparative analysis, Mann-Whitney U-tests or Spearman rank correlation were performed and antibody data presented either as median or geometric means + 95% confidence intervals. Kruskal-Wallis was performed with post-hoc Dunn's analysis for comparative groups and Wilcoxon's matched-pairs signed rank test for paired responses. Logistic regression of clinical variables was tested for associations with positive antibody response after each vaccine dose. Chi-square analysis was used to compare proportions of responders and Kaplan-Meier for time to first treatment with Gehan-Breslow-Wilcoxon test reported. Analysis was performed using Graphpad prism v9.1.0 for Mac (San Diego, California USA) and SPSS Statistics v27.0 for Windows (Armonk, NY: IBM Corp.)

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