Methods

Study Design

This study was conducted at a single site at Oregon Health and Science University. The protocol was approved by the Oregon Health and Science University Institutional Review Board (OHSU IRB# 21230). All participants gave written informed consent before enrolling in the study. This vaccine arm of the cohort is part of a larger biobank study on human immune response during and following acute viral infection and vaccination against novel viruses. SARS-CoV-2 vaccines were administered through standard procedures as part of the Oregon's COVID-19 Vaccination Plan and not as part of the study. On initial enrollment, demographics, CLL disease characteristics, and treatment details were collected (Table 1), and baseline laboratory values were obtained (Table 2), including SARS-CoV-2 spike antibody titer, serum IgG, a complete blood count, and multicolor flow cytometry measuring immune cell populations (Table 2). One-month following completion of 2-dose mRNA vaccination series, follow up draws were completed and vaccine-specific immune response was evaluated. Study protocol includes additional serology studies at 3 months, 6 months, and 1 year following vaccine.

Participants

Eligible participants were adults 18 years or older with confirmed diagnosis of CLL or small lymphocytic lymphoma (SLL) who did not have a history of previous SARS-CoV-2 infection or vaccination. Subjects were excluded if they had received anti-CD20 therapy within 6 months of enrollment. Prior intravenous immune globulin (IVIG) therapy was not an exclusion criterion.

Healthy subject controls

Age and gender matched controls who received a two-dose mRNA (Pfizer-BioNTech) vaccine in January – February 2021were enrolled in a vaccine study approved by the Oregon Health and Science

University Institutional Review Board. Serum from twelve age/gender matched controls (13 – 28 days following 2nd dose) and PBMCs (247 – 267 days following 2nd dose) from six age/gender matched controls were used for determining RBD and measles specific antibody responses in immunocompetent subjects.

Statistical Analysis

Mean and median lymphocyte panel laboratory findings were determined for each positive and negative antibody response, cellular immune response, and MBC frequency. Group means were compared using an independent t-test, except where applicable and noted, the Satterthwaite test statistic was used instead. Mean, median, and independent group t-tests were also calculated for IgG and ALC values collected at the time of vaccination. The relationship between prior CD20 mAB treatment and current treatment status with antibody, MBC frequency, and cellular immune responses were similarly assessed using a chi-square test, or Fisher's exact, where applicable. All statistical analyses were performed in SAS version 9.4.

Sample processing

After obtaining informed consent, at the time points indicated, 10mL of whole blood was collected for serum (BD Vacutainer® Red Top Serum Tubes) and 40mL of whole blood was collected for PBMCs and plasma (BD Vacutainer® Lavender Top EDTA Tubes). Plasma and serum samples were centrifuged for 10 minutes at 1000 x G, heat inactivated and stored at -20C. PBMCs were isolated and stored in liquid nitrogen until needed.

Antigen-specific plasma endpoint ELISA:

ELISAs were performed as previously described[35], Briefly, ninety-six well ELISA plates (3590, Corning) were coated with 100 μL recombinant RBD protein (Provided by Dr. David Johnson) at a

concentration of 0.5 µL/mL or 50 µL of measles virus antigen (MyBioSource, MBS239121) at a concentration of 4 µg/mL prepared in PBS and the plates were incubated overnight at 4°C. Coating antigen was removed, plates were washed once with PBS-T containing 0.05% Tween (wash buffer) and blocked for 1 hour at RT with 5% milk prepared in PBS-T containing 0.05% Tween (dilution buffer). Plasma in dilution buffer, 100 µL of 1:50 (RBD) or 1:30 (measles) dilution was added to each well. Plasma samples were serially 3-fold diluted in dilution buffer. Plates were incubated at RT for 1 hour. The plates were washed 3 times with wash buffer and 100 µL of 1:3000 dilution of anti-human IgG (H+L) HRP (Novus, NBP1-73319) detection antibody was added and incubated at RT for 1 hour. After rinsing (3X) with wash buffer, 100 µL of colorimetric detection reagent containing 0.4 µg/ml o-phenylenediamine and 0.01% hydrogen peroxide in 0.05 M citrate buffer (pH 5) were added and the reaction was stopped after 20 minutes by the addition of 100 µL 1 M HCl. Optical density (OD) at 492 nm was measured using a CLARIOstar ELISA plate reader. Antibody titers were determined by logarithmic transformation of the linear portion of the curve with an endpoint of 0.1 optical density units.

MBC limiting dilution analysis:

PBMCs were thawed and resuspended in LDA media, RPMI 1640 (Gibco), 1×Anti-Anti (Corning), 1X non-essential amino acids (HyClone), 20 mM HEPES (Thermo Scientific), 50µMβ-ME, and 10% heat-inactivated FBS (VWR). Cells were cultured in serial 2-fold diluted doses (10 wells per dose), starting with 3-5 x 10^5 PBMCs per well at the highest dose. In 96-well round-bottom plates in a final volume of 200µl per well[36]. Cells were incubated with IL-2 (Prospec) 1000U/ml and R848 (InvivoGen) 2.5ug/m.[37] To determine background absorbance values, supernatants were used from 8 wells unstimulated PBMCs only. Plates were incubated at 37 °C and 5% CO^2 for 7 days. Stimulation was determined by running total IgG ELISAs. Antigen-specific MBC frequencies were calculated by assaying LDA supernatants by

antigen-specific ELISAs. MBC precursor frequencies were calculated by the semi-logarithmic plot of the percent of negative cultures versus the cell dose per culture, as previously described [38] and frequencies were calculated as the reciprocal of the cell dilution at which 37% of the cultures were negative for antigen-specific IgG production. Cell doses which yielded 0% negative wells were excluded, since this typically resides outside of the linear range of the curve and artificially reduced the MBC precursor frequency. For subjects with low frequency of antigen-specific antibody secreting cells frequency was determined by number of positive wells divided by the total number of IgG positive secreting wells, multiplied by one million, giving a frequency per million PBMCs stimulated.

Antigen-specific ELISA for LDA:

Ninety-six well half-well ELISA plates (Greiner Bio-one) were coated with 50uL of 0.5 ug/mL antigen in PBS, recombinant RBD protein (provided by Dr. David Johnson) and measles virus antigen (MyBioSource, MBS239121) . Plates were incubated overnight at 4ºC, washed once with PBS-T containing 0.05% Tween (wash buffer) and blocked for 1 hour with 5% milk prepared in PBS-T containing 0.05% Tween (dilution buffer). 20 uL of LDA supernatants were added to each well. Plates were incubated at room temperature (RT) for 1 hour, washed 4 times with wash buffer, and 50 uL of 1:3000 dilution of anti-human IgG-HRP (BD Pharmingen, 555788) detection antibody was added and incubated at RT for 1 hour. Plates were washed 4 times with wash buffer, 50 uL of colorimetric detection reagent containing 0.4 mg/ml o-phenylenediamine and 0.01% hydrogen peroxide in 0.05 M citrate buffer (pH 5) were added and the reaction was stopped after 20 minutes by the addition of 50 uL 1 M HCl. Optical density (OD) at 492 nm was measured using a CLARIOstar ELISA plate reader. Positive wells were determined as wells 2-fold above background (unstimulated PBMC wells).

Spike-specific simulation and intracellular cytokine staining

PBMCs were thawed at 37°C, washed and resuspended in RPMI1640 (Corning) supplemented with 5% FBS (Hyclone), Glutamine (Corning), HEPES (Lonza) and Pen Strep (Gibco). Cells were stimulated at 1 million cells/well in 200 µl in 96 well round bottom plates (Corning) at 37°C/6% CO_2 with 2 peptide pools (peptides 1-90 and 91-181 at 0.5 µg/ml of each peptide) of overlapping (10AA) 17mers representing the SARS-CoV-2 Spike protein (BEI Resources). All conditions contain a final DMSO concentration of 0.045%. Positive control wells were stimulated with 0.04 µg/ml anti-CD3 (HIT3a NA/LE, BD Biosciences) in media containing 0.045% DMSO or incubated with 0.045% DMSO media alone to assess spontaneous production of cytokines. After 1 hour, Brefeldin A (Sigma) was added to a final concentration of 2 µg/ml and stimulations were continued for 5 hours. Intracellular cytokine staining was performed as described previously.[39,40] After stimulation, cells were stained overnight at 4°C with anti-CD8, anti-CD4+ (2ST8.5H10 and L200 BD Biosciences) and Aqua live/dead stain (InVitrogen) in PBS + 1% FBS + 0.1 mg/ml MsIgG. Following overnight incubation, cells were fixed with 2% formaldehyde in PBS. After staining with anti-IFNγ and anti-TNFα (4S.B3 and Mab11 from eBioscience)) in PermWash cells were washed with PermWash, PBS + 1% FBS and fixed with 2% formaldehyde in PBS. Data was acquired on an LSR Fortessa (Becton Dickenson) and analyzed using FlowJo software (Becton Dickenson). Cytokine expression in medium +DMSO alone cultures was subtracted from peptide-stimulated cultures to calculate peptide-specific cytokine expression. Responses to both peptide pools were added together to yield the total frequency of SARS-CoV-2-specific cytokine producing CD4+ and CD8+ T cells.

	N= 16 (median, IQR)
Age	64.5 (60-75)
Sex	
Male	10
Female	6
Race	
White	15
Asian/White	1
Treatment status	
Treatment naive	4
Active treatment	8
Observation after prior treatment	4
Previous lines of treatment (n=12)	1 (0-4)
Current treatment (n=8)	
BCL2 inhibitor + anti-CD20	1
BCL2 inhibitor + BTK inhibitor	1
BTK inhibitor	6
Time since previous anti-CD20 (n=11)	
6-12 months	2
>12 months	9
Previous IVIG (n=6)	
≤ 6 months	0
>6 months	6
IGHV (n=8)	
Mutated	5
Unmutated	3
Baseline IgG (n=10)	466 (85-918)
Baseline ALC (n=14)	2.91 (0.33-87)
Rai stage at diagnosis	
0	9
I	5
II	1
III	0
IV	1

Supplemental table 1. Summary subject demographic table. Summary data for 16 CLL subjects included in this study at the time of enrollment.

			TNFa+IFNg+ Spike-specific response cells per 10^6 T-cells	
Subject ID	Treatment	Visit	CD4	CD8
1	N	Pre	0	0
		Post vaccination	0	0
2	N	Pre	0	0
		Post vaccination	0	**235**
3	C	Pre	**4**	164
		Post vaccination	**126**	79
4	N	Pre	0	**51**
		Post vaccination	0	0
5	C	Pre	**54**	0
		Post vaccination	**60**	**106**
6	C	Post vaccination	**72**	**62**
7	O (6)	Pre	**2**	0
		Post vaccination	**488**	**440**
8	C	Pre	**205**	0
		Post vaccination	**26**	2
9	C	Pre	**12**	**66**
		Post vaccination	**583**	**647**
10	C	Pre	1	0
		Post vaccination	**25**	0
11	N	Pre	**15**	0
		Post vaccination	**220**	**75**
12	O (6-12)	Pre	16	38
		Post vaccination	**451**	**894**
13	C	Pre	0	**51**
		Post vaccination	**48**	40
14	O (>12)	Post vaccination	**701**	0
15	C	Post vaccination	0	101
16	O (>12)	≧Post vaccination	**23**	**31**
	BOLD	2 fold background		

Supplemental table 2. T-cell specific response per subject, pre andpost vaccine. CD4 and CD8 spike-specific T-cell response persubject per 10^6 T-cells. N: Treatment Naïve, C: Currently on treatment, O (6): Observation, last treatment within 6 months, O (6-12): Observation, 6-12 months since last treatment, O (>12): Observation, more than 12 months since last treatment.

	Response										
	Antibody			CD4			CD8			MBC	
	Pos (n=4)	Neg (n=11)		Pos (n=10)	Neg (n=5)		Pos (n=8)	Neg (n=7)		Pos (n=1)	Neg (n=14)
B-cells (CD19+) %											
Mean (SD)	23.3 (39.9)	34.4 (36.2)	p=0.6	23.3 (31.0)	47.5 (43.6)	p=0.2	32.4 (36.0)	30.2 (39.0)	p=0.9	8.01 (NA)	33.1 (36.8)
Median [min, max]	5.1 [0.1, 82.9]	30.3 [0, 96.2]		5.1 [0, 82.9]	61.6 [0.4, 96.2]		19.2 [0.004, 82.9]	3.0 [0, 96.2]		8.01 (NA)	16.7 [0, 96.2]
B-cells (CD19+) #, log[a]											
Mean (SD)	5.6 (3.5)	6.4 (3.9)	p=0.7	5.9 (3.7)	6.6 (4.1)	p=0.7	6.4 (3.8)	6.0 (3.9)	p=0.9	4.09 (NA)	6.4 (3.8)
Median [min, max]	4.1 [3.0, 9.6]	7.9 [0, 10.7]		6.8 [0, 9.9]	8.2 [1.9, 10.7]		7.5 [0, 9.7]	5.5 [1.9, 10.7]		4.09 (NA)	7.9 [0, 10.7]
IGD+27-naive B-cells of B-cell %											
Mean (SD)	29.9 (42.9)	24.6 (32.1)	p=0.8	24.2 (34.4)	29.7 (35.8)	p=0.8	17.1 (31.5)	36.2 (35.7)	p=0.3	93.6 (NA)	21.2 (29.1)
Median [min, max]	10.9 [4.1, 93.6]	11.4 [0, 91.5]		7.5 [0, 93.6]	21.3 [0.09, 91.5]		7.1 [0, 93.6]	24.1 [0, 91.5]		93.6 (NA)	10.7 [0, 91.5]
IGD+27+non-switched B of B-cell %											
Mean (SD)	45.3 (43.8)	40.8 (43.3)	p=0.9	44.9 (44.1)	36.4 (41.4)	p=0.7	49.8 (48.7)	33.2 (34.1)	p=0.5	1.48 (NA)	44.9 (41.9)
Median [min, max]	42.1 [1.5, 95.7]	19.8 [0, 100]		41.4 [0, 100]	19.8 [0.09, 87.8]		52.2 [0, 100]	19.8 [0, 73.2]		1.48 (NA)	43.0 [0, 100]
IGD-27+Switched Memory B of B-cell %											
Mean (SD)	5.3 (8.1)	8.4 (18.0)	p=0.7	2.8 (5.3)	17.3 (25.1)	p=0.3[c]	8.2 (20.6)	6.9 (9.1)	p=0.9	3.77 (NA)	7.9 (16.3)
Median [min, max]	2.0 [0, 17.3]	1.5 [0, 59.2]		0.9 [0, 17.3]	3.6 [0.5, 59.2]		0.3 [0, 59.2]	2.4 [0, 22.5]		3.77 (NA)	1.2 [0, 59.2]
B1 B-cells(CD5+CD19+) %											
Mean (SD)	19.5 (37.7)	32.8 (35.1)	p=0.5	20.5 (28.2)	46.9 (43.6)	p=0.2	30.5 (35.3)	28.0 (37.4)	p=0.9	0.8 (NA)	31.3 (35.4)
Median [min, max]	0.96 [0.08, 76.2]	30.4 [0.006, 95.9]		1.0 [0.006, 76.2]	60.0 [0.2, 95.9]		15.6 [0.007,76.2]	2.2 [0.006, 95.9]		0.8 (NA)	16.3 [0.006, 95.9]
B1 B-cells(CD5+CD19+) #, log[b]											
Mean (SD)	4.6 (4.3)	7.1 (3.5)	p=0.3	6.3 (3.4)	6.4 (4.3)	p=0.96	7.2 (3.2)	5.7 (4.2)	p=0.5	1.79 (NA)	6.8 (3.5)
Median [min, max]	2.4 [1.8, 9.5]	8.0 [1.4, 10.7]		7.3 [1.8, 9.6]	8.2 [1.4, 10.7]		8.2 [1.8, 9.7]	5.1 [1.4, 10.7]		1.79 (NA)	8.0 [1.4, 10.7]

[a]Sample size: antibody (n=3, 9), CD4 (n=7, 5), CD8 (n=6, 6), MBC (n=11)

[b]Sample size: antibody (n=3, 8), CD4 (n=6, 5), CD8 (n=5, 6), MBC (n=10)

[c]Satterthwaite test statistic

Supplemental table 3. Specific B-cell populations (%) stratified by antibody, CD4, CD8, and MBC responders and non-responders.

	Response											
	Antibody			CD4			CD8			MBC		
	Pos (n=4)	Neg (n=12)		Pos (n=10)	Neg (n=6)		Pos (n=8)	Neg (n=8)		Pos (n=1)	Neg (n=15)	
Prior CD20 mAB, n (col %)[a]	3 (75.0)	8 (66.7)	p=1.0	8 (80.0)	3 (50.0)	p=0.3	5 (62.5)	6 (75.0)	p=1.0	0 (0.0)	11 (73.3)	p=0.31
Treatment, n (col %)												
Naïve[a]	1 (25.0)	3 (25.0)	p=1.0	1 (10.0)	3 (50.0)	p=0.12	2 (25.0)	2 (25.0)	p=1.0	0 (0.0)	4 (26.7)	p=1.0
Active[a]	1 (25.0)	7 (58.3)	p=0.57	5 (50.0)	3 (50.0)	p=1.0	3 (37.5)	5 (62.5)	p=0.62	0 (0.0)	8 (53.3)	p=1.0
Observation[a]	2 (50.0)	2 (16.7)	p=0.24	4 (40.0)	0 (0.0)	p=0.23	3 (37.5)	1 (12.5)	p=0.57	1 (100.0)	3 (20.0)	p=0.25
IgG mg/dL, log[b]												
Mean (SD)	6.4 (0.19)	5.6 (0.92)	p=0.15	6.1 (0.76)	5.4 (1.03)	p=0.27	6.5 (0.24)	5.4 (0.84)	**p=0.03**	6.4 (NA)	5.8 (0.90)	
Median [min, max]	6.4 [6.3, 6.7]	5.8 [4.4, 6.8]		6.3 [4.6, 6.8]	5.4 [4.4, 6.4]		6.5 [6.3, 6.8]	5.6 [4.4, 6.4]		6.4 (NA)	6.1 [4.4, 6.8]	
ALC												
Mean (SD)	4.8 (8.17)	17.2 (26.6)	p=0.38	6.1 (9.81)	27.3 (34.3)	p=0.19[c]	6.3 (8.07)	21.8 (31.7)	p=0.22[c]	0.75 (NA)	14.9 (24.3)	
Median [min, max]	0.9 [0.3, 17.0]	4.4 [0.3, 87.0]		1.5 [0.3, 30.0]	13.4 [0.4, 87.0]		2.3 [0.3, 21.0]	3.8 [0.3, 87.0]		0.75 (NA)	2.9 [0.3, 87.0]	

[a]Fisher's exact

[b]IgG sample size: antibody (n=3, 8), CD4 (n=7, 4), CD8 (n=4, 7), MBC (n=1, 10)

[c]Satterthwaite

Supplemental table 4. Clinical markers, prior CD20, current treatment group, serum IgG at baseline as well as ALC at baseline, stratified by antibody, CD4, CD8, and MBC responders and non-responders.