ORIGINAL ARTICLE

Cellular and humoral immune response to SARS-CoV-2 mRNA vaccines in patients treated with either Ibrutinib or Rituximab

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Abstract

Patients treated with B-cell-targeting therapies like Rituximab or Ibrutinib have decreased serological response to various vaccines. In this study, we tested serological and cellular response to SARS-CoV-2 mRNA vaccines in 16 patients treated with Ibrutinib, 16 treated with maintenance Rituximab, 18 patients with chronic lymphocytic leukaemia (CLL) with watch and wait status and 21 healthy volunteers. In comparison with the healthy volunteers, where serological response was achieved by 100% subjects, patients on B-cell-targeting therapy (Ibrutinib and Rituximab) had their response dramatically impaired. The serological response was achieved in 0% of Rituximab treated, 18% of Ibrutinib treated and 50% of untreated CLL patients. Cell-mediated immunity analysed by the whole blood Interferon-γ Release immune Assay developed in 80% of healthy controls, 62% of Rituximab treated, 75% of Ibrutinib treated and 55% of untreated CLL patients. The probability of cell-mediated immune response development negatively correlates with disease burden mainly in CLL patients. Our study shows that even though the serological response to SARS-CoV-2 vaccine is severely impaired in patients treated with B-cell-targeting therapy, the majority of these patients develop sufficient cell-mediated immunity. The vaccination of these patients therefore might be meaningful in terms of protection against SARS-CoV-2 infection.

Keywords SARS-COV-2 · Vaccination · Ibrutinib · Rituximab · Cellular immune response

Abbreviations

BTKi	Bruton tyrosine kinase inhibitor
CBC	Complete blood count
CLL	Chronic lymphocytic leukaemia
mRNA	Messenger ribonucleic acid
mAbs	Monoclonal antibodies
SARS-COV-2	Severe acute respiratory syndrome coronavirus 2
RB	Rituximab–Bendamustine
RFC	Rituximab–Fludarabine–Cyclophospha-mide
MCL	Mantle cell lymphoma
FL	Follicular lymphoma
R-CHOP	Rituximab and Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone
APL	Acute promyelocytic leukaemia
AML	Acute myeloid leukaemia
HL	Hodgkin lymphoma
DLBCL	Diffuse large B-cell lymphoma
WaW	Watch and wait

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Introduction

Haematological malignancies induce immune disturbances. [1] The immune defects are commonly worsened by treatment with Fludarabine, Rituximab and other agents that induce long-term paralysis of the immune system. [2, 3] Those patients not

only have decreased immune response against infections, but also their ability to develop sufficient serological response to vaccination is decreased. This immune deficiency has been well documented in patients treated with B-cell-targeting therapies (either Rituximab or Ibrutinib). The mechanism of action of both drugs is considerably different. [4] Rituximab depletes CD20 expressing B cells by inducing antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity and apoptosis by down-regulation of anti-apoptotic proteins. [5] As the CD20 is expressed only on B cells, but not on stem cells or mature plasma cells, the treatment preserves established humoral immunity, whereas the capacity to generate humoral immune response to recall antigens is impaired [4]. Ibrutinib inhibits B-cell proliferation and survival by irreversibly binding the protein Bruton's tyrosine kinase. The treatment promotes apoptosis, inhibits proliferation and prevents CLL cells from responding to survival stimuli [6, 7]. The treatment allows for partial reconstitution of normal B cells and humoral immunity in patients with CLL; nevertheless, the humoral response to vaccines is compromised. [8–12]

Even though the mechanisms of action of Ibrutinib and Rituximab differ, both groups of patients show insufficient serological response to both bacterial (pneumococcal) and viral (hepatitis B, influenza) vaccines. [8–11, 13–15]

In this study, both serological and cell-mediated immune response to SARS-CoV-2 mRNA vaccines [16, 17] in patients treated with B-cell-targeting therapy was investigated. Based on previous research, we anticipated the impairment of serological response to vaccinations. [18, 19]

In addition to antibodies, the vaccine generates virus-specific T cells that are required for protective immunity to SARS-CoV-2 and that might play crucial role in patients with B-cell-targeting therapies. [20–23] Even if both Rituximab and Ibrutinib target primarily B cells, they might have a minor impact on T cells, as well. Ibrutinib restores both T-cell number and function in CLL patients by binding on interleukin-2-inducible kinase (ITK) and by reducing the expression of exhaustion and inhibitory markers such as PD-1 (programmed death 1) and CTLA-4 (cytotoxic T-lymphocyte antigen 4) [24]. The ITK binding subverts Th2 immunity thereby potentiating Th1-based immune responses. [25]

The capacity of the vaccine to generate cell-mediated immunity in the patients treated with either Ibrutinib or Rituximab was yet to be systematically investigated.

Material and methods

Population

71 adult subjects were recruited into the retrospective cohort study (age 26–86 years, median 69 years). All subjects

signed informed consent approved by local ethical authorities (Ethical Committee of the Faculty Hospital Kralovske Vinohrady, decision EK-VP/24/0/2021 dated 5 May 2021). All subjects filled in a questionnaire focused on side effects of vaccination and underwent blood sampling for CBC with differential, blood biochemistry, serology testing for COVID-19, flow cytometry analysis and QuantiFERON analysis. Further patient data are described in Results section.

Intervention

All subjects completed SARS-CoV-2 (64 Comirnaty by Pfizer/BioNTech or 7 Spikevax by Moderna) vaccination. Median time between the last vaccine dose and study examination was 36 days. The time did not significantly differ between groups of patients.

Comparison

The study included 16 patients treated with Ibrutinib (age 59–78 years, median 72 years), 16 patients treated with maintenance Rituximab (age 38–86 years, median 64 years), 18 patients with yet untreated, active chronic lymphocytic leukaemia—watch and wait status (age 52–80 years, median 71 years), and 21 healthy vaccinated volunteers (age 26–81 years, median 57 years) as the comparison group.

Outcome

Serology

Antibody response was measured in the clinical laboratory using a commercial sandwich immunoassay, and IgG antibodies to the Spike protein were measured by the quantitative Atellica IM SARS-CoV-2 IgG (sCOVG) (REF 11,207,386) test in a Siemens Atellica Solution analyser (Siemens Healthcare GmbH, Germany).

Cellular immunity

SARS-CoV-2-specific T-cell response was assessed in the clinical immunology laboratory by a whole blood Interferon-Gamma Release immune Assay (IGRA) that uses two Qiagen proprietary mixes of SARS-CoV-2 S protein (Ag.1 and Ag.2) selected to activate both CD4$^+$ and CD8$^+$ T cells, following manufacturer's instructions. Briefly, venous blood samples were collected directly into the QuantiFERON® tubes containing Spike peptides as well as positive and negative controls. Whole blood was incubated at 37 °C for 16–24 h and centrifuged to separate plasma. IFN-γ (IU/ml)

was measured in these plasma samples using ELISA (QuantiFERON Human IFN-γ SARS-CoV-2, Qiagen) test.

Flow cytometry

Fresh peripheral blood was analysed by flow cytometry. Surface staining was performed with the following mAbs: anti-CD4 (clone OKT4), anti-CD8 (clone RPA-T8), anti-CD19 (HIB19), anti-CD45RO (clone UCHL1) and anti-CD69 (clone FN50) were purchased from Thermo Fisher Scientific; anti-CD161 (clone HP-3G10) and anti-TCR Vα7.2 (clone 3C10) were purchased from Sony Biotechnology, and anti-CD3 (clone UCHT1) was purchased from BD Biosciences. The cell populations were defined as follows. T cells: $CD3^+CD19^-$; B cells: $CD3^-CD19^+$, NK cells: $CD3^-CD19^-CD161^+$, MAIT cells: $CD3^+CD161^{++}TCRV\alpha7.2^+$ (Supplementary data). Cells were acquired with a Navios EX Flow Cytometer (Beckman Coulter) and analysed using Kaluza Analysis Software (Beckman Coulter).

Time

The subjects were recruited into the study between May 2021 and August 2021.

Statistical analysis

The normal distribution assumption of the samples was tested by Kolmogorov–Smirnov and Shapiro–Wilk tests. [26] According to these tests of normality, the normal distribution of the values enabling the use of parametric tests was found in following parameters: Age, Time last vaccine—examination, % MAIT cells, MAIT cells $\times 10^9$/L. Levene's 2-tailed test for equality of variances was then used. The value ≤ 0.05 were considered statistically significant.

For all other parameters (Time diagnosis—examination, Time the last chemotherapy—examination, Time the start of Ibrutinib/anti-CD20—examination, Lymphocytes $\times 10^9$/L, % T cells, T cells $\times 10^9$/L, % B cells, B cells $\times 10^9$/L, % NK cells, NK cells $\times 10^9$/L, % MAIT cells, MAIT cells $\times 10^9$/L) was the hypothesis of normal distribution rejected (tested by Kolmogorov–Smirnov and Shapiro–Wilk tests) and non-parametric test (Mann–Whitney U Test) was thus used to retain or reject the null hypothesis. The value ≤ 0.05 was considered statistically significant.

Results

The vaccinated subjects

71 subjects with completed SARS-CoV-2 vaccination were recruited: 21 healthy volunteers, 18 patients with yet untreated CLL, 16 patients treated with Ibrutinib and 16 patients treated with Rituximab (Table 1).

The "healthy volunteers" group contained 7 disease-free haematological patients treated more than 5 years ago with curative intent for curable diagnosis (APL, AML, HL, DLBCL).

The Ibrutinib group contained 15 CLL and 1 MCL patients. The majority of the patients previously received several lines of cytotoxic therapies (mainly RB and RFC) and the Ibrutinib represented 2.5th line of treatment (median).

The Rituximab group contained 13 patients with FL and 3 patients with MCL. In all of the patients, the Rituximab was maintenance therapy of the first line treatment (mainly R-CHOP and RB). The median time between last dose of chemotherapy and the study examination was 16 months in this group.

The flow cytometry analysis revealed significant differences in the groups reflecting the original disease activity and the effect of the treatments. Obviously, the B-cell count was the most striking difference among the groups.

All 71 patients completed the vaccination with mRNA vaccines, either Pfizer/BioNTech (64 subjects) or Moderna (7 subjects). The time between the last vaccine and study examination was shorter in Rituximab group but did not differ among other groups. The vaccines induced mild either local (typically local pain) or general (typically fatigue) side effects in the majority of the subjects in similar frequency within all groups. None of the subjects developed serious side effects.

Humoral response to the vaccination

The vaccine induced serological response (defined as S-anti-SARS-CoV-2 IgG exceeding cut-off value according to laboratory practice) in 21 of 21 studied healthy subjects. Expectedly, none of maintenance Rituximab-treated patients developed serological response. The response rate was also very low in Ibrutinib group (3 of 16). The serological response was observed in 9 of 18 subjects in "WaW CLL" group (Fig. 1A).

Table 1 Demographic, clinical, epidemiologic and immunologic characteristics of enrolled subjects

	Healthy	WaW CLL	Rituximab	Ibrutinib
	Demography			
Males/Females	7 + 14	11 + 7	6 + 10	8 + 8
Age (years; median; range)	57 (26–81)	71 (52–80)	64 (38–86)	72 (59–78)
Time diagnosis—examination (months; median; range)	NA	97 (0–206)	23 (14–44)	117 (17–182)
Haematologic disease	NA	18 CLL	13 FL	15 CLL
			3 MCL	1 MCL
Time start of B depletion therapy—examination (months; median; range)	NA	NA	14 (6—29)	18 (4—51)
Time the last cytotoxic treatment—examination (months; median; range)	NA	NA	16 (9—31)	33 (0—93)
Previous lines of treatment	NA	NA	1 (1–1)	2.5 (1–6)
	The immune system			
Total lymphocytes (cellsx10exp3/uL); (median; range)	1.67 (0.8–2.86)	30.03 (1.23 – 233.09)	1.49 (0.66–2.92)	3.34 (0.84 – 257.51)
Total T cells (cellsx10exp3/uL); (median; range)	1.15 (0,24—2,19)	2.33 (0.67 – 8.87)	1.05 (0.02 – 2.16)	1.39 (0.22 – 3.47)
Total B cells (cellsx10exp3/uL); (median; range)	0.1 (0.01 – 0.31)	26.47 (0.07 – 218.87)	0.001 (0.00007 – 0.06)	0.89 (0.01 – 242.49)
Total NK cells (cellsx10exp3/uL); (median; range)	0.02 (0.0007 – 0.09)	0.14 (0.001 – 1.57)	0.01 (0.001 – 0.11)	0.05 (0.03 – 0.24)
% CD4 + /CD3 + (range)	61.8 (29.15 – 78.45)	54.27 (34.91 – 75.57)	37.84 (10.87 – 80.57)	57.7 (21.58 – 80.85)
Total IgM (G/L); (median; range)	0.88 (0.58 – 1.7)	0.37 (0.08 – 1.22)	0.37 (0.08 – 0.84)	0.28 (0.09 – 0.96)
Total IgG (G/L); (median; range)	10.69 (6.9 – 20.05)	7.59 (3.38 – 12.11)	7.66 (3.26 – 11.37)	6.5 (2.34 – 14.11)
Total IgA (G/L); (median; range)	2.06 (0.15 – 3.83)	1.21 (0.15 -3.5)	1.33 (0.23 -2.73)	0.77 (0.15 – 5.78)
	COVID-19			
Previous COVID-19	9 / 21 (42%)	1 / 18 (5%)	0	0
Vaccine (company)	4 Moderna	1 Moderna	1 Moderna	1 Moderna
	17 Pfizer-BioNTech	17 Pfizer-BioNTech	15 Pfizer-BioNTech	15 Pfizer-BioNTech
Time the last vaccine—examination (days; median; range)	48.5 (5—123)	35 (6—89)	19 (3—67)	45 (0—111)
Mild local side effects of vaccination	9 / 21 (42%)	6 / 18 (33%)	10 / 16 (62%)	10 / 16 (62%)
Mild general side effects of vaccination	11 / 21 (52%)	3 / 18 (16%)	3 / 16 (18%)	3 / 16 (18%)

FL—follicular lymphoma; MCL—mantle cell lymphoma, WaW—watch and wait, CLL—chronic lymphocytic leukaemia

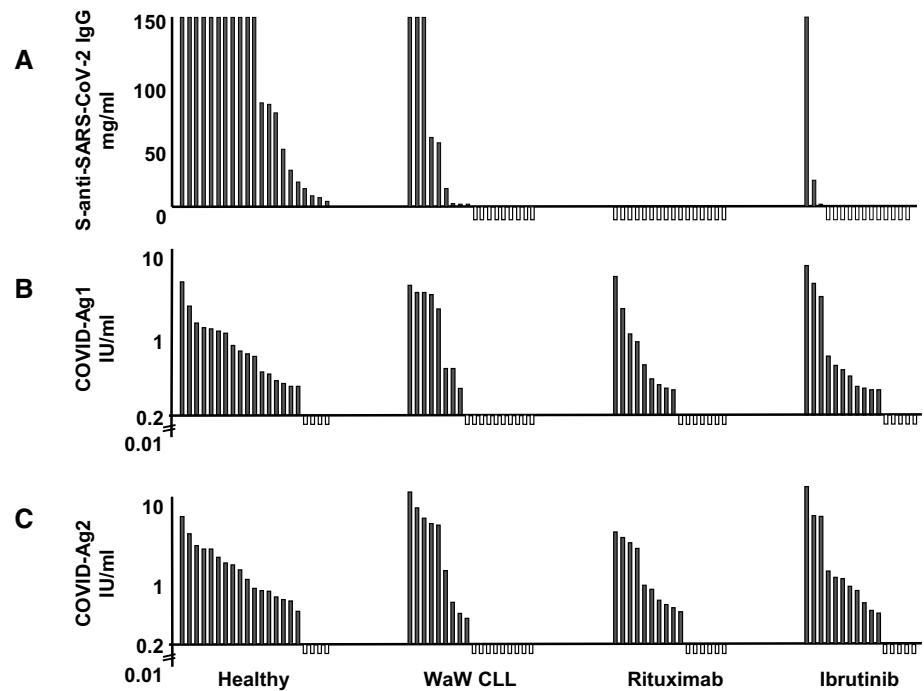## Cellular response to the vaccination

The cellular response was assessed by using a commercial QuantiFERON SARS-CoV-2 kit by Qiagen. The kit analyses CD4+ (Ag1) and CD4+/CD8+ (Ag2) specific immunity upon to the stimulation of whole blood with the mix of SARS-CoV-2 peptides. Using the recommended cut-off of 0.2 IU/ml, the positive response of CD4+ cells was detected in 80% (17/21) of the healthy subjects, 44% (8/18) subjects in the "WaW CLL" group, 56% (9/16) in the Rituximab and 68% (11/16) in the Ibrutinib group (Fig. 1B).

In agreement with a previous report [27], the Ag1 and Ag2 response showed a high concordance rate. Using the cut-off of 0.2 IU/ml, the positive response of both CD4+ and CD8+ cells was detected in 80% (17/21) of the healthy subjects, 50% (9/18) subjects in the "WaW CLL" group, 62% (10/16) in the Rituximab and 68% (11/16) in the Ibrutinib group (Fig. 1C).

Positive cellular response defined as positivity of either Ag1 or Ag2 or both was achieved in 80% (17/21) of the healthy subjects, 55% (10/18) subjects in the "WaW CLL" group,

Fig. 1 Humoral and cellular immunity to SARS-CoV-2 mRNA vaccine

62% (10/16) in the Rituximab and 75% (12/16) in the Ibrutinib group.

Factors associated with positive cellular response to SARS-CoV-2 mRNA vaccines

The study also tested factors that predict positive cellular response to the SARS-CoV-2 vaccine. The subjects were divided according to the cellular response to the negative ("Cellular negative") and positive ("Cellular positive") groups and compared in various clinical and laboratory parameters. Due to the low sample size, the comparison of the "Cellular negative" versus "Cellular positive" patients was performed not only within WaW, Rituximab and Ibrutinib groups separately, but also in a pooled analysis. Data of Rituximab and Ibrutinib patients were pooled, and data of CLL patients from Ibrutinib and WaW groups were analysed as CLL group, etc. (Table 2).

The pooled data of all patients showed that positive cellular response to SARS-CoV-2 vaccine was associated with younger age (72 years in "Cellular negative" versus 66 years in "Cellular positive" subjects, p = 0.01). The time between vaccination and study examination was significantly shorter in "Cellular positive" subjects across various subject groups (Table 2).

The flow cytometry analysis showed that responding subjects had lower total lymphocyte count and lower percentage of B cells at the expense of the percentage of T cells. Unlike the absolute cellularity of T cells, which was identical in both groups (Cellular positive and Cellular negative, respectively), the total amount of B cells was significantly higher in "Cellular negative" subjects in Ibrutinib-treated and CLL patients (Table 2).

Discussion

The pandemic of SARS-CoV-2 killed nearly 5 million of people worldwide and has significant impact on the lives of the whole planet. The most powerful weapon against the pandemic is the vaccination that significantly reduces the mortality and morbidity caused by the virus. [28] The effect of the vaccination is routinely monitored by the examination of the antibodies (anti-Spike protein). Immunocompromised and haematologic patients (mainly those receiving B cell-depleting therapies) are unable to produce antibodies, thus making the assessment of vaccination efficacy complicated. This was confirmed by our work that showed that none of anti-CD20-treated patients and only 18% of Ibrutinib-treated patients (3 from 16) produced anti-S antibodies. This data is identical with what has been observed by Herishanu and colleagues (none of the patients exposed to anti-CD20 antibodies < 12 months before vaccination and 16% patients treated with BTKi serologically responded) [19] and similar to data of Roeker and colleagues (32% of the patients exposed to anti-CD20 antibodies < 12 months before vaccination and 32% patients treated with BTKi serologically responded). [18]

Table 2 Factors associated with positive cellular response to the m-RNA vaccine

Cellular negative versus Cellular positive (≥ 0.2 IU/ml). Median value + p value if significant

	Rituximab	Ibrutinib	WaW	CD20 + Ibr	CD20 + Ibr + WaW	All subjects	CLL
Age (years; median)	71 vs 64	69 vs 73	72 vs 73	69 vs 68	72 vs 69	72 vs 66 (0.01)	72 vs 73
Time last vaccine—examination (days; median)	38 vs 16	78 vs 39	42 vs 29	45 vs 31	45 vs 30 (0.02)	45 vs 35	46 vs 36
Time diagnosis—examination (days; median)	610 vs 787	2698 vs 3618	1999 vs 3330	693 vs 1449	896 vs 1666	NA	2698 vs 3618
Time the last chemotherapy—examination (days; median)	462 vs 640	1065 vs 1400	NA	521 vs 929	NA	NA	NA
Time the start of Ibrutinib/anti-CD20—examination (days; median)	390 vs 494	501 vs 626	NA	422 vs 573	NA	NA	NA
Lymphocytes (10exp9/L; median)	0.9 vs 1.7	65.4 vs 2.2 (0.01)	50.6 vs 30	1.7 vs 2	13.1 vs 2.5 (0.02)	3.3 vs 2.1 (0.01)	50.5 vs 7.27 (0.01)
% T cells/ lymphocytes (median)	77.7, vs 80.7	4.4 vs 48.8 (0.05)	4.7 vs 12.9	57.6 vs 73.6	7.9 vs 51.8 (0.05)	14.8 vs 65.6 (0.009)	4.6 vs 27.75 (0.006)
T cells (10exp9/L; median)	0.7 vs 1.2	2.0 vs 1.0	2.2 vs 2.7	0.9 vs 1.2	1.8 vs 1.8	1.7 vs 1.2	2.09 vs 2.11
% B cells/ lymphocytes (median)	0.1 vs 0.1	88.9 vs 23.5 (0.04)	93.9 vs 69.7	1.7 vs 2.8	80.8 vs 13.6 (0.03)	55.0 vs 8.2 (0.01)	93.6 vs 47.6 (0.001)
B cells (10exp9/L; median)	0 vs 0	61.5 vs 0.41 (0.02)	47.5 vs 26.4	0.01 vs 0.02	10.2 vs 0.2 (0.02)	1.8 vs 0.2 (0.01)	47.5 vs 4.5 (0.01)
% NK cells/ lymphocytes (median)	1 vs 0.6	0.2 vs 1.8	0.7 vs 0.6	0.8 vs 0.8	0.8 vs 0.7	1 vs 0.8	0.37 vs 0.76
NK cells (10exp9/L; median)	0.09 vs 0.14	0.078 vs 0.048	0.476 vs 0.141	0.015 vs 0.017	0.043 vs 0.049 (0.03)	0.043 vs 0.026 (0.01)	0.18 vs 0.11 (0.02)
% MAIT cells/ lymphocytes (median)	1.4 vs 0.6	0.5 vs 0.7	0.8 vs 1.1	1.0 vs 0.6	0.9 vs 1.1	1.1 vs 1.0	0.74 vs 0.89
MAIT cells (10exp9/L; median)	0.008 vs 0.008	0.01 vs 0.07	0.026 vs 0.043	0.01 vs 0.08	0.014 vs 0.015	0.015 vs 0.014	0.01 vs 0.01

vs: versus, MAIT: Mucosal-Associated Invariant T cells

Experimental data, however, show that an important role in the defence against viruses including SARS-CoV-2 is played by T cells that are also abundantly generated by the vaccines. [20–23] To analyse the cellular immune response, we have chosen commercially available whole blood Interferon-γ Release immune Assay that uses mixes of SARS-CoV-2 S proteins selected to activate both CD4^{+} and CD8^{+} T cells. This test showed certain concordance with anti-S antibody response in healthy subjects. [27, 29] Our analysis showed that significant fraction of both

Ibrutinib- and Rituximab-treated patients develop cellular response to the vaccination (75% and 62%, respectively). This is not completely surprising, as similar data were reported either by case reports or by small patient series. For example, Hueso and colleagues [30] analysed T-cell response by ELISPOT in 3 anti-CD20-treated patients and reported reactivity in all of them. Malard and colleagues [31] used ELISPOT to show that the haematological patients develop a T-cell response to the BNT162b2 vaccine. The anti-B-cell treatment did not affect the probability of developing the response. One should emphasize that the studies vary by methods used to analyse T-cell response and peptide pool selection. Their comparison is complicated, but the similarities are striking.

The probability of developing the cellular response increased with higher percentage of T cells. It seems intuitive, as larger T-cell pool would have higher probability of containing SARS-CoV-2-specific T cells and generating IFN-γ upon specific stimulation. However, the cellular response to vaccination does not correlate with absolute amount of T cells. Instead, it inversely correlates with the amount of B cells in Ibrutinib-treated and CLL groups suggesting that the generation of the cellular immune response to vaccine is hampered by the disease burden. It is not surprising as malignant B cells (typically CLL) inhibit cellular immunity. [32] Moreover, the analysis of CLL patients (WaW + Ibrutinib) showed that the cellular immune response inversely correlates with the fraction and total amount of CD69 positive B cells (not shown). CD69 overexpression significantly correlates with disease burden and prognosis (advanced Rai stages, larger lymphadenopathy, splenomegaly, shorter progression-free survival and overall survival). [33] Finally, common markers of disease activity, such as LDH and beta-2-microglobulin, were both increased (not statistically significantly) in non-responding CLL patients.

An interesting observation is provided by the comparison of WaW and Ibrutinib-treated patients. The proportion of responding patients is slightly higher in the Ibrutinib group suggesting that Ibrutinib can overcome the negative impact of CLL on the cellular immunity. It has indeed been shown that Ibrutinib restores T-cell number and function in CLL patients by various mechanisms including targeting of ITK, PD-1 or CTLA-4. [24, 34]

Our analysis across practically all groups showed that responding patients have shorter time from vaccination to analysis than non-responding patients. It suggested that the cellular response weakens through the time as it was raised by epidemiologic and immunological studies. [35–38]

In conclusion, our work shows that humoral immune response to SARS-CoV-2 vaccine is severely impaired in patients treated with Rituximab and Ibrutinib. Significant fraction of these patients mount cellular immune response

to the vaccine. The capacity to develop cellular response is compromised by disease burden in CLL patients.

The immune response was evaluated 1–192 days (median 38 days) after the second dose of mRNA vaccine. Each bar corresponds to one subject in the study. (A).

Serological response to vaccination is defined as S-anti-SARS-CoV-2 IgG exceeding cut-off index < 1,00, with uppermost value measurable in clinical laboratory being cut-off index 150. According to internal laboratory standard cut-off value 1 is equal to 1,00U/ml. Negative subjects are schematically marked by white colour below the line.

IFN-γ response (IU/ml) to SARS-CoV-2 peptides by $CD4^+$ T cells (B) and $CD4^+$ plus $CD8^+$ T cells (C) was measured by ELISA. Cut-off value of 0.2 IU/ml was used for determination of positivity, with negative subjects schematically marked by white colour below the line.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethical approval The research has been performed in accordance with the Declaration of Helsinki and was approved by local ethical authorities (Ethical Committee of the Faculty Hospital Kralovske Vinohrady, decision EK-VP/24/0/2021 dated 5th May 2021).

Consent of publication The work has not been published previously, and it is not under consideration for publication elsewhere.

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