CLINICAL SCIENCE

Immunogenicity and safety of a fourth COVID-19 vaccination in rituximab-treated patients: an open-label extension study

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ABSTRACT

Objectives Patients under rituximab therapy are at high risk for a severe COVID-19 disease course. Humoral immune responses to SARS-CoV-2 vaccination are vastly diminished in B-cell-depleted patients, even after a third vaccine dose. However, it remains unclear whether these patients benefit from a fourth vaccination and whether continued rituximab therapy affects antibody development.

Methods In this open-label extension trial, 37 rituximab-treated patients who received a third dose with either a vector or mRNA-based vaccine were vaccinated a fourth time with an mRNA-based vaccine (mRNA-1273 or BNT162b2). Key endpoints included the humoral and cellular immune response as well as safety after a fourth vaccination.

Results The number of patients who seroconverted increased from 12/36 (33%) to 21/36 (58%) following the fourth COVID-19 vaccination. In patients with detectable antibodies to the spike protein’s receptor-binding domain (median: 8.0 binding antibody units (BAU)/mL (quartiles: 0.4; 13.8)), elevated levels were observed after the fourth vaccination (134.0 BAU/mL (quartiles: 25.5; 1026.0)). Seroconversion and antibody increase were strongly diminished in patients who received rituximab treatment between the third and the fourth vaccination. The cellular immune response declined 12 weeks after the third vaccination, but could only be slightly enhanced by a fourth vaccination. No unexpected safety signals were detected, one serious adverse event not related to vaccination occurred.

Conclusions A fourth vaccine dose is immunogenic in a fraction of rituximab-treated patients. Continuation of rituximab treatment reduced humoral immune response, suggesting that rituximab affects a second booster vaccination. It might therefore be considered to postpone rituximab treatment in clinically stable patients.

Trial registration number 2021-002348-57.

INTRODUCTION

COVID-19 vaccination is a critical component in the management of the COVID-19 pandemic. Despite the recent variants of concern (VOC) Omicron and Delta, the currently available vaccines are still effective in preventing severe disease courses and death, although at a reduced level compared with preceding variants.1–2 Most importantly, due to mutations in the spike protein, VOC exhibit a higher degree of vaccine evasion, resulting in higher repertoire of antibodies required for effective virus neutralisation.3–6 However, booster vaccinations improve protection against Delta and Omicron variants.7–8 Patients under immunosuppressive therapy with rituximab, a B-cell-depleting antibody against the CD20 surface antigen, have impaired humoral responses after primary vaccination, depending on the number of detectable peripheral B-cells.9–13 A booster dose given to these patients improved humoral responses, nonetheless overall seroconversion rate and antibody titers were significantly
lower than in healthy individuals. Additionally, therapy with rituximab itself is associated with worse COVID-19 outcomes, such as the requirement of invasive mechanical ventilation or mortality. Therefore, improving the level of protection against COVID-19 in this patient population is of utmost importance. The American College of Rheumatology guidelines suggest discussing optimal timing of dosing and vaccination prior to rituximab treatment. The EULAR recommends that rituximab or any other B-cell-depleting therapy should be scheduled in a way to optimise vaccine immunogenicity. However, due to a lack of high-level evidence, no specific recommendations are given. Currently, no data are available for the immunogenicity or safety of a fourth vaccination or how the continuation of rituximab treatment affects vaccine responses. We, therefore, investigated the immunogenicity and safety of a fourth vaccine dose in rituximab-treated patients and analysed the effect of continued rituximab treatment on vaccine immunogenicity.

METHODS

Trial design and participants
In this prospective open-label extension study, rituximab-treated patients received a fourth dose (second booster) with an mRNA-based vaccine. In the main study, patients who did not seroconvert after primary vaccination with an mRNA-based vaccine had received their third vaccination with either an mRNA (BNT162b2, Pfizer–BioNTech or mRNA-1273, Moderna) or a vector-based vaccine (ChAdOx1 nCoV-19, Oxford–AstraZeneca). In the current trial, an mRNA-based vaccine was used as the fourth vaccination, in accordance with their primary vaccination (figure 1A). The most important exclusion criteria were previous COVID-19 infection and known allergies to vaccine components. Medical history regarding SARS-CoV-2 infections was verified before enrolment. Details can be found in the supplementary study protocol. The trial was registered on Eudra-CT (Number 2021-002348-57).

Interventions
Patients included in the main trial were invited to a fourth vaccination 12 weeks after the third dose. At screening, concomitant medications, demographics and hypersensitivity reactions to previous SARS-CoV-2 vaccines were recorded. The vaccination was applied at the baseline visit. Immunogenicity and safety were assessed at week 1 and week 4 after vaccination. Serum samples obtained during screening visit, as well as visits 3 and 4 were stored below −70°C at the Biobank of the Medical University of Vienna, a centralised facility for the preparation and storage of biomaterial with certified quality management (certified according to International Organization for Standardization (ISO) 9001:2015). Peripheral blood mononuclear cells (PBMCs) were isolated at screening and visit three by density gradient centrifugation and stored in the vapour phase of liquid nitrogen.

The vaccination compound was open label and selected according to the primary vaccination series. Vaccination with mRNA-1273 was carried out using the full dose (100 µg).

Assessment
Study outcomes included seroconversion rates, SARS-CoV-2 antibody levels at week 4 (overall and stratified for patients with different numbers of peripheral B-cells) and cellular immune responses at week 1. T-lymphocyte restimulation potential to SARS-CoV-2 antigens was assessed before and 1 week after the fourth vaccination. Laboratory assessors were blinded to patient
characteristics. Safety is presented as solicited adverse events over the first 7 days as reported by the patients using a paper-based diary. Adverse events and changes in the immunosuppressive treatment were assessed over a period of 28 days. Antibodies against platelet factor 4 (PF4) were routinely assessed at week 1 and 4 after fourth vaccination.

Assessment of CD19+ peripheral B-cells
Flow cytometry (FACS Canto II, Becton Dickinson, San Jose, California, USA) was used to determine immunological phenotypes of lymphocyte subsets. Hereby, the whole blood staining was done previous to lysis (Becton Dickinson). A combination of the following monoclonal antibodies (all provided by Becton Dickinson) was applied: fluorescein isothiocyanate-labelled anti-CD3, phycoerythrin (PE)-labelled anti-CD16+56+, peridinin-chlorophyll–protein–cy5.5-labelled anti-CD4, PE–Cy7-labelled anti-CD19, allophycocyanin (APC)–Cy7-labelled anti-CD8, V450-labelled anti-human leucocyte antigen–DR, V500-labelled anti-CD45 and APC-labelled anti-CD14. CD19+ B-cells are expressed as percentage among total lymphocytes.

Anti-SARS-CoV-2 antibody testing
Quantitative assessment of antibodies to the receptor-binding domain (RBD) of the viral spike (S) protein was performed by an Elecsys Anti-SARS-CoV-2 S immunoassay. The detection range is between 0.4 and 2500.0 binding antibody units (BAU)/mL. A concentration greater than 0.8 BAU/mL was considered positive. Analysis was performed on a Cobas e801 (Roche Diagnostics, Medical University of Vienna (certified acc. to ISO 9001:2015 and accredited acc. to ISO 15189:2012).

T-cell responses
For T-cell stimulation (see below), PepMix SARS-CoV-2 peptide pools were acquired from JPT (Berlin, Germany). The S peptides are split into two subpools S1 (aa 1-643) and S2 (aa 633-1273). Peptides were dissolved in dimethyl sulfoxide and diluted in AIM V medium for use in enzyme-linked immunosorbent spot (ELISpot) assays as described previously.

For ex vivo T-cell IFN-γ ELISpot assay, PBMCs from patients before and after the fourth vaccination were thawed and processed on the same day. A total of 1–2×107 cells per well were incubated with SARS-CoV-2 peptides (2 µg/mL; duplicates), AIM-V medium (negative control; 3–4 wells) or phytohemagglutinin (L4414; Sigma; 0.5 µg/mL; positive control) in 96-well plates coated with 1.5 µg anti-IFN-γ (1-D1K, Mabtech) for 24 hours. After washing, spots were developed with 0.1 µg biotin-conjugated anti-IFN-γ (7-B6-1, Mabtech), streptavidin-coupled alkaline phosphatase (Mabtech, 1:1000) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma). Spots were counted using a Bio-Sys Bioreader 5000 Pro/S/BR177 and Bioreader software generation V10. Data were calculated as spot-forming cells (SFCs) per 108 PBMCs after subtracting the spots from the negative control (mean spot numbers from three to four unstimulated wells).

Statistical analysis
All subjects vaccinated with a fourth dose who completed week four were included in the immunogenicity analysis. Seroconversion rates and increase in antibody concentrations were analysed and displayed in graphical form. Antibody levels were also examined towards peripheral B-cell status and whether rituximab therapy was continued between third and fourth dose. Cellular immunity is shown over three timepoints and in respect to the third dose applied. Given the fixed sample size, no formal sample size calculation was conducted and therefore trial outcomes and safety data are presented descriptively only. ‘R’ V4.0.3 (R Development Core Team, Vienna, Austria) was used for the entire analysis. Following packages were used: ‘ggplot2’ and ‘ggbeeswarm’ for creating plots as well as ‘tableone’ to create baseline tables.

Patient and public involvement
Patients and/or the public were not involved in the design, conduct, or reporting, or dissemination plans of this research

RESULTS
Patient characteristics
Overall, 55 patients who completed the main study were screened, of whom 37 patients consented to participate in the extension study to receive a fourth vaccine dose. Twenty nine were vaccinated with BNT162b2 and 8 with mRNA-1273, according to their primary vaccination, except for one patient who was switched to BNT162b2. Among these patients, 50% had received the ChAdOx1 nCoV-19 vaccine and 50% an mRNA vaccine as a booster. One patient experienced a SARS-CoV-2 infection and was therefore excluded from the immunogenicity analysis. Overall, 36 patients subsequently presented at follow-up visits and completed the trial 4 weeks after vaccination (figure 1). Patients continued their immunosuppressive therapy including rituximab following the EULAR guidelines at the treating physician’s discretion. Patient characteristics of all analysed patients are presented in table 1.

Humoral immune response
At screening, 12/36 patients (33%) had detectable anti-RBD antibodies; thus, the frequency of patients who seroconverted increased to 21/36 (58%) at week 4 after the fourth vaccination. Detectable anti-RBD antibodies were maintained between third and fourth vaccination. Accordingly, 9/24 (38%) of the patients who initially did not seroconvert after three vaccinations developed anti-RBD antibodies on receiving a fourth vaccination (figure 2A). Levels of antibodies were higher after an additional booster vaccination, increasing from median 0.4 BAU/mL (quartiles: 0.4; 8.1) at screening to 12.4 BAU/mL (quartiles: 0.4; 197.3) at week four in the total study population (figure 2B). In patients with detectable antibodies before vaccination (n=12), antibody levels increased from median 11.6 BAU/mL (quartiles: 8.1; 25.5) to 344.5 BAU/mL (quartiles: 119.0; 1387.8). Patients with no detectable antibodies at baseline, but who seroconverted on a fourth dose (n=9), had a median antibody concentration of 43.8 BAU/mL (quartiles: 22.8; 163.0) 4 weeks after the fourth dose, indicating further immunogenicity of a fourth vaccination. Anti-RBD antibody levels were lower in patients with CD19+ peripheral B-cells<1% (n=26) than in those with B-cells≥1% (n=10). Furthermore, all patients with<1% peripheral B-cells and detectable anti-RBD antibodies at week four (n=11) already had detectable antibodies before the fourth dose, except for two. All patients with≥1% detectable peripheral B-cells (n=10) had antibodies at week 4, irrespective of their AB levels at screening (figure 2C), supporting the relevance of detectable peripheral B-cells for antibody production.

Overall, 15/36 (42%) of the patients received rituximab treatment between the third and the fourth vaccination. Patients who did not seroconvert on three vaccinations and continued rituximab treatment (n=9) did not develop anti-RBD antibodies
Epidemiology

Table 1  Baseline patients characteristics.

<table>
<thead>
<tr>
<th>n</th>
<th>Age, years</th>
<th>Sex: female</th>
<th>Diagnosis</th>
<th>Patients with detectable B-cells</th>
<th>Months between last RTX and fourth dose</th>
<th>Concomitant medication</th>
<th>Third vaccine dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>36</td>
<td>62.1 (14.0)</td>
<td>25 (69.4%)</td>
<td>Rheumatoid arthritis 14 (38.9%)</td>
<td>14 (38.9%)</td>
<td>7.4 (5.8)</td>
<td>Any csDMARD</td>
<td>ChAdOx1 nCoV-19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Connective tissue disease 12 (33.3%)</td>
<td></td>
<td></td>
<td>Mycophenolate mofetil</td>
<td>BNT162b2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IgG4-related disease 1 (2.8%)</td>
<td></td>
<td></td>
<td>Leflunomide</td>
<td>mRNA-1273</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Multiple sclerosis 2 (5.6%)</td>
<td></td>
<td></td>
<td>Hydroxychloroquine</td>
<td>Patients with SARS-CoV-2-S AB at screening</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Vasculitis 7 (19.4%)</td>
<td></td>
<td></td>
<td>Methotrexate</td>
<td>Level of SARS-CoV-2-S AB at screening</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Azathioprine</td>
<td>Data are presented as n (%), mean±SD or median (quartiles), RTX, csDMARDs defined here as concomitant treatment with at least one of the following: methotrexate, mycophenolate mofetil, azathioprine, leflunomide, hydroxychloroquine—one patient had a combination of two csDMARDs, SARS-CoV-2-S AB: SARS-CoV-2 spike antibody. csDMARD, conventional synthetic disease-modifying antirheumatic drug; RTX, rituximab. csDMARD, conventional synthetic disease-modifying antirheumatic drug; RTX, rituximab.</td>
</tr>
</tbody>
</table>

300) vs 1880 BAU/mL (quartiles: 1311; 2449), respectively (figure 3B), suggesting significant effects of rituximab treatment on antibody production in patients who received a fourth vaccination.

Cellular immune response SARS-CoV-2-specific T-cell responses have been analysed over a period of 12 weeks before the fourth vaccination. The effect of a fourth vaccination was evaluated at week 1 (figure 4A). Overall, a decrease of the cellular immune response between week 1 (median 388 per 10⁶ SFC (quartiles: 45; 861)) and week 12 (median 38 per 10⁶ SFC (quartiles: 11; 110)) after third vaccination was observed. A fourth dose led to an only modest increase (median 56 per 10⁶ SFC (quartiles: 10; 533)) (figure 4B). However, when analysing patients who received a

Figure 2  Humoral immune response to fourth COVID-19 vaccination in rituximab-treated patients. Antibodies to the receptor-binding domain (RBD) of the viral spike (S) protein were determined using an anti-SARS-CoV-2 immunoassay. (A) Fraction of seroconverted patients based on the presence of detectable anti-RBD antibodies (B) Anti-RBD antibody levels in patients at screening (n=36) and at week 4. (C) Anti-RBD antibodies grouped in patients according to the percentage of CD19" peripheral B-cells. Median is shown, colour indicating detectable antibodies before a fourth dose. Wk: week.

Figure 3  Humoral immune response in patients based on the time of last rituximab treatment. Antibodies to the receptor-binding domain of the viral spike (S) protein were determined using an anti-SARS-CoV-2 immunoassay in patients who (A) did not seroconvert on three vaccinations (n=24) and (B) seroconverted patients (n=12). Colours indicate whether rituximab was applied between third and fourth vaccination. Log scale was used in (A), Wk: week.

Figure 4  SARS-CoV-2-specific T-cell responses. (A) SARS-CoV-2-specific T-cell responses were determined by ELISpot assay from peripheral blood mononuclear cells (PBMCs) stimulated with spike subunit S1 and S2 peptide pools one and 12 weeks after the third vaccination as well as 1 week after the fourth vaccination. (B) Average of SFCs/10⁶ PBMCs from S1 and S2 peptide pools are shown for three time points. Y-axis indicates the number of spot forming cells (SFCs) per 10⁶ PBMCs. Data show the sum of average SFCs/10⁶ PBMCs from S1 and S2 peptide pools. (C) Composite ELISpot results divided into patients who received a third vaccination with either an mRNA or vector-based vaccine. The median and IQR are shown.
third vaccination with an mRNA or vector-based vaccine separately, we could observe a faster decline of SARS-CoV-2-specific cells in patients with a homologous mRNA-based vaccination regime (n=18) as compared with patients who received a vector-based regime as a third vaccination, suggesting that heterologous vaccination induces a more stable cellular immune response (figure 4C). However, this did not affect cellular immune responses after the fourth vaccination.

Reactogenicity

Adverse events were monitored using a paper-based patient diary throughout the first 7 days after vaccination and by an interview-based assessment at week 4. Prevalence of systemic reactogenicity was comparable between BNT162b2 and mRNA-1273 vaccinated patients, except for arthralgia and headache; arthralgia was reported by 4/7 (57%) as compared with 11/29 (38%) of patients vaccinated with mRNA-1273 and BNT162b2, respectively. Nausea only occurred in BNT162b2-vaccinated patients (5/29, 17%). Headache was prevalent in 5/7 (71%) of mRNA-1273, compared with 13/28 (45%) BNT162b2 vaccinated patients. Local pain and local pruritus were more often reported with mRNA-1273 than with BNT162b2 (6/7, 86% vs 14/29, 48% and 2/5, 40% vs 2/29 7%, respectively) (online supplemental figure 1). No thrombocytopenia or antibodies against PF4 were observed after an additional booster vaccination. One serious adverse event, hospitalisation due to lower back pain unrelated to vaccination, occurred during follow-up. No disease flares requiring a change of immunomodulating therapy were reported during the trial. None of the patients experienced an anaphylactoid reaction or neurological complication. One patient developed COVID-19 after the fourth vaccination (four doses of BNT162b2, no humoral vaccine response at the time of infection). The patient received sotrovimab as part of our routine clinical care after testing positive for SARS-CoV-2 and only suffered from mild COVID-19 associated symptoms. No hospitalisation was required.

DISCUSSION

In this open-label extension trial, we found an increase in seroconversion rates as well as in antibody levels after a fourth vaccination in rituximab-treated patients who mounted no or low antibody titers after their third vaccination. However, the antibody response was vastly diminished depending on the numbers of peripheral B-cells and the timing of the rituximab treatment in relation to the fourth vaccination. A modestly enhanced cellular immune response was observed.

Preliminary results of a fourth vaccination in healthy individuals from Israel indicate that a fourth dose of an mRNA dose restores antibody titers. Patients under rituximab therapy have markedly reduced vaccine seroconversion rates and antibody concentration, mainly depending on the number of peripheral B-cells, which is also consistent with results after the third vaccination. Data on the fourth vaccination in immunosuppressed patients are sparse. Increased seroconversion rates and elevated antibody titre in kidney transplant patients with a possible improvement of the vaccination response after a temporary hold of immunosuppressive therapy were observed after four vaccinations. In a case series of 18 patients with autoimmune diseases, 2 patients under mycophenolate mofetil therapy remained without humoral immune response after four vaccine doses. In the present extension trial, we could reduce the rate of vaccine non-responders from almost 7 of 10 to about 4 of 10 rituximab-treated patients. Low antibody levels were observed in patients who received rituximab treatment between the third and fourth vaccination, suggesting that rituximab hampers seroconversion rates and exerts an adverse effect on the ability to booster SARS-CoV-2-specific humoral immune responses in these patients. This will be especially important for the use of possible variant vaccines to boost responses in the future, although first results of Omicron-specific vaccine doses report little advantage as compared with standard vaccination in animal models. We have previously reported that cellular immune response can be mounted in B-cell-depleted patients. Consecutive analysis revealed a drop in the cellular immune response 12 weeks after the third vaccination. We observed only moderate effects of a fourth vaccination on the cellular immune response. Subgroup analysis revealed a higher stability of a cellular immune response in those who received a heterologous vaccination in line with previously published data. In the current trial, patients with different rheumatic diseases as well as two patients with multiple sclerosis (MS) have been included. Although a sufficient humoral immune response to COVID-19 vaccination has been reported in patients with MS, we cannot exclude disease-specific effects. A fourth dose of the mRNA vaccine has shown a favourable safety profile. Reactogenicity of the vaccine dose over 7 days was in the expected range of our previous trial. Although no serious adverse events were observed in any group, reactogenicity was more pronounced in patients who received a fourth vaccination with mRNA-1273 as compared with BNT162b2, which is in line with previous trials in healthy individuals on a third vaccination.

The major limitation of our study is the relatively small number of patients vaccinated; thus, further investigations are required to confirm our results. Furthermore, it still needs to be determined how antibodies (or their absence) are linked to protection against symptomatic infection with SARS-CoV-2 in these patients, especially with respect to novel VOCs. Recent data have shown, that memory T-cells with cross-reactive potential can exert protection against SARS-CoV-2 by rapid expansion.

Comedication with conventional synthetic DMARDs and corticosteroids can lead to a reduced immunogenicity after COVID-19 vaccination. Larger patient cohorts would be needed to decipher different effects of DMARD cotherapy in addition to rituximab. Our data show that a fourth vaccination dose in this high-risk population of patients is safe and can also increase seroconversion rates and antibody levels. Most importantly, as rituximab also seems to hamper the ability to boost previously seroconverted patients, the continuation of rituximab treatment should be carefully considered even in patients with a detectable vaccine response. Non-responders should be evaluated for therapy with monoclonal antibodies as prophylaxis or post exposure to improve COVID-19 outcome in this high-risk group of patients.

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Contributors All authors contributed to manuscript preparation. MB, DA, DS, DM, and SW contributed to the study design. DM, HR, LXA, ES and DS contributed
to data analysis, TP, HH and KS performed antibody measurements. JHH, MM and PH contributed to cellular assays, MB, DA, JSS, DM and LKH contributed to the primary manuscript draft. ES, DM, MB, PM, AK, FK, TH and TD contributed to patient recruitment, RT determined leukocyte subsets. MB acts as guarantor and accepts full responsibility for the work.

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**Competing interests**

PM reports speakers fees from AbbVie, Janssen and Novartis and research grants from AbbVie, BMS, Novartis, Janssen, MSD and UCB. MB reports commercial or non-commercial consultancy fees from AbbVie, Amgen, Lilly, Merck, Novartis, Pfizer, Roche and Sanofi. JSR reports about grants, consulting and personal fees from AbbVie, Astra-Zeneca, Lilly, Novartis, Amgen, Astral, Bristol-Myers Squibb, Celgene, Celltrion, Chugai, Gilead, ILTO, Janssen, Merck Sharp & Dohme, Novartis-Sandoz, Pfizer, Roche, Samsung and UCB. DM received support for meeting attendances from Pfizer. HH received grants from Gilek Health, BlueSky Immunotherapies and Neutrion. AK reports about speaker and consulting fees from AbbVie, Amgen, Bristol-Myers Squibb, Eli Lilly, Gilead, Janssen, Merck Sharp and Dohme, Novartis and Pfizer. All other authors declare no competing interests.

**Patient and public involvement**

Patients and/or the public were not involved in the design, conduct, or reporting, or dissemination plans of this research.

**Patient consent for publication**

Not applicable.

**Ethical approval**

The trial was performed in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki. The trial was approved by the University of Vienna ethics committee in September 2021 (EK4: 1481/2021). All patients provided their written informed consent. All trial visits were conducted in a single centre (Vienna General Hospital).

**Provenance and peer review**

Not commissioned; externally peer reviewed.

**Data availability statement**

Data are available upon reasonable request. Anonymous patient data are available under specific conditions. Proposals will be reviewed and approved by the sponsor, scientific committee and staff on the basis of scientific merit and absence of competing interests. Once the proposal has been approved, data can be transferred through a secure online platform after the signing of a data access agreement and a confidentiality agreement.

**Supplemental material**

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