Evaluation of IL- 2 and IFN γ levels in recovered COVID 19 Patients and Vaccinated Participants, Comparative Study in AL-Diwaniyah, Iraq

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ABSTRACT
The coronavirus disease 2019 (COVID-19), is a highly contagious transmittable disease caused by a recently discovered coronavirus, pathogenic SARS-CoV-2. Coronavirus is considered one of the diseases arisen recently, Thus, The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has consequently resulted in a notable rate of morbidity and mortality. There are hopes that vaccination generated immunity or infection generated immunity or from herd achieved through both natural SARS-CoV-2 infection and vaccination, can control it. So, this study aimed to evaluated of IL- 2 and IFN γ levels in COVID-19 recovered patients, vaccination and hybrid (infected and vaccinated) for three months after infection or vaccination. This study was conducted on 165 COVID-19 Iraqi recovered patients, hybrid and vaccinated (males and females) with age ranged between 15-75 years, and 85 matched healthy control group. The nasopharyngeal swabs was used to confirm recovery via Real-Time Polymerase Chain Reaction (RT-PCR) technique. Blood samples collected for ELISA (enzyme-linked immunosorbent assay). There was significant difference in mean age ($p = 0.007$) among groups and the highest mean age was for recovered group (37.95 ±14.81) years followed by hybrid group(35.80 ±14.96)years and vaccinated group (33.93 ±16.06)years , the least mean age was for control group (29.84 ±12.20) years. While, there was no significant difference in the frequency distribution of subjects according to sex among study groups ($p = 0.899$). In addition there was significant increase ($P< 0.001$) 0f IL-2 level in COVID-19 recovered patients that taken vaccines group (210.37 ±36.51 pg/mL) followed by recovered patients (181.52 ±24.54 pg/mL) and vaccinated persons (178.81 ±17.50 pg/mL)as compared to healthy control group(148.91 ±7.14 pg/mL). There was significant difference in mean of IFN-gamma level ($p < 0.001$) and the level was highest in infected and vaccinated group(213.73 ±77.46 pg/mL) followed by vaccinated (188.99 ±33.31 pg/mL)and recovered groups (186.99 ± 46.11 pg/mL) then lastly by control group(145.06 ±5.34 pg/mL).Moreover, there was a strong positive correlation between IL-2 and IFN-γ ($r = 0.534$),($P<0.001$) in COVID-19 recovered group .

1. Introduction

The novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the cause of COVID-19, has led to over 120 million ill patients and nearly 2.7 million deaths. The immune system's response plays a crucial part in the disease. Therefore, preventing more COVID-19-related morbidity and mortality is of utmost importance to the international society [1]. To achieve this goal, effective vaccination against the novel virus is an essential strategy to maintain health services and public life while reducing social constraints [2].

The immune system is a major player in the pathogenesis of COVID-19 because the causative virus (SARS-CoV-2) is able to induce dysregulated innate and adaptive immune responses that are ultimately associated with widespread damage to tissues and organs[3]. This response is typically accompanied by the release of mediators of inflammation, which include high levels of cytokines linked to immune system activation. Additionally, high levels of cytokines have been strongly associated with severe illness that results from the infection of respiratory system by viral infection [4]

IFN-γ has been shown to have profound effects on both innate and adaptive immunity that facilitate host protection, were originally described as agents interfering with the replication of viruses and eliciting potent anti-viral activity[5]. During SARS-CoV-2 infection, both the innate and adaptive immune responses are required for successful virus clearance and must be adequately controlled to minimize immunopathological damage[6]. The expression of IFN-γ tends to be slightly lower in severe cases than in moderate cases, mainly due to the decrease in CD4+, CD8+, and NK lymphocytes [7].

Although, the numbers of CD4+ and CD8+ T cells are decreased, the IFN-γ producing ability of both them is increased, especially the increased ability of CD4+ T cells. Therefore, the IFN-γ–producing ability of CD4+ T cells was also remarkably increased in extremely severe patients[8]. The hyper function of CD4+ T cells will initiate macrophage activation syndrome, which leads to cytokine storm in extremely severe patients. The majority of cytokines that are known to be associated with cytokine storm IFN- γ were only significantly elevated in the late stages of severe COVID-19 illness [9] High levels of secretion of IFN-γ and monocyte chemoattractant protein-1 (MCP-1) have been reported in patients with COVID-19. These inflammatory cytokines may activate the T-helper type 1 cell response and Th1 activation is a key event in the activation of specific immunity [9,10] .

IL-2 is a growth factor for cellular expansion of specific T cells and the generation of effector and memory T cells [11]. As has previously been detected upon in vitro stimulation of PBMCs from both acute and convalescent COVID-19 patients, intracellular staining revealed that both CD8+ T cells and CD4+ T cells are sources of this cytokine, but CD4+ T cells appear to be the main producers[12,13] . Immune mediators including interleukins were demonstrated to play an important role in the development of COVID-19 [14]. High levels of inflammatory cytokines and chemokines such as IL-2 with an increased number of neutrophils and eosinophils may induce immune abnormalities in patients with COVID-19 [15].Compared with healthy donors, the concentrations of recombinant antigen peptide pools induced-IL-2 and -IFN-γ in convalescent individuals were much higher, suggesting that IL-2 and IFN-γ had induced SARS-CoV-2-specific responses [16] . Thus ,this study aimed to evaluated the levels of circulating cytokine (IL- 2 and IFN γ) of COVID-19 recovered patients, vaccination and hybrid that infected and vaccinated for three months after infection or vaccination.

2. Methodology

The study subjects

This study was conducted on 165 (COVID-19 Iraqi recovered patients, vaccinated and infected and vaccinated (males and females) with age ranged between 15-75 years, and 85 aged and gender matched healthy control group. Swabs for **r**RT-PCR test to confirm negative of participants and blood samples collected for ELISA(enzyme-linked immunosorbent assay) in AL-Diwaniyah-Teaching-Hospital, during the period from February 2022 to August 2022. Diagnoses of COVID-19 were according to the international criteria. The recovery patients confirmed by use nasopharyngeal swabs via Reverse Transcriptase–PCR technique and the clinical examination was performed by internal medicine specialist physician. This work was approved by medical ethics committee of Iraqi Ministry of Health.

Exclusion criteria:

The exclusion in COVID-19 recovered patients and control groups are following cases; pregnant women, patients younger than 15 years old, patients with chronic disease (uncontrolled hypertension, diabetes, and autoimmune disease), smokers, and patients with liver or kidney diseases.

ELISA assay of IL-2 and IFN-γ

Estimation the level of Human IL-2 and Human IFN-γ was done dependent on **ELISA** Kit's procedure (Elabscience/USA)

Statistical analysis:

Data were collected, summarized, analyzed and presented using statistical package for social sciences (SPSS) version 23 and Microsoft Office Excel 2010. Quantitative (numeric) variables were first evaluated for normality distribution using Kolmogorov-Smirnov test, and then accordingly normally distributed numeric variables were expressed as mean (an index of central tendency) and standard deviation (an index of dispersion), in addition to range. The following statistical tests were used:(Chi-

square test ,One way analysis of variance (ANOVA) ,Independent samples student t-test and Pearson test).

3. Results and discussion

Demographic characteristics of patients and control subjects

Assessing virus-specific immune memory over at least a 3-month period is likely necessary to ascertain the durability of immune memory to SARS-CoV-2. From 1st February to 1st August 2022, 165 patients (including Sixty COVID-19 recovered patients confirmed by negative PCR, Forty-five recovered patients that taken vaccine and Sixty vaccinated) ,males and females with age ranged between 15-75 years, and 85 aged and sexes matched healthy control group had their samples tested at AL-Diwaniyah-Teaching-Hospital in AL-Diwaniyah-Province.

3.1.1 Age and Sex of participants

This study distributed COVID-19 patients as recovered, vaccinated and hybrid that (infected and vaccinated) according to age groups. There was significant difference in mean age (p = 0.007) among groups and the highest mean age was for group recovered (37.95 ±14.81) years followed by infected and vaccinated group(35.80 ±14.96)years and vaccinated group (33.93 ±16.06)years , the least mean age was for control group (29.84 ±12.20) years. While, there was no significant difference in the frequency distribution of subjects according to sex among study groups (*p* = 0.899) as presented in Table (1).

Table 1: Comparison of mean age and sex among studied groups and control group

Characteristic	Control group *n* = 85	Vaccinated group *n* = 60	Infected and vaccinated group *n* = 45	Recovered group *n* = 60	*p*
Age (years)					
Mean ±SD	29.84 ±12.20 C	33.93 ±16.06 B	35.80 ±14.96 B	37.95 ±14.81 A	0.007 O **
Range	15 -61	15 -73	17 -71	17 -73	
<20 years	22 (25.9 %)	13 (21.7 %)	3 (6.7 %)	5 (8.3 %)	0.023 C *
20-40 years	43 (50.6 %)	27 (45.0 %)	26 (57.8 %)	30 (50.0 %)	
>40 years	20 (23.5 %)	20 (33.3 %)	16 (35.6 %)	25 (41.7 %)	
Sex					
Male, n (%)	47 (55.3 %)	35 (58.3 %)	24 (53.3 %)	31 (51.7 %)	0.899 C NS
Female, n (%)	38 (44.7 %)	25 (41.7 %)	21 (46.7 %)	29 (48.3 %)	

n: number of cases; C: Chi-square test; NS: not significant

n: number of cases; **O**: one way **ANOVA** test; **C**: chi-square test; ***:** significant at $p \leq 0.05$; ****:** significant at $p \leq 0.01$; Capital letters were used to present level of significance following performance of post hoc **LSD** (least significant difference) multiple comparison test in such a way that different letters indicated significant difference and similar letters indicated no significant difference; letter (A) takes the highest mean value

Comparison of immunological markers among studied groups and control group

Interleukin-2 levels

Statistical analysis of the current study observed significant increase (P< 0.001) 0f IL-2 level in COVID-19 recovered patients that taken vaccines group (210.37 ±36.51 pg/mL) followed by recovered patients (181.52 ±24.54 pg/mL) and vaccinated persons (178.81±17.50 pg/mL)as compared to healthy control group(148.91 ±7.14 pg/mL) as shown in table(2).

Table 2: Comparison of Interleukin-2 among studied groups and control group

Characteristic	**Control group *n* = 85**	**Vaccinated group *n* = 60**	**Infected and vaccinated group *n* = 45**	**Recovered group *n* = 60**	***p***
IL-2					
Mean ±SD	148.91 ±7.14 C	178.81 ±17.50 B	210.37 ±36.51 A	181.52 ±24.54 B	<0.001 O ***
Range	120.14 -180.31	138.54 -249.46	150.03 -324.74	136.81 -277.63	

n: number of cases; **O**: one way **ANOVA** test; **SD**: standard deviation; ***: significant at $p \leq 0.001$; Capital letters were used to present level of significance following performance of post hoc **LSD** (least significant difference) multiple comparison test in such a way that different letters indicated significant difference and similar letters indicated no significant difference; letter (A) takes the highest mean value

IFN-γ (Interferon gamma) levels

There was significant difference in mean of IFN-gamma level ($p < 0.001$) and the level was highest in infected and vaccinated group followed by vaccinated and recovered groups then lastly by control group, as shown in Table 3.

Table 3 : Comparison of INF-gamma levels among studied groups and control group

Characteristic	**Control group *n* = 85**	**Vaccinated group *n* = 60**	**Infected and vaccinated group *n* = 45**	**Recovered group *n* = 60**	***p***
INF-gamma					
Mean ±SD	145.06 ±5.34 C	188.99 ±33.31 B	213.73 ±77.46 A	186.99 ± 46.11 B	<0.001 O ***
Range	134.91 - 182.67	130.57 -339.31	136.65 -562.02	133.18 -435.24	

n: number of cases; **O**: one way **ANOVA** test; **SD**: standard deviation; ***: significant at $p \leq 0.001$; Capital letters were used to present level of significance following performance of post hoc **LSD** (least significant difference) multiple comparison test in such a way that different letters indicated significant difference and similar letters indicated no significant difference; letter (A) takes the highest mean value.

Correlations between interleukin-2 and interferon gamma

The correlation coefficient (r value) between IL-2 and IFN-γ in Covid-19 recovered group as presented in table (4) reviewed a strong positive correlation between IL-2 and IFN-γ (r = 0.534),(P<0.001).

Table 4 : Correlations among markers in recovered group

Characteristic		**IL2**	**INF-γ**
IL-2	*r*		0.534
	p		<0.001 ***
INF-γ	*r*		
	p		

r: correlation coefficient; ***: significant at $p \leq 0.001$;

4. Discussion

Age and Sex of participants

There is a significant difference in the mean age (p = 0.007) between the groups in the current study. However, there was no significant difference in the participants' frequency distribution by sex. A related study conducted in Iraq looked into the relationship between COVID-19 and age in recovered patients. It revealed statistically significant differences in age between the groups under study, but it also found no significant differences in sex between COVID-19 recovered patients[17] .

Approximately the same proportion was also found in a study on Indian nationals, where the mean age of COVID-19 recovered patients was 36.85 ± 18.51 years [18]. Additionally, previous studies conducted in Iraq shown that COVID-19 could affect people of any age [19,20].Additionally, the current investigation verified that there was no significant difference between the sexes, which was consistent with other studies that found around the same proportion [21,22].

Each participant group was divided into three age classes separated by twenty years. The majority of participants in all studied groups are between the ages of twenty and forty, accounting for roughly(50.0 %, 57.8 %,45.0 % and 50.6 %) of the groups that were recovered, recovered and vaccinated, vaccinated only, and healthy control, respectively.

As table (1) above illustrates, the age range of 20–40 years has the greatest number of participants. Since the elderly are thought to be the most susceptible to death, the majority of those who have recovered are young, according to current research. When age is one of the major risk factors for death and severe COVID-19, however the processes underlying these effects remain not fully understood [23].

However, when comparing younger uninfected controls and mild COVID-19 cases, many investigations are complicated by age, with older individuals often being associated with moderate and severe COVID-19 [24]. According to earlier research [25,26,27] the majority of hospitalizations for COVID-19 occur in adults in their sixth decade of life, who also have a higher risk of death than younger adults. Differences between present results and prior studies on age related effects in immunity may also be due to our cohort including more young and middle-aged adults 38 years old.

Furthermore, comparing the percentages of the two sexes in the groups under study, men made up a higher percentage of COVID-19 recovered patients (51.7%) and those who recovered after taking the vaccination (53.3%) than did women (48.3% and 46.7%, respectively. These findings were consistent with an Iraqi study conducted in Baghdad that found that roughly the same percentage of recovered patients were male (54.4%) as opposed to female (45.6%) [28].

As well as more Iraqi research conducted by [29, 30, 21,19] .In contrast, a different Iraqi study conducted in Duhok found that 44% of COVID-19 cases were in males and 56% in females [31] While individual investigations, including as the current study, have shown varying incidences of COVID-19 in males and females, it appears that males are more likely to be affected than females[32,33]. There are some differences between males and females may explain why COVID-19 was more preponderant in males than females in some studies, and why females perform better outcomes in terms of disease prevalence and mortality. In this context, three relevant determinants were addressed and included X-chromosome-related immune functions, possible influence of sex hormones, and some behavioral patterns related to sex [34].

Men may have more social relationships than women, but there may also be differences in the study's sample size, demographic region, race, education level, marital status, and occupation that account for the study's mean difference. Males are more susceptible to contracting SARS-CoV-2 infection than females, according to clinical features, and males also exhibit a higher severity of SARS-CoV-2 illness than females.

This study provided evidence on sex differences in the determinants of willingness to get the COVID-19 vaccine. In the present study, the percentage of people who were willing to get the COVID-19 vaccine was lower among women than among men (41.7 % vs. 58.3%). Notable, that Iraqi population's high approval of COVID-19 vaccination shows a good understanding of the role of vaccines in pandemic control.

The pandemic had a significant influence on Iraqi's work, income, and daily lives. Since the outbreak of COVID-19, Iraq has taken substantial efforts and applied public health initiatives to address these challenges, and these actions have significantly halted the disease's spread[35]. In Iraq, the adult population showed a high acceptability of the COVID-19 vaccine. Vaccine hesitancy was higher among those with lower income education[36]. A cross-sectional study of Japanese citizens aged 20–65 years, observed that the percentage of people who were willing to get the COVID-19 vaccine was lower among women than among men (33.0% vs. 41.8%) [37].

Various factors are related to COVID-19 vaccination intentions. Previous studies have reported that age, gender, race, education level, influenza vaccination status, psychological factors, such as confidence in the vaccines importance, and fear of COVID-19 transmission are associated with the willingness to get the COVID-19 vaccine [38,39,40].

Interleukin-2 levels

Significant differences of IL-2 level were observed in all groups, the COVID-19 recovered patients that taken vaccine recorded the highest percentage (210.37 ±36.51 pg/mL) compared with the control group, which recorded the lowest percentage (148.91 ±7.14 pg/mL). These results were similar to different studies which were recorded approximately the same proportion. Iraqi study in Wasit Province reported the mean level of IL-2 was (70.46±22.76 pg/mL) in control group and (213.92±74.27pg/mL)in convalescent patient [41].

However, considering the importance of IL-2 in the formation of the memory T-cell compartment, numerous studies have indicated that enhanced IL-2 responses may potentially imply a general improved development of memory T-cells following vaccination [42, 43]. As well as [43] demonstrated that the levels of cytokine-producing T-cells were remarkably stable between three and twelve months post infection, and were comparable to IFNγ+ and IFNγ+IL-2+ T-cell responses but lower than IL-2+ T-cell responses at three months after vaccination.

Another Iraqi study, however, reported by[44], found that the group that received two doses of the BNT162b2 mRNA vaccine had higher serum levels of IL-2 than the symptomatic recovered COVID-19 group. This suggests that the BNT162b2 mRNA vaccine promotes favorable B and T cell responses and a clearer cellular and humoral immune response.

According to reports, patients with COVID-19 had higher levels of IL-2 or its receptor IL-2R, which [9] found to be correlated with the severity of the illness. T cell proliferation as well as the production of effector and memory T cells are significantly influenced by IL-2. While elevated IL-2 family levels in COVID-19 are harmful and may result in severe inflammation in patients, elevated IL-2 can also increase cellular immunity in those who are not presently infected by activating T cells[45].

It is involved in adaptive immunity and increases glucose metabolism to promote the proliferation and activation of T, B, and NK cells [46]. Hence, IL-2 participates in the prevention of autoimmune diseases and is essential to control immune responses and maintain self-tolerance. The absence of this interleukin has been associated with a poor control of effector cells and the consequent development of autoimmunity[47,11]

Furthermore, compared to IFN-γ generating T cells, the recovered patients had a notably higher proportion of IL-2-producing SARS-CoV-2-specific T cells. In patients who recover with COVID-19, the predominance of CD4+ T lymphocytes rather than CD8+ T lymphocytes as a manifestation of immunological memory may be explained by the increased incidence of IL-2-producing SARS-CoV-

2-specific cell clones [48]. Notably, memory T-cell formation, proliferation, and maintenance in response to a particular antigen depend on IL-2 release. In order to acquire a better understanding of memory T-cell responses and protection following vaccination as well as during convalescence, it is therefore possible to analyze the production of this cytokine against particular SARS-CoV-2 antigens [49,50].

IL-2 can represent a good protection biomarker as it has a central role in the maintenance of memory T-cell populations and their effector functions, being secreted mainly by memory CD4+ T-cells and enhancing the activity of both NK and CD8+ T-cells. In this sense, knowing the roles that both cytokines play in the adaptive response against SARS-CoV-2, would allow an understanding of immune protection against the virus and reinfection, as reported for other viruses [51].

After vaccination of participants with a prior confirmed SARS-CoV-2 infection, IL-2 responses were significantly higher than vaccinated participants without previous infection[52]. Finally, the changes in serum cytokines with SARS-CoV-2 indicated the host's immune responses against the coronavirus inflammation seem to be different from what has been seen with other viral pathogens[53].

IFN-γ (Interferon gamma) levels

Results of the current study found a significant elevation (P<0.001) in Interferon gamma level in COVID-19 recovered patients that taken vaccines, vaccinated and recovered as compared with apparently healthy control group as denoted in table 4-7. In addition the COVID-19 recovered patients that taken vaccine recorded the highest percentage (213.73 ±77.46 pg/mL) compared with the control group, which recorded the lowest percentage (145.06 ±5.34 pg/mL).

The result found to be similar to a recent Iraqi study in Duhok province that measured IFN-γ level in COVID-19-recovered individuals and vaccinated participant without pervious infection using enzyme-linked immunosorbent assays and observed that IFN-γ level in COVID-19-recovered individuals that taken vaccine was significantly higher than in vaccinated only without pervious infection [54]. Due to increased vaccination rates and the continued spread of the severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) virus, many people are developing "hybrid immunity" to the virus[55].

Similar findings have been reported using different methods for measuring IFN-γ concentration and indicated that recovered individuals had a much stronger IFN-γ response following vaccination than vaccinated participants without a history of infection [52, 56]. Vaccinated recovered subjects promote stronger immune responses than vaccinated naive subjects due to naturally acquired immunity [57]. In a research by [58] it was shown that the SARS-CoV-2 spike and peptide pools triggered the release of IFN-gamma in COVID-19 recovered effector antigen-specific CD4+ effector T cells, but not in naive donors, and COVID-19 recovered patients mount a greater IFN-gamma response compared to naive participants .

A local Iraqi study in Karbala province showed that previous infection with coronavirus disease-2019 seems to have no effect on IgG and IFN-γ levels after vaccination[55] this study incompatible with the present study. Moreover , many studies measuring the specific IFN-γ released by T-cells in SARS-CoV-2 infected peoples, showing that IFN-γ increased during convalescence compared to the acute disease phase[59,60] While, study in Erbil city, Kurdistan Region of Iraq found the levels of IFN-γ were significantly lower in the recovery group than the severe case of the COVID-19 group [53].

Notably, it has been previously reported that there is a good correlation between humoral and IFN-γ T-cell responses. Additionally, humoral responses are typically more prevalent in vaccinated and convalescent individuals. These findings indicate that IFN-γ T-cell responses can provide a great overview of the specific cellular immunity against SARS-CoV-2 [61,59].IFN-γ secretion is involved in multiple functions, including increasing antigen presentation, inducing antiviral status (prevention of viral multiplication and promotion of apoptosis) and stimulating the expression of numerous genes

related to the inflammatory process[5].

It has also been shown that IFN-g influences humeral responses by regulating the Ig isotypes that B cells generate and by promoting long-lived antibody-secreting cells [62]. IFN-γ secretion is a potent defense against SARS-CoV-2, and numerous studies have shown that low IFN-γ production is strongly associated with an increased risk of fatal COVID-19 outcomes and an inability to control an initial SARS-CoV-2 infection [63,64].

Correlations between interleukin-2 and interferon gamma

The current study found that IL-2 level strong positively correlated with IFN-γ level ($r = 0.534$),($P<0.001$),in Covid-19 recovered group. As reported before, recovered people exhibited a higher response of both IFN-γ and IL-2 cytokines as compared to acute COVID-19 patients. IFN-γ/IL-2 dual detection shows promise in describing and evaluating the immunization status and supporting patient management in this regard [65].

Notably, patients who experienced a mild COVID-19 course after developing a prompt cellular response that produced IFN-γ and IL-2. This observation aligns with the widely accepted notion that effective viral infection control necessitates the early development of virus-specific T cells and the subsequent production of neutralizing antibodies [66].

Furthermore, the percentage of positive responses rose when IL-2 and IFN-γ were combined, highlighting the information that is missed when assessing IFN-γ alone. According to reports for other viruses, this means that understanding the functions that both cytokines play in the adaptive response against SARS-CoV-2 will enable comprehension of immune protection against the virus and reinfection [50,51].

4. Conclusion and future scope

In conclusion, there are no specific age to COVID-19 infection, it occurs in both sexes. Previous SARS-CoV-2 infection and hybrid immunity both provided greater IL-2 level than vaccination alone. The functions that both cytokines play in the adaptive response against SARS-CoV-2 were seen in the current study. This information will help to establish novel vaccination procedures and follow-ups for monitoring protection, as well as understand immune protection against the virus and reinfection. Furthermore, the current study hypothesized that a combined analysis of the two cytokines would aid in the management of SARS-CoV-2 infection by offering details on the immunological state in various clinical contexts. Therefore, the main objective of this study was to assess the dual IFN-γ and IL-2 detection, using ELISA technique, in recovered patients, vaccinated and hybrid that infected and vaccinated.

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