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Comparative study of IL- 2, IL- 6 levels for recovered COVID-19 patients and vaccinated participants

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***Abstract*---**SARS-CoV-2 transmission and hospitalization can be reduced primarily by vaccination. BNT162b2-mRNA vaccine induced both humoral and cellular immune responses against spike proteins. The aim of this cross-sectional study conducted in Wasit Governorate/Iraq is to compare IL-2 and IL-6 levels for recovered COVID-19 patients and participants who were vaccinated with BNT162b2 mRNA vaccine, we used systems serology to investigate the effects and benefit of the BNT162b2 mRNA COVID-19 vaccine on the level of the immune response. A total of 160 male and female blood specimens were collected from the various hospitals and vaccination centers throughout the governorate and divided into four groups: 40 healthy control participants, 40 symptomatic recovered COVID-19 patients (after 1-3 months), 40 participants vaccinated with the 1st dose of BNT162b2 mRNA (after 21 days of 1st dose), and 40 participants vaccinated with the 2nd dose of BNT162b2 mRNA (after 20-30 days of 2nd dose), aged between (18-61) years old, serum cytokines levels were assayed by ELISA method. By comparing the levels of IL-2 and IL-6 in all our studied groups, our findings found that the groups vaccinated with two doses of BNT162b2 mRNA vaccines was recorded the highest response level compared to the symptomatic recovered COVID-19 group. Meaning that there is a clearer cellular and humoral immune response and the BNT162b2 mRNA vaccine contributes to favorable B cell, and T cell responses.

***Keywords*---**SARS-CoV-2, BNT162b2 mRNA vaccine, IL-2, IL-6.

Introduction

Over 2.6 million people have died as a result of the current COVID-19 pandemic, however the approval and widespread use of many COVID-19 vaccine platforms has given hope that the current pandemic will be brought under control (Parry *et al.*, 2021). Following the sequencing of the severe acute respiratory syndrome coronavirus-2, a COVID-19 vaccine based on an experimental mRNA platform was developed in less than a year. The BNT162b2 mRNA vaccine it was the first vaccine to be approved for emergency use by both the FDA and the EMA, Due to its effectiveness in healthy individuals (Polack *et al.*, 2020; Chagla, 2021). Anti-COVID-19 vaccines (BNT162b2) is one of the world's most commonly used. This vaccine is based on a lipid nanoparticle-formulated, nucleoside-modified mRNA that encodes a prefusion stabilized form of the spike (S)-protein generated from the SARS-CoV-2 strain (Föhse *et al.*, 2021). BNT162b2 delivers the precise genetic information of the immunogen to antigen presenting cells and elicits potent cellular and humoral immune responses (Sahin *et al.*, 2020). Cytokines and chemokines have a pivotal role in the development and maintenance of adaptive immunity, in response to both infection and vaccination (Bergamaschi *et al.*, 2021). The T cell response after vaccination is characterized by a coordinated production of all Th1 cytokines, with IFN-γ correlating with both TNF-α and IL-2, IL-2 is essential for the homeostatic maintenance of a functional specific T cell response (Agrati *et al.*, 2021), and involved in the generation of memory T cells as well as the maintenance of regulatory T cells, resulting in a well-balanced immune response (Malek and Bayer, 2004; Harty and Badovinac, 2008). IL6 classical signaling is active in the acquired antibody mediated adaptive immune system being a regulator of B cell transition to antibody-producing plasma cells and T cell differentiation (George, 2019).

Material and Method

Study design and sample collection

The current study was carried out in Wasit Province, Iraq, as a cross-sectional study between October 16, 2021, and January 12, 2022. The specimens was collected from Al- Zahraa teaching hospital, Vaccination Centers at Al Zahraa teaching hospital and Teba Health Center, also from different geographical residences in the governorate. We collected 160 specimens of whole blood (3ml) from each participant of four studied groups in the gel tube, the separated serum was distributed into aliquots in Eppendorf tubes and then frozen at -80°C, forty specimens were collected from a healthy control participants who had no history of COVID-19 symptoms and had not been vaccinated with BNT162b2 mRNA vaccines , forty specimens were collected from mildly symptomatic COVID-19 recovered patients (1 to 3 months after recovery) and the results of RT- PCR was positive during the infection with COVID-19 and negative after recovery with IgG-positive and IgM-negative antibodies and had not been vaccinated, forty were collected from participants vaccinated with the first dose of Pfizer vaccine who had no history of COVID-19 symptoms, and forty specimens were collected from participants vaccinated with the second dose of Pfizer vaccine who had no history of COVID-19 symptoms. The Participants ages were ranged between 18-61 years

old and from both sexes (female to male ratio was 1:1). The required information was taken from the participants based on pre-prepared questions for each group.

Exclusion criteria:

Any patient having IgM with IgG antibodies positive for SARS-CoV2 antigen was excluded from the healthy control group. Patients on steroids or immunosuppressive drugs, pregnant women, and anyone with a medical history of hyperlipidemia, hypertension, tonsillitis, or diabetes were all ruled out.

Anti-SARS-CoV-2 Antibody assessment (IgG, IgM)

IgM and IgG antibodies were detected using immune chromatography methods (rapid lateral flow assay) (Khibio company) to verification of exposure to COVID-19 in human whole blood sample qualitatively. LFAs are an appealing choice for monitoring disease prevalence and measuring vaccination response because of their cost-effectiveness, scalable production method, and appropriateness for self-testing (Trombetta et al., 2021).

ELISA

Measure of Intrleukin-2 and Intrleukin-6 through enzyme-linked immune sorbent assay (ELISA) technique. We used double kits of ELISA based on the biotin double antibody sandwich technology to assay the human IL-2, IL-6, 96 well ELISA plates (Shanghai Company) were pre-coated with IL-2, IL-6 monoclonal antibody. We add prepared samples (40 µl) and standards solution (50 µl) which previously diluted (2400ng/L for IL-2 and 640ng/L for IL-6), then add (10 µl) anti- IL-2, IL-6 antibodies labeled with biotin to unite with (50 µl) streptavidin-HRP, which forms immune complex and incubated for 60 minutes at 37°C. Remove the unbound enzymes after incubation and washing by substrate wash buffer. Add (50 µl) of the substrates A, B and stop solution to each well. Then the solution will turn blue and change into yellow with the effect of acid. We measuring the absorbance (OD) of each of the well one by one under 450nm wavelength.

Ethical Approval

Ethical approval was obtained from the Ethical Committee at scientific research by ethical approval of both health ministry and higher education and scientific research ministry in Iraq. Informed consent was obtained from all the participants.

Statistical Analysis

Data entered with an Excel sheet and analyzed by using SPSS V 28.0. for Windows. Descriptive statistics (frequencies, mean ± standard deviation with tables and graphs) and inferential statistics (Shapiro-Wilk test of Normality, Independent T-test, ANOVA, Pearson/ Spearman Correlation Coefficient and ROC Analysis) were used P-value of ≤0.05 considered statistically significant.

Results

The demographic characteristics of the study participants are shown in table (1). The study comprised a total of 160 participants and distributed into four groups, 40 healthy control participants, 40 mildly symptomatic recovered COVID-19 patients (after 1-3 months), 40 participants vaccinated with the first dose of BNT162b2 mRNA (after 21 days), and 40 participants vaccinated with the second dose of BNT162b2 mRNA (after 20-30 days), The participants were divided into four age groups, each separated by eleven years, the highest number of convalescent participants fall in (18 -28 years) age group making about 45% with mean age 34.3 years, while the highest number of un-exposed participants fall in age group (29-39 years) making about 40% with mean age 31.9 years, and the highest number of vaccinated participants with the first dose fall in (18 -28 years) age group making about 50% with mean age 29.6 years, while the highest number of vaccinated participants with the second dose fall in age group (29-39 years) making about 35% with mean age 32.8 years. With regard to sex, the male and female make about 25% in all groups.

Table 1: Demography characteristics of the studied groups (N = 160)

Demography		Control	Recovered COVID1-19 Patients	Vaccinated Participants 1st Dose	Vaccinated Participants 2nd Dose
Sex (n=160)	male (n=80)	20 (25%)	20 (25%)	20 (25%)	20 (25%)
	female (n=80)	20 (25%)	20 (25%)	20 (25%)	20 (25%)
Age, y, by category (18-61)	18-28 (n=70)	14 (35%)	18 (45%)	20 (50%)	18 (45%)
	29-39 (n=52)	16 (40%)	10 (25%)	12 (30%)	14 (35%)
	40-50 (n=26)	6 (15%)	8 (20%)	8 (20%)	4 (10%)
	51-61 (n=12)	4 (10%)	4 (10%)	0	4 (10%)
Mean age ± SD*		31.9±11.03	34.3±12.07	29.6±8.49	32.8±10.51

* Standard Deviation

In table (2), the ANOVA test indicated that IL-2 mean levels were statistically significantly different in the studied groups ($P<0.001$). The control group have the lower mean value of IL-2 (167.5 ng/L), and both vaccinated groups have the higher mean level of IL-2 (186 ng/L). The post hoc pairwise comparison revealed that the mean value of IL-2 in control group was statistically significantly lower than in all other groups ($P\leq0.004$), the recovered group also have IL-2 mean level lower than both vaccinated groups, but there is no IL-2 mean difference between those vaccinated with (1st dose and 2nd dose) groups.

Table 2: Mean differences (range) of Interleukins (IL-2) and pairwise comparison in all groups (n=160).

Markers	Groups	n	Mean±SD* (range)	ANOVA	Post hoc**
IL-2 (ng/L)	Control	40	167.5±6.69 (155-182)	F=61.3 Sig. <0.001	Ctr. vs Rec P 0.004 Ctr. vs D1 P <0.001 Ctr. vs D2 P <0.001 Rec vs D1 P <0.001 Rec vs D2 P <0.001 D1 vs D2 P 0.980
	Recovered	40	174.3±10.1 (160-194)		
	Vaccinated 1st dose	40	186.2±6.76 (170-197)		
	Vaccinated 2nd dose	40	186.8±6.28 (171-200)		

* Standard Deviation.
** Post hoc multiple comparison, Games-Howell Test.

In table (3), the ANOVA test indicated that IL-6 mean levels were statistically significantly different in the studied groups (P<0.001). The control group have the lower mean value of IL-6 (34.9 ng/L) compare to recovered and both vaccinated groups which have the higher mean level of IL-6 (38.2 ng/L), (53.0 ng/L), (68.4 ng/L) respectively. The post hoc pairwise comparison revealed that the mean value of IL-6 in control group was statistically significantly lower than in all other groups (P≤0.088), and the mean value of IL-6 in vaccinated groups with 2nd dose group was statistically significantly higher than in all other studied groups.

Table 3: Mean differences (range) of Interleukins (IL-6) and pairwise comparison in all groups (n=160).

Markers	Groups	n	Mean±SD* (range)	ANOVA	Post hoc**
IL-6 (ng/L)	Control	40	34.9±5.22 (25-45)	F=151.5 Sig. <0.001	Ctr. vs Rec P 0.088 Ctr. vs D1 P <0.001 Ctr. vs D2 P <0.001 Rec vs D1 P <0.001 Rec vs D2 P <0.001 D1 vs D2 P <0.001
	Recovered	40	38.2±7.27 (24-53)		
	Vaccinated 1st dose	40	53.0±9.12 (35-68)		
	Vaccinated 2nd dose	40	68.4±9.28 (52-98)		

* Standard Deviation.
** Post hoc multiple comparison, Games-Howell Test.

Correlations

In control group, IL-6 uncorrelated with IL-2, but in recovered group IL-6 has significant moderated positive correlation with IL-2. In vaccinated group with 1st dose, IL-6 has insignificant weak positive correlation with IL-2 while in vaccinated group of 2nd dose, there is significant moderated positive correlation between IL-2 and IL-6, table (4).

Table 4: Correlation of IL-6 with IL-2 in studied groups.

Groups	Immunological markers		IL-2
Control	IL-6	r*	0.054
		Sig.	0.740
		n	40
Recovered	IL-6	r	0.634
		Sig.	<0.001
		n	40
Vaccinated 1st dose	IL-6	r	0.273
		Sig.	0.088
		n	40
Vaccinated 2nd dose	IL-6	r	0.382
		Sig.	0.015
		n	40
*Correlation Coefficient			

Potential markers of diagnostic value (ROC Curve Analysis)

To evaluate the diagnostic value of immunological markers (IL-2 and IL-6) in recovered COVID-19 patients and vaccinated participants with two doses of BNT162b2 mRNA vaccine compare to healthy control participants, ROC curves were drawn, figure (1). A graph of Roc Curve (receiver operating characteristic curve) showing the performance of a classification model at all classification thresholds and the AUC (Area Under the ROC Curve) measures the entire two-dimensional area underneath the entire ROC curve from (0,0) to (1,1) (Carrington *et al.*, 2021).

In this study the area under the ROC curve for IL-2 [AUC = 0.879 (95% CI: 0.827-0.930)] was able to identify recovered COVID-19 patients and vaccinated participants with two doses in compare to control with ≥ 178.517 ng/L as the optimal cut-off, having 72.5% sensitivity and 50% specificity figure (1).

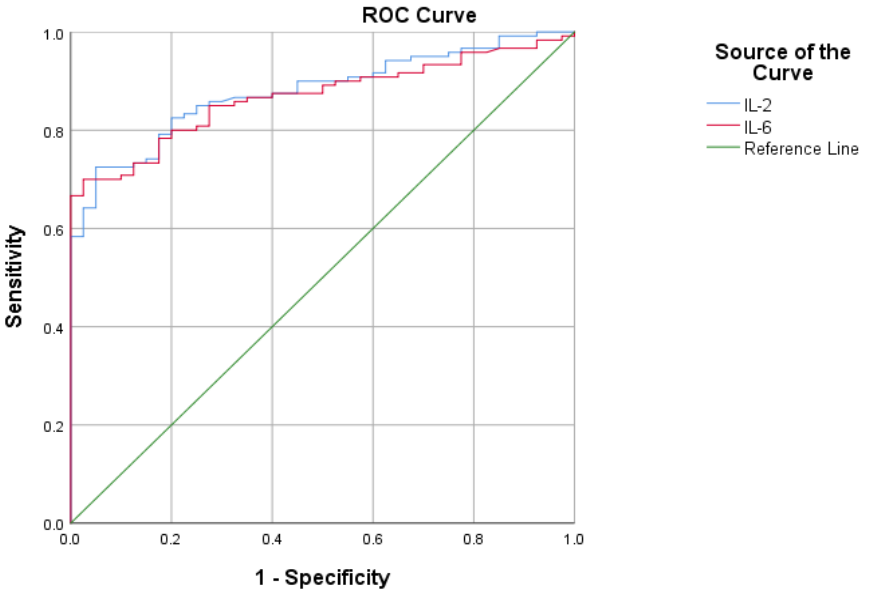

Figure 1: ROC curve to determine IL-2 and IL-6 cut-off in control group compare to other studied groups.

IL-6 [AUC = 0.868 (95% CI: 0.814-0.922)] was able to identify recovered COVID-19 patients and vaccinated with two doses in compare to control with ≥ 44.396 ng/L as the optimal cut-off, having 70% sensitivity and 25% specificity, figure (1).

Discussion

Immunological markers
Interleukin 2 (IL-2)

A study presented by (Lozano-Rodríguez *et al.*, 2022) showed that between naive subjects and individuals recovered from COVID-19 early after BNT162b2 mRNA vaccination (14 days) , with a more pronounced activation of CD4+ T cells in naive subjects. This was revealed by a higher induction of cytokine production (IL-2, IL-4, IL-6, IL-10, and tumor necrosis factor alpha [TNFa]) and proliferation after re-stimulation, also observed a consistent IL-2 production in naive individuals early after vaccination two doses, these results are consistent with our study.

As the researchers (Harty and Badovinac, 2008; Dennehy *et al.*, 2021) pointed out, increased IL-2 responses might potentially imply a general improved generation of memory T-cells following vaccination, given the significance of IL-2 in the creation of the memory T-cell compartment, and (Dennehy *et al.*, 2021) showed that the levels of cytokine-producing T-cells were remarkably stable between three and twelve months after 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.

While another study presented by (Payne *et al.*, 2021) in the United Kingdom showed that, after giving a primary dose, delaying administration of a second dose of BNT162b2 COVID-19 vaccine up to 6– 14 weeks continues to provide strong protection and contributes to favorable antibody (high levels of NAb), B cell, and T cell (enrichment of CD4+ T cells expressing interleukin-2 [IL-2]) responses compared with the conventional 3- to 4-week regimen in both naive participants and pre-existing SARS-Cov-2.

The current study observed that there is high levels of IL-2 in serum of naïve participants vaccinated with 1st dose of BNT162b2 mRNA vaccine than the recovered COVID-19 patients, while a study done by (Angyal *et al.*, 2021) in the United Kingdom who revealed that the donors no evidence of previous SARS-CoV-2 infection, a single dose of vaccine generates comparable antibody and T-cell levels to those detected weeks or months after natural infection and this contrast with our study.

Interleukin 6 (IL-6)

In the present study, we noticed that there was moderate increased in the level of IL-6 for convalescent COVID-19 patients compare to healthy control group, while a study performed by (Zhang, Li and Li, 2020) found that higher cytokine levels of IL-6 in recovered COVID-19 patients.

A research by (Bergamaschi *et al.*, 2021) has shown that the level of IL-6 in naive vaccine recipients was transient increase at day 2 followed by rapid downregulation close to baseline levels by day 8 after the 1st vaccination, but the level of IL-6 at day 23 (1 day after the 2nd vaccination) showed elevated levels and higher significant than those at day 2, while a study performed in university of Copenhagen and Aarhus university, Denmark, by (Ostrowski et al., 2021) noted that increased in the level of IL-6 and IL-10 after vaccination with both Oxford/AstraZeneca and mRNA vaccine (BNT162b2) (median of 11 days (range 8-16) post-vaccination).

A study presented by (Karimabad *et al.*, 2021) for the coronary artery disease patients were found increase in the levels of IL-6 after second vaccination with a BNT162b2 mRNA (Pfizer/BioNTech) vaccine at day 2, and this study consistence with our finding of increased levels of IL-6 after 2nd dose of Pfizer vaccine but at (20-30) days, and this confirm the association of IL-6 with the vaccination and will be resulting in antibody development.

Our study on the circulating levels of cytokines implies that cytokine modulation could be a biomarker for effective vaccination and antibody generation, and the role of these cytokines upon vaccination became more apparent after the 2nd vaccination.

Correlation between immunological markers (IL2 and IL-6)

In terms of cytokine production, CD4+ T-helper (Th) 1 and Th2 cells are two types of T cells. Th1 cells contribute to the cellular immune response by secreting IL-2,

IFN-, and TNF-. Th2 cells, on the other hand, release IL-4, IL-6, and IL-10, which promote humoral immune responses (Okada et al., 2008).

Among recovered COVID-19 patients IL-2 were positively correlated with IL-6 compare to control group and this consistent with a studies by (Wen et al., 2020; Zhang, Li and Li, 2020) they found that T cell-B cell interactions in COVID-19 patients induce T cells to release IL-2, which promotes B cell growth, which might explain the T-B cell association. Also a study presented by (Allamy,2021) observed a correlation between IL-2 and IL-6, this allows for a more accurate assessment of convalescent patients' immunological function.

The present study revealed a differential immune response for cellular and humoral immunity between naive subjects vaccinated with two doses of BNT162b2 mRNA (Pfizer/BioNTech) vaccine and individuals recovered from COVID-19 after 3 months and our study consistence with a study performed by (Lozano-Rodríguez et al., 2022) showed that a higher induction of cytokines production and proliferation and only naive individuals showed a robust induction of most of the cytokines analyzed IL-2, IL-6.

Conclusion

Our finding show that two doses of BNT162b2 mRNA (Pfizer/BioNTech) vaccine are effective in elicit IL-2 and IL-6 stimulation than natural infection with COVID-19 after recovery.

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