Effect of BNT162b2 and CoronaVac boosters on humoral immunity of individuals previously fully vaccinated with CoronaVac against SARS-CoV-2: A longitudinal study

by

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Koç University

Graduate School of Health Sciences

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Date: June 29th,2022

To KUISCID

ABSTRACT

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Zeynep Ece Kuloğlu

Master of Science in Immunology

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It is essential to know about the immune response levels after booster doses of the two different types of vaccines, the mRNA and the inactivated, currently used against COVID-19. For this purpose, it was aimed to determine the effects of BNT162b2 (BNT) and CoronaVac (CV) boosters on the humoral immunity of individuals who had two doses of CV vaccination.

For the estimation of humoral immune responses, live SARS-CoV-2 virus was isolated from nasopharyngeal swabs of SARS-CoV-2 PCR test positive patient and grown on the Vero E6 cells. After the confirmation of growth and estimation of the TCID50 for the virus, the virus was used in the PRNT50 experiments. For the study the healthcare workers who had no history of Covid-19 infection and were immunized with two doses of inactivated vaccine CoronaVac were included. Blood samples from these individuals were collected at different time points. From whole blood samples, PBMCs and sera were isolated. PBMCs were used for the flow cytometry experiments and sera were used for the neutralizing antibody experiments.

In the neutralizing assay, the PRNT50 titer was calculated a dilution in which half of the virus at 10^{-3} PFU/ml concentration was inactivated and was selected for everyone in all time points. The percentage of B cell subtypes and proportion of memory B cells were estimated for each time point by staining with specific B cell markers (Anti-CD19, Anti-CD20, Anti-IgG, Anti- CD27, Anti- CD14, Anti-CD38 and Anti-CD56). After staining, samples were run by Attune flow cytometry.

After the experiments, a 3.38-fold increase in neutralizing antibody geometric mean titers (Neutralizing Antibody, GM, 78.69) was found one month after BNT162b2 booster and maintained at the third month (Neutralizing antibody, GM, 80). Nevertheless, in the CoronaVac booster group, significantly lower neutralizing antibodies, GMT than BNT162b2 after 1 month and 3 months were observed (21.44 and 28.44, respectively) ($p<0.001$).

In the B cell part, significantly higher expression of memory B cell and memory B cells expressing IgG was observed in the BNT162b2 booster group when compared with CoronaVac booster group both in 1st and 3rd month after booster vaccination. The ratio of effector memory B cells 1st month after vaccination in the CoronaVac booster group was significantly higher than BNT162b2 booster group ($p=0.0263$).

ÖZETÇE

CoronaVac ile SARS-CoV-2'ye karşı tam olarak aşılanmış bireylerde BNT162b2 ve CoronaVac hatırlatıcı dozlarının hümoral bağışıklık üzerindeki etkisi: Boylamsal bir çalışma

Zeynep Ece Kuloğlu

İmmünoloji, Yüksek Lisans

Haziran 29, 2022

Şu anda COVID-19'a karşı kullanılan iki farklı aşı türü olan mRNA ve inaktive edilmiş aşıların hatırlatıcı dozlarından sonraki bağışıklık tepkisi düzeylerini bilmek önemlidir. iki doz CV aşısı olan bireylere uygulanan BNT162b2 (BNT) ve CoronaVac (CV) hatırlatma dozlarının hümoral bağışıklığı üzerindeki etkilerinin belirlenmesi amaçlanmıştır.

Hümoral bağışıklık tepkilerinin belirlenmesi için, canlı SARS-CoV-2 virüsü, SARS-CoV-2 PCR testi pozitif hastanın nazofaringeal sürüntülerinden izole edildi ve Vero E6 hücrelerinde üretildi. Viral üremenin konfirmasyonundan ve TCID50'nin belirlenmesinden sonra virüs nötralizan antikor deneylerinde kullanıldı.

Çalışmaya Covid-19 enfeksiyonu öyküsü olmayan ve iki doz inaktif aşı CoronaVac ile aşılanan sağlık çalışanları dahil edildi. Bu kişilerden farklı zaman aralıklarında kan örnekleri alındı. Tam kan örneklerinden PMNKH'ler ve serum ayrıştırıldı. Akış sitometrisi deneyleri için PMNKH''ler ve nötralizan antikor deneyleri için serumlar kullanıldı.

Nötralizasyon testlerinde, 10^{-3} PFU/ml konsantrasyonunda virüsün yarısının inaktive edildiği serum dilüsyonu PRNT50 olarak belirlendi. B hücresi alt tiplerinin yüzdesi ve hafıza B hücrelerinin oranı spesifik B hücresi belirteçleri (Anti-CD19, Anti-CD20, Anti-IgG, Anti-CD27, Anti-CD14, Anti-CD38 ve Anti-CD56) ile boyandı . Boyamadan sonra örnekler Attune akış sitometrisi ile okutuldu.

BNT162b2 booster uygulamasından bir ay sonra nötralizan antikor geometrik ortalama titrelerinde (GOT, 78.69) 3.38 katlık bir artış bulundu ve bu titre üçüncü ayda sabit kaldı (GOT, 80). Bununla birlikte CoronaVac booster grubunda, 1 ay ve 3 ay sonra BNT162b2'ye göre önemli ölçüde daha düşük düzeyde nötralize edici antikorlar gözlendi (GOT sırasıyla 21.44 ve 28.44) ($p<0.001$).

B hücresi kısmında, booster aşılamadan sonra hem 1. hem de 3. ayda CoronaVac booster grubu ile karşılaştırıldığında BNT162b2 booster grubunda hafıza B hücresi ve IgG eksprese eden hafıza B hücrelerinin ekspresyonu önemli ölçüde daha yüksek gözlendi. Hatırlatma dozundan 1 ay sonra, CoronaVac booster grubundaki efektör hafıza B hücreleri oranı BNT162b2 booster grubundan istatiksel olarak anlamlı düzeyde yüksek bulundu.($p=0.0263$)

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ABBREVIATIONS

ACC	Acceleration
ACE-2	Angiotensin Converting Enzyme-2
APC	Antigen Presenting Cell
ATCC	American Type Culture Collection
BNT	BNT162b2
BMI	Body Mass Index
BSA	Bovine Serum Albumin
BSL-3	Biosafety Level-3
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CD14	Cluster of Differentiation 14
CD19	Cluster of Differentiation 19
CD20	Cluster of Differentiation 20
CD27	Cluster of Differentiation 27
CD38	Cluster of Differentiation 38
CD56	Cluster of Differentiation 56
Covid-19	Coronavirus disease-19
CPE	Cytopathic Effect
CR2	Complement Receptor 2
CV	CoronaVac
CD	Deceleration
DdDp	DNA Dependent DNA Polymerase
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
dsDNA	Double stranded DNA
dsRNA	Double stranded RNA
DPBS	Dulbecco's phosphate-buffered saline
EBV	Epstein-Barr Virus
EDTA	Ethylenediamine Tetra acetic Acid

FAB	Fragment antigen-binding
FBS	Fetal Bovine Serum
FC	Fragment crystallizable
FDA	Food and Drug Administration
GI	Gastrointestinal
GISAID	Global Initiative on Sharing All Influenza Data
GM	Geometric mean
GMT	Geometric mean titer
HIV	Human Immunodeficiency Virus
ICTV	International Committee on Taxonomy of Viruses
ICU	Intensive Care Unit
IFN	Interferon
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgD	Immunoglobulin D
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-2	Interleukin-2
ILC	Innate Lymphoid Cell
MHC	Major Histocompatibility Complex
ml	Millilitre
mRNA	Messenger RNA
NET	Neutrophil Extracellular Trap
NLR	Nod-like Receptor
nsp	Non-structural Protein
ORF	Open Reading Frame
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Unit
PMN	Polymorphonuclear
PRNT50	Plaque Reduction Neutralizing Test 50
qPCR	Quantitative PCR
RBD	Receptor Binding Domain

RdRp	RNA dependent RNA polymerase
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute Medium
SARS- CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SIFV	*Sulfolobus islandus* Filamentous Virus
sp	Structural Protein
ssDNA	Single Stranded DNA
ssRNA	Single Stranded RNA
SST	Serum separator tube
TCID50	Tissue Culture Infectivity Dose 50
TCR	T Cell Receptor
TLR	Toll-like Receptor
TMV	Tobacco Mosaic Virus
TMPRSS2	Transmembrane Serine Protease 2
TNF	Tumour Necrosis Factor
UTR	Untranslated Region
VLP	Virus Like Particle
VTM	Viral Transport Medium
WHO	World Health Organization

Chapter 1:

INTRODUCTION

SARS-CoV-2 is the causative agent of the Covid-19. In Covid-19 pandemic, it is essential to estimate the immune responses to various Covid-19 vaccinations in order to estimate the efficacy of these vaccines. The neutralizing antibody responses show a positive correlation with vaccine efficacy. So far cellular and humoral immune responses to primary vaccination and booster vaccination have been investigated. However, the immune responses of the BNT162b2 or CoronaVac booster in the individuals previously fully vaccinated with CoronaVac have not yet been fully elucidated. In the following section, to focus on the humoral immune responses of the booster vaccination, general information about viruses, SARS-CoV-2 and immune responses were introduced.

1.1 Viruses

Viruses are one of the infectious microorganisms consisting of a nucleic acid segment, RNA or DNA and a protein segment that envelops the nucleic acid. Viruses can also be described as the obligate intracellular parasites. They do not show any metabolic activity and they are not susceptible to antibiotics (Taylor, M. W., 2014).

Viruses cannot replicate their genetic material on their own. To replicate, viruses should first infect the other cells, which are called host cells. They require the host cell's mechanisms to replicate their genome and make copies of themselves. These mechanisms are transcription and translation. The life cycle of a virus consists of 6 stages (Figure 1.1). These stages are viral attachment, penetration, uncoating, macromolecule synthesis (replication and translation), assembly, and release (Cann, A. J., 2008).

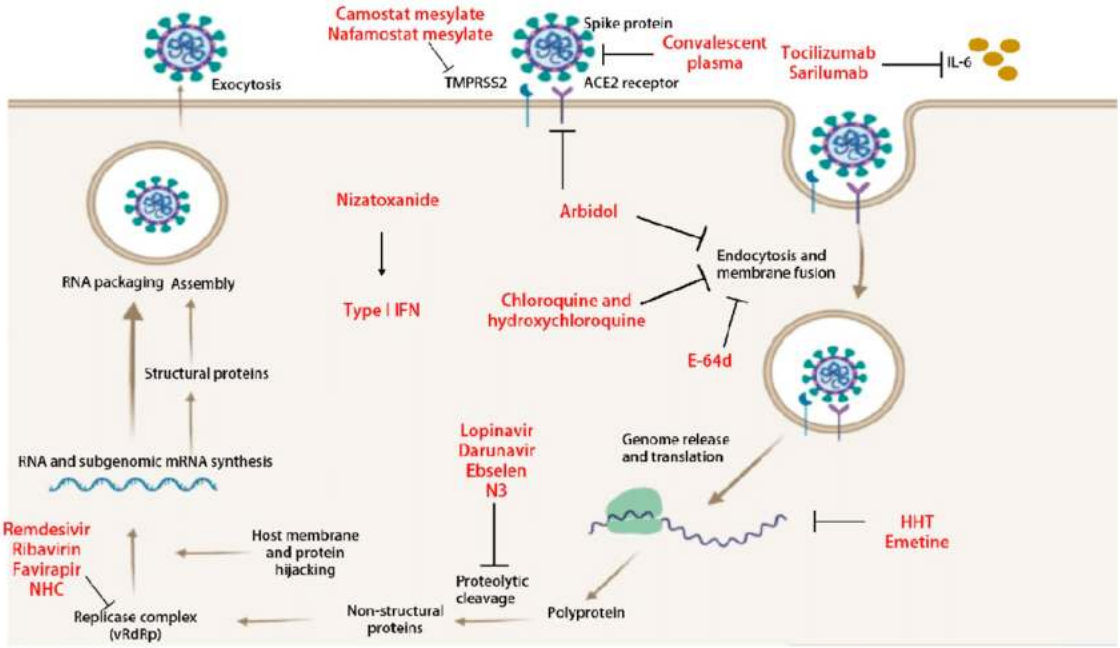


Figure 1.1: The stages of the viral life cycle. The stages consist of viral attachment, penetration, uncoating, macromolecule synthesis (replication and translation), assembly and release. These stages correspond to the lytic life cycle of a virus (revised from Neerukonda, S., and Katneni U., 2020).

In the viral attachment stage, the glycoproteins located on the virus surface interact with the receptors found on the host cells. This interaction facilitates the viral entry. Different viruses infect different host cells by different interactions. For example, EBV (Epstein-Barr Virus) infects the B cells by CR2 (complement receptor 2) on the cells. On the other hand, HIV infects the helper T cells by CD4 (cluster of differentiation 4) on the cell surface (Fenner, F., et. al., 1984). In the penetration stage, viruses enter the host cell. In this stage viruses use 3 main different strategies. These strategies are membrane fusion, receptor mediated endocytosis and viropexis. In the membrane fusion strategy, enveloped viruses use the fusion proteins on their surface. The envelope is fused with the membrane of the host cell. This is not a common strategy used by viruses. The receptor mediated endocytosis is the most common strategy used by the viruses. This interaction is mediated by the clathrin proteins. By the formation of coated vesicles, the viruses are transported to the target area in the host cells. This mechanism is a normal process used by the cells (endocytosis). The last strategy is viropexis, in this strategy small naked RNA viruses can slip through the cell membrane. This strategy is also rare for viruses. In the uncoating stage, the viral genome is released into the host cell. This step is mediated by the low pH created by the fusion of the endosomes with lysosomes (Dimitrov, D. S., 2004). The 4th

stage, macromolecule synthesis consists of 2 main steps. Replication and protein synthesis. After the release of the genome, viruses transcribe their genome by using the host transcription mechanisms. This step can occur in the cytoplasm or nucleus based on the type of the virus. For the DNA viruses this step occurs in the nucleus by the help of DdRp and for the RNA viruses it occurs in the cytoplasm by the help of RdRp. The synthesis sequence occurs as the following, early mRNA synthesis which is required for the shut off the host cell replication machinery and formation of non-structural proteins, replication of genome and late mRNA and protein synthesis which is required for the formation of structural proteins (viral proteins and capsid proteins). Viruses also use the translation machinery of the host cell to produce their proteins. This stage always occurs in the cytoplasm. In the assembly stage the replicated viral genome is brought together with the proteins to form the new viruses. In the last stage, newly formed viruses are detached from the host cell either with budding or host cell lysis. Mostly ~ 95% of the released virions are not infectious (Rampersad, S. and Tennant, P., 2018)

There are different classification methods for the viruses. Based on the International Committee on Taxonomy of Viruses (ICTV) system, the viruses are classified according to their structure (ICTV, 2020). Based on the Baltimore system the viruses are classified according to their nucleic acid content, strandedness, sense, and method of replication. Even though the ICTV system is the official taxonomy system, The Baltimore system is also commonly used. The Baltimore system classifies the viruses into 7 groups (Figure 1.2), as shown in following,

Class I: dsDNA viruses

Class II: ssDNA viruses (+) sense DNA

Class III: dsRNA viruses

Class IV: (+) ssRNA viruses (+) sense RNA

Class V: (−) ssRNA viruses (−) sense RNA

Class VI: ssRNA-RT viruses (+) sense RNA with DNA intermediate in life cycle

Class VII: dsDNA-RT viruses (Mahmoudabadi, G. and Phillips, R., 2018)

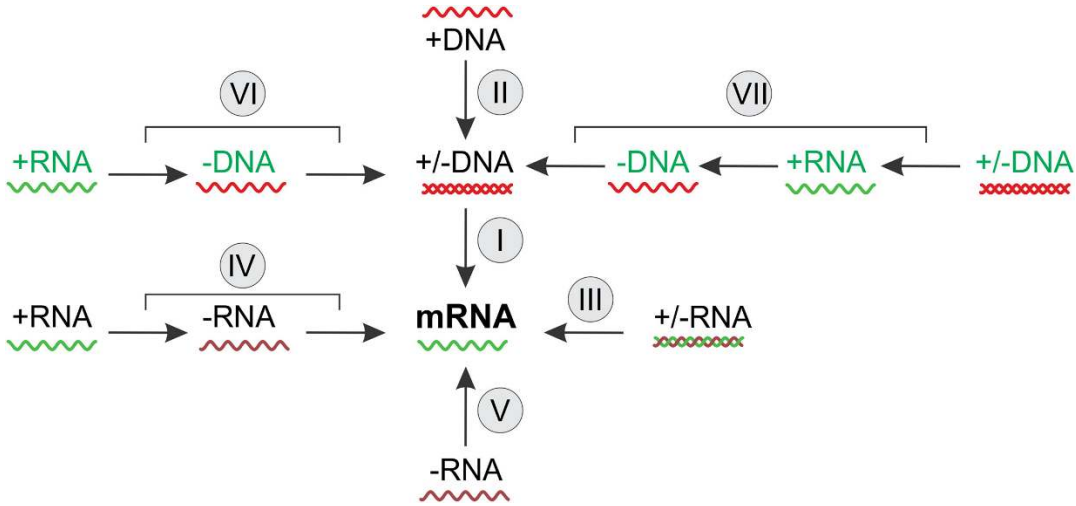


Figure 1.2: The classification of viruses based on the Baltimore System. In this system, viruses are classified according to their nucleic acid content, strandedness, sense, and method of replication. Viruses are divided into 7 distinct groups in this system (revised from Koonin, E.V., et al., 2021).

DNA and RNA viruses have differences according to the classification and replication strategies. DNA viruses are divided into 3 groups. These groups are double stranded DNA viruses, single stranded DNA viruses and complicated DNA viruses. ds-DNA viruses can be enveloped or non-enveloped (naked). Non-enveloped DNA viruses can have circular or linear DNA. ss-DNA viruses are enveloped, and complicated DNA viruses are non-enveloped. RNA viruses are divided into 3 groups based on the polarity of the genome. These groups are + sense ss-RNA viruses, - sense ss-RNA viruses, and ds-RNA viruses. + sense ss-RNA viruses can be enveloped or nonenveloped. – sense ss-RNA viruses are enveloped, and ds-RNA viruses are non-enveloped. Based on the replication strategies, RNA viruses replicate their genome in the cytoplasm, in other words their target in the host cell is cytoplasm. DNA viruses replicate their genome in the nucleus, in other words their target in the host cell is the nucleus. However, there are exceptions, for example Influenza is an RNA virus, yet the replication of the genome takes place in the nucleus of the host cell. DNA viruses use DdRp (DNA dependent RNA polymerase) and RNA viruses use RdRP (RNA dependent RNA polymerase) enzymes, transcriptase for the replication of the genome (Liu, D., et. al., 2018).

Viruses are capable of escaping the host immune system whereas immune responses are trying to get rid of the virus. Different viruses have developed different strategies to

escape. When the virus efficiently escapes from the host immune responses and continues to spread in the body and after among people. In this case different situations can occur. These situations are endemic, epidemic, pandemic, and outbreak. Even though, these situations are not specific to a virus, these terms can be used for the infections caused by different microorganisms. An outbreak is a term used for the unexpected increase in the number of cases of a specific infectious disease in a particular area or the occurrence of the infectious disease in a novel area. Endemic is a term used for an infectious disease effects particular group of people or a particular county. Epidemic is a term used for an infectious disease that affects large populations, within a community or a country. A pandemic a kind of epidemic spread to multiple countries or continent (Famulare, M.and Hu, H., 2015)

So far during human history many major pandemics were reported caused by the viruses. Some of the examples of these pandemics are H1N1 pandemic in 1918, 1957-1958 H2N2 pandemic, 1963 H3N2 pandemic and 2009 H1N1 pandemic. Nowadays scientists are debating whether the pandemic is over and in many countries the precautions required to stop the spread of virus are being lifted (Piret, J. and Boivin, G., 2021).

1.2 SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus)

The SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus) is the reason for Covid-19. It was first identified in Wuhan; China in December 2019. The virus has spread worldwide since first identification. In January 2020, after the occurrence of the virus in many countries and different continents, The World Health Organization (WHO) Emergency Committee declared the pandemic for Covid-19. So far since the first identification 512,607,587 confirmed cases and 6.243.038 deaths were reported (WHO,2022). SARS-CoV-2 is an enveloped positive strand RNA virus. Based on the classification, it belongs to the family *Coronaviridae*, the subfamily *Coronavirinae* and the genera *Betacoronaviruses.* SARS-CoV-2 belongs to the *Betacoronavirus* b2 lineage (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020). SARS-CoV-2 is a + strand ss-RNA virus. The RNA is approximately 29.9kb in

size, 29,903 nucleotides (Figure 1.3). The RNA consists of 14 open reading frames (ORFs). In these ORFs, 27 proteins are coded. These proteins are both structural proteins (sp) and non-structural (nsp). At the 5'UTR of the RNA, a replicase complex is found. This replicase complex consists of ORF1a and ORF1b. ORF1a encodes for the proteins nsp1 to nsp10. ORF1b encodes for the nsp1 to nsp16. 4 additional genes are found downstream of the replicase complex. The genes encode the Spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. After these genes, poly-A tail is located on the 3'UTR end of the genome (Rastogi, M., et. al., 2021).

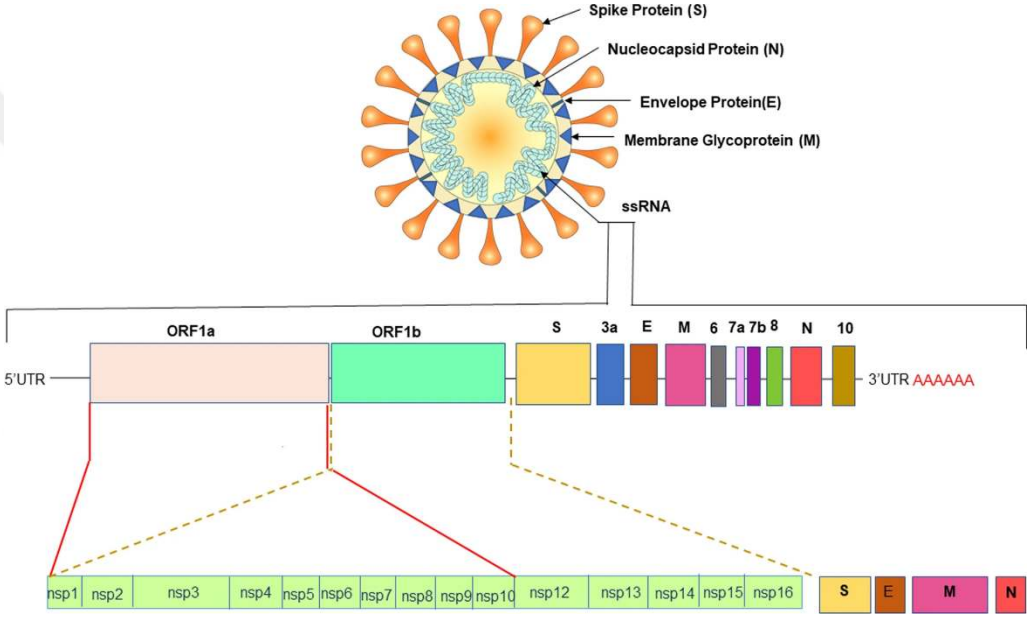


Figure 1.3: The structure and genomic organization of the SARS-CoV-2. The structure of the virus consists of spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins in the outer part and + strand ss-RNA in the inner part. The genome encodes for the replicase complex, structural, and non-structural proteins (revised from Rastogi, M., et. al., 2021).

The virus infects the cells with the Spike-ACE-2 (angiotensin converting enzyme-2) receptor interaction. The RBD (receptor binding domain) of the Spike protein is responsible for this interaction. The ACE-2 receptor is found on the cell membrane of many human cells, especially on lung epithelial cells which are in the inner lining of the lung. The interaction between the Spike protein and the ACE-2 receptor enables viral entry to host cells (Yang, J., et. al, 2020). Putative interaction between RBD region and

ACE-2 receptor is facilitated by the activation of the Spike protein by the transmembrane serine protease 2 protein (TMPRSS2) of furin protein which are located on the surface of the host cells. For the effective binding, these mentioned proteins are required. After the entry the SARS-CoV-2 replicates its RNA and produces its proteins by using the host transcription and the translation systems (Petrovszki. D., et. al., 2022). After the replication and production process, viruses increase in number and keep the colonization and the infection in the host (Figure 1.4).

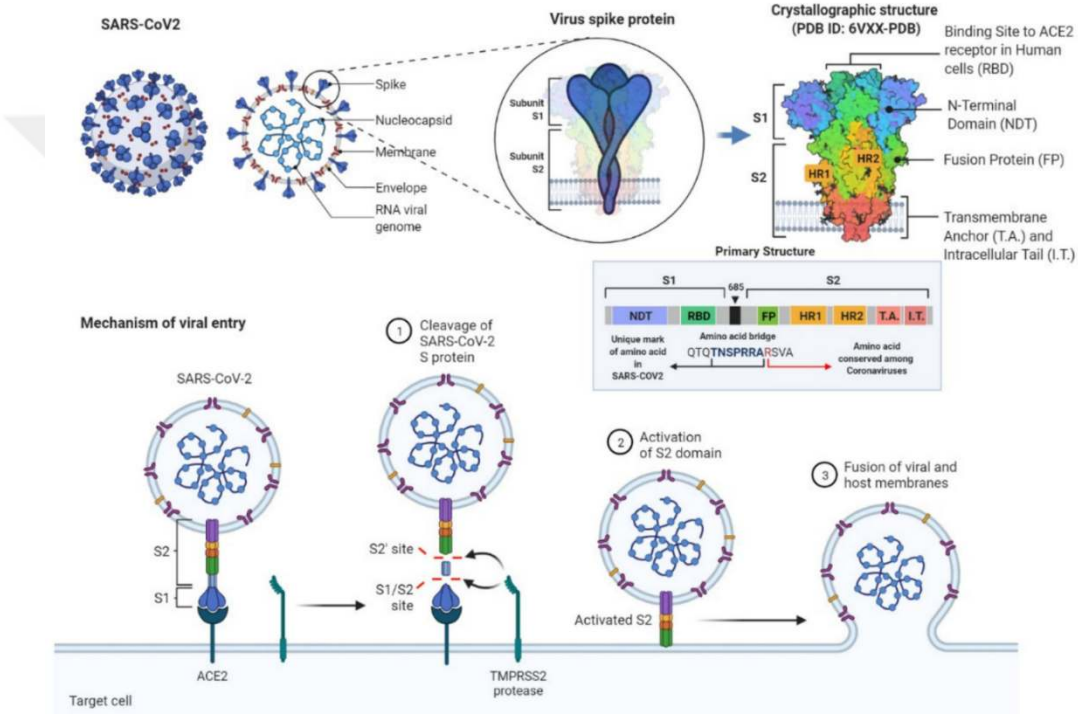


Figure 1.4: The viral attachment of the SARS-CoV-2 with Spike- ACE-2 interaction and viral entry mechanism of SARS-CoV-2 to the host cell with the fusion strategy (revised from Petrovszki. D., et. al., 2022).

The genome of the SARS-CoV-2 shows 96% genome similarity with a bat Coronavirus. Since the first identification, it was concluded that the primary host or the putative host of SARS-CoV-2 is bats (miniopterus bat coronavirus HKU8). With a direct contact, the virus was transmitted to an intermediate host presumably *Manis pentadactyl*. After that with another direct contact, the virus was transmitted to the incidental host *Homo sapiens*. Presence of ACE-2 receptor in the incidental host facilitated the infection. Later that human-to-human transmission with droplets and direct contact was confirmed.

The original virus was named as Wuhan strain or wild type SARS-CoV-2 virus (Zhao, J., et. al., 2020)

After the Wuhan strain (wild type), the virus kept evolving and different variants emerged. The variants are different from the wild-type virus (Wuhan Strain) because of the changes in the genetic material. The new variants have different Spike protein structures. Because of these differences in the protein structures, the protectivity of the vaccines decreases against the variants. The variants evolved to be more transmissible and cause more severe symptoms. So far there are 5 major variants which are identified. These are Alpha (B.1.1.7), Beta (B.1.315), Gamma (P.1), Delta (B.1.617.2) and Omicron (B. 1.1.529). Omicron variant also have 2 different subgroups. These are BA.1 and BA.2. Both of these lineages were first detected in November 2021. Different from the other variants, Omicron has a different evolutionary path (Hirabara, S. M., et. al., 2022). (Figure 5).

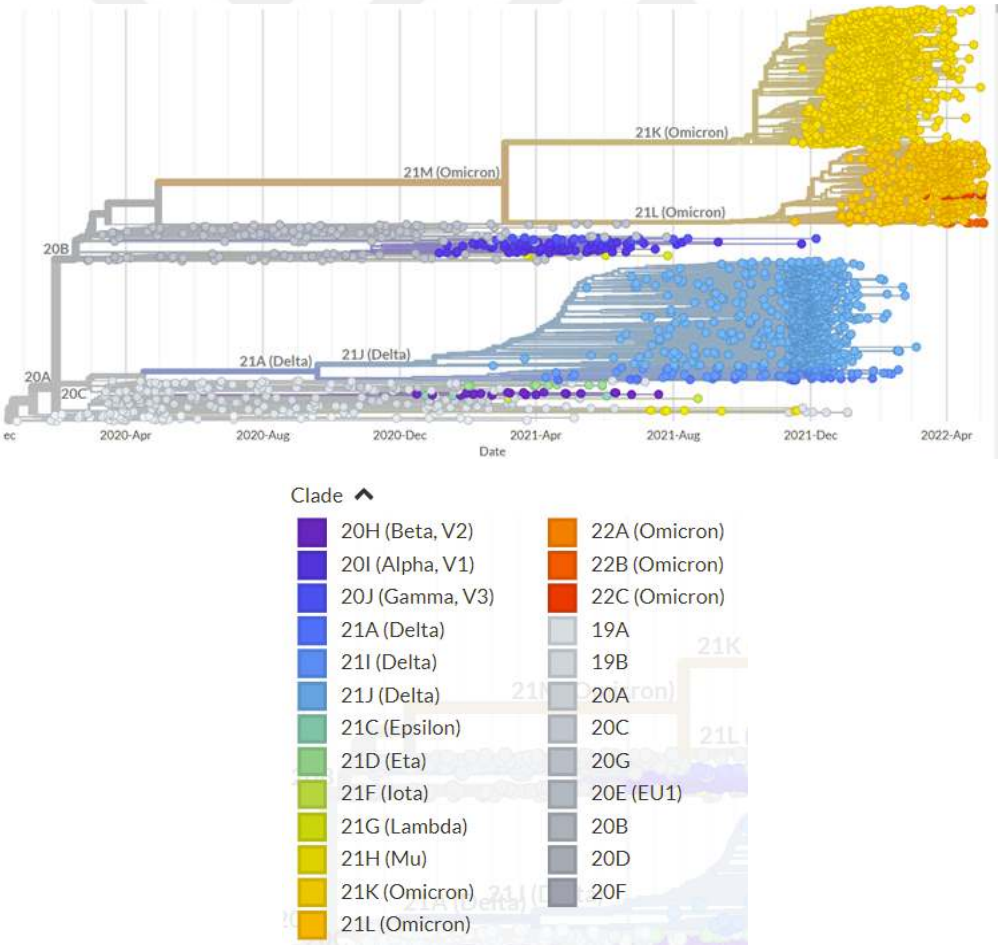


Figure 1.5: The evolutionary path of SARS-CoV-2 variants. The phylogenetic tree starting from the first identification of SARS-CoV-2 until April 2022. The variants were

represented with different colors. The Omicron variant lineage emerged from a different line than other variants of concern. (Adapted from GISAID, Nextstrain.org, 2022)

1.3 Covid-19 (Coronavirus disease-19)

The clinical outcome of the SARS-CoV-2 infection can be different for each individual. Some people don't show any symptoms, this is the asymptomatic group. If an individual shows symptom, the disease can be mild-moderate or severe. General symptoms are cough, fever, muscle and joint pain, headache, loss of taste and/or smell, and fatigue. In some individuals, gastrointestinal symptoms such as vomiting, and diarrhea are also observed. In more severe cases, hospitalization may occur with different symptoms such as difficulty breathing or shortness of breath, loss of speech or mobility, or confusion, chest pain and pneumonia (Wu, Y., et. al., 2020). In Turkey different options are available for Covid-19 treatment. According to the Covid-19 Treatment guide released by the Ministry of Health, there are different treatment options present. Remdesivir which is the only FDA approved drug, also Oseltamivir, Lopinavir/Ritonavir, and Favipiravir are used in the treatment. Recently, Molnupiravir has been added to the treatment options and is being used for hospitalized patients. Since the beginning of the pandemic monoclonal antibody treatment and convalescent plasma treatment have been used for the severe cases (Ministry of Health of Turkey, Treatment options of Covid-19 outpatients,2021).

In the literature, in previous studies it was stated that approximately 6-8 months after Covid-19 infection (Wild Type), the IgG antibody levels decrease. It was investigated that different IgG subtypes all show a decrease in titers. At first shortly after the administration of the inactivated vaccine, CoronaVac, the antibody titers are detected in high levels, both for IgG types and neutralizing antibodies. After the CoronaVac vaccine the IgG antibody levels start to decrease between 4-6 months. Approximately after 8 months, neutralizing antibody levels and IgG levels decrease under the protective levels. Lastly, according to latest studies and in our laboratory study, approximately 8 months after 2 doses of mRNA vaccine, BioNTech, the IgG and neutralizing antibody levels start to decrease. During this 8-month period antibody levels relatively stay stable. However,

because of the waning immunity, the booster doses started to be administered to increase the protectivity (Pérez-Then, E., et. al., 2021; Khoury, D. S., et. al., 2021; Coa, Y., et. al., 2022). Additionally, it was stated that the IgG levels are not fully correlated with the protectivity levels. The reason is that not all of the IgG antibodies have the neutralizing capacity. The gold standard test for the detection of the neutralizing antibody titers is Plaque Reduction Neutralization Test (PRNT50). Even though PRNT50 is the gold standard, it is not a common practice routinely used in the clinic laboratories. The reasons for this situation are technicalities and lack of BSL-3 (Biosafety level-3) facilities. Mostly it was shown that IgG levels are neutralizing antibody levels and are positively correlated. However, at the individual level, this correlation may change. For different viruses such as the smallpox, the influenza, and the polio in previous studies, this hypothesis was proven. Again, for the SARS-CoV-2, the positive correlation was shown between neutralizing antibody and IgG, yet this situation is not valid for all individuals (Yalçın, T. Y., et. al., 2022)

Another importance about the neutralizing antibodies is that for the estimation of the efficacy of the vaccines, neutralizing antibody levels are used. There is a positive correlation between the neutralizing antibody levels and the efficiency of the vaccines. Because of this reason for the Phase trials of different vaccines, PRNT50 tests are vastly used (Chen, X., et. al., 2022).

1.4 Immune System & Responses

The immune responses against a microorganism or foreign body are generated by the cells of the immune system. Immune cells are generated from the hematopoietic stem cells. Hematopoietic stem cells are multipotent which means that they can differentiate into different cell types, and they have the property to self-renew by cell division. The cells of the innate immune system are neutrophils, eosinophils, basophils, and monocytes (macrophages). The cells of the adaptive immune system are lymphocytes, B and T cells. Natural killer cells also play important roles in the immune system and responses. They are referred to as the connection between innate and adaptive immune systems (Bonilla, F. A. & Oettgen, H. C., 2010; Beutler, B., 2004) (Figure 1.6).

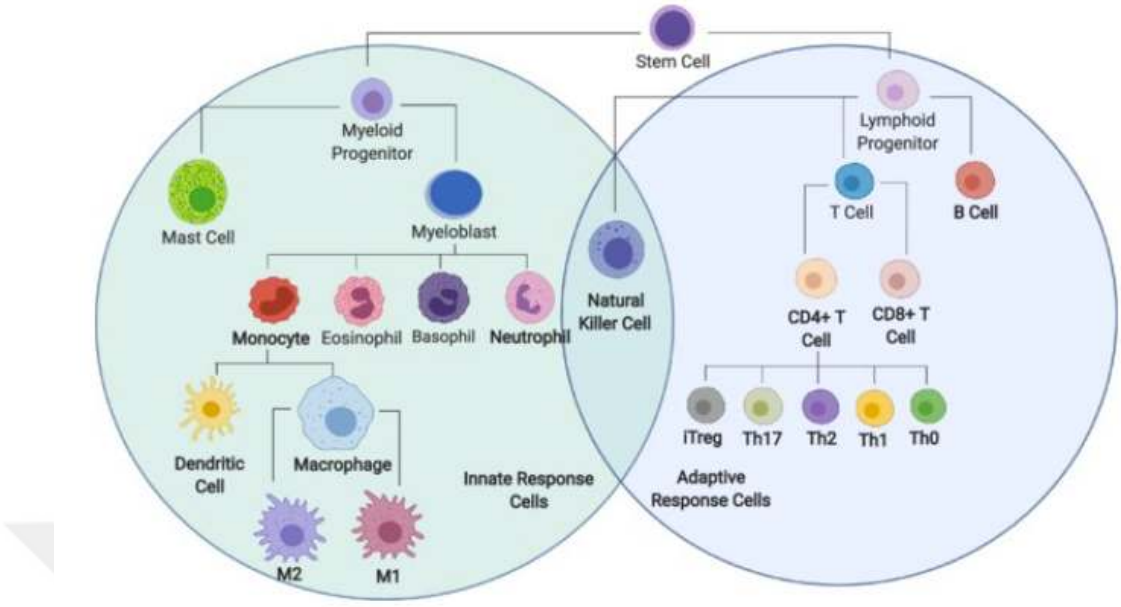


Figure 1.6: The cells of the immune system. Starting from the stem cell, myeloid and lymphoid lineage and each differentiated cell are shown. The cells are classified based on being innate or adaptive. (Revised from Torang, A., et. al., 2019)

Form the myeloid progenitor, in the first differentiation step mast cells and myeloblast cells are produced. From the myeloblast cells, monocytes, eosinophils, mast cells, basophils, and neutrophils are differentiated (Ibáñez -Cabellos, J. S., et. al., 2019). Monocytes are differentiated to the dendritic cells or monocytes. Neutrophils are the first cell type which comes to the site of infection when a pathogen enters the body. Neutrophils also known as the polymorphonuclear (PMN) cell are one of the granulocytes. They have granules in their cytoplasm and when released the enzymes in the granules are toxic to bacteria and fungi. These enzymes cause the formation of reactive oxygen species (ROS) and facilitate the burst of the pathogen (Ziegler-Heitbrock, L., 2015). Neutrophils can also kill the pathogens by phagocytosis. They are short lived, but they are activated rapidly. They also play a role in the inflammatory reactions during the infection. Recent studies also revealed that when neutrophils encounter larger pathogens, NET formation occurs to kill the pathogens (Witko-Sarsat, V., et. al.,2000; Boeltz, S., et. al., 2019). Eosinophils are the other type of granulocytes. They attack multicellular parasites. Eosinophils secrete the proteins and free radicals outside the cell. These proteins and free radicals help to kill the parasites. However, these molecules can also cause the allergic reaction because they also damage the tissues and other normal

cells of the cells. Because of this reason hyperactivation of eosinophils causes allergies and normally the activation is strictly regulated (Hogan, S. P., et. al., 2008). Basophils are also granulocytes. They attack multicellular parasites by releasing the histamine content in their cytoplasm (Min, B., et. al., 2012). Mast cells found in the mucous membranes and connective tissues. This cell type is responsible for both immune responses and wound healing process. By the help of cytokines and granules secreted from the mast cells, an inflammatory cascade is activated. Secreted cytokines act as messengers and conduct the communication between the immune cells. Cytokines facilitate the recruitment of neutrophils and macrophages to the site of infection. Monocytes are one of the phagocytic cells. These cells are able to migrate to the side of infection. In the site of infection these cells are differentiated to the macrophages. These cells are both responsible for phagocytosis and tissue repair as M1 and M2 macrophages subtypes (Krystal-Whittemore, M., et. al., 2016; Italiani, P. & Boraschi, D., 2014).

The cells of the adaptive immune system are lymphocytes. Lymphocytes are B and T cells. Those cells are produced in the bone marrow, but they get mature in different sites of the body. B cells mature in the bone marrow and T cells mature in the thymus. B cells are responsible for the humoral immune responses and T cells are responsible for the cellular immune responses in the adaptive immune system (Cooper, M., et. al., 2006). B cells are responsible for the recognition of pathogens and the production of antibodies. After the production of antibodies, they are mostly secreted to the bloodstream. The structure of an antibody contains two chains. These chains are light chain and heavy chain. There are also two distinct regions of an antibody, which are FAB (Fragment antigen binding) and FC (fragment crystallizable) regions. The FAB region is located at the end of ‘Y’ shape. In this region there are variable regions. Variable regions are responsible for binding to the antigen. These regions in different antibodies bind to the specific pathogens. The changes in the variable regions, provides antibodies to recognize pathogens (Chiu, M. L., et. al., 2019).

Based on their properties, the antibodies can be divided into 5 groups. These groups are IgG, IgE, IgA, IgM and IgD. An antibody can be responsible for many functions in the immune system. Some of these immune activities are the neutralization of the biological effect of a pathogen, the activation of the classical pathway of the complement system, the opsonization of the pathogens and enhancing the phagocytosis of the pathogens, the direct cell lysis. At the same time, antibodies have properties such as

immune regulation. In the allergic conditions the IgE antibodies are produced against the allergen. IgA is the antibody associated with mucosal immunity. The only type of antibody which is not secreted to the bloodstream is IgD. After the production of the IgD, antibodies stay on the B cell membrane, and they play roles in the B cell maturation. It is also believed that the IgD may also have a role in the B cell selection and homeostasis. The first antibody produced against a pathogen is the IgM antibody. It has a pentameric structure and binding affinity of this antibody to the pathogen is weak. In the late stages of an infection IgG antibodies are produced. The binding of this antibody to the pathogen is high, and IgG is mainly responsible for the long-term protection of the pathogen after an infection or vaccination. In this point IgG antibodies have a significant importance. Other antibodies than IgG, are degraded shortly after they are secreted to the bloodstream, their half-life is shorter than IgG. The reason for this situation is the FC-FC receptor interaction. The IgG antibodies go back to the circulation, and they escape from the phagocytic degradation (Forthal, D. N., 2014; Sela-Culan, I., et al., 2013).

The T cell responses are the second part of the adaptive immune responses. Based on the cell markers, there are two types of T cells, the helper T cells also referred to as CD4+ T cells and the cytotoxic T cells also referred to as CD8+ T cells. The helper T cells are responsible for the activation of the B cells. With this activation B cells produce the specific antibodies against the pathogens. T helper cells also secrete cytokines. Cytokines are small protein molecules secreted from various cells. They are responsible for the communication between the immune cells. The most important of these cytokines are IL-2 (Interleukin-2) and TNF-α (Tumor necrosis factor-α). By these cytokines, other immune cells can be activated. The activation leads to the clearance of the pathogens. Similar to B cell responses, the T cell responses are specific to the pathogens. The second T cell types is cytotoxic T cells. These cells are responsible for the direct killing of the pathogens. In order to generate the specific cytotoxic T cells, first antigens must be introduced to the T cells. This presentation is done by the help of other immune cells which are called the antigen presenting cells (APCs). The APCs take the pathogen from the site of infection by phagocytosis and lyse the pathogen. After the phagocytosis, the small pieces of the pathogen, mostly small peptide molecules are introduced to the T cells. The interaction is done by the MHC (major histocompatibility complex) Class I/II located on the surface of the APCs. The MHC Class I//II interacts with the TCR (T cell receptor) located on the surface of the T cells. This interaction activates the cytotoxic T cells and

facilitates the direct killing of pathogens by the T cells (Huang, J, et. al., 2012). Natural killer (NK) cells originate from the lymphoid origin. They are responsible for the communication between innate and adaptive immune responses. Even though the natural killer cells have a lymphoid origin they mostly belong to the innate immune cells, and they are located to the innate lymphoid cell family (ILCs). Just like the T lymphocytes, NK cells have cytotoxic properties. They are responsible for the killing of the virus infected cell or tumor cell. This process occurs when the NK cells are activated because of the lack of a specific interaction with the abnormal cells (Vivier, E., et. al., 2008).

Against a virus, both innate and adaptive immune responses are generated. The immune responses involve interferon (IFN) α/β responses to interfere with the replication system of the infected cell to stop the viral replication, NK cells responses to direct killing of infected cells, CD4+ Helper T cell to expand the primary CD8+ T cells, CD8+ T cells for direct killing of infected cells and antibodies for both neutralization and protection (Rouse, B. T. & Sehrawat S., 2010)

1.5 Vaccines

A vaccine is a substance which is given to humans or animals to promote the immune responses, production of antibodies for the protection against a specific type of agent, mostly against viruses. For developing a vaccine there are 4 main methods that can be used. The most common and traditional method is inactivation. In this method, the virulent or viruses are inactivated with a physical or chemical agent. With this method, the infectivity of the virus is destroyed but immunogenicity is retained. CoronaVac vaccine developed by Sinovac is an inactivated/killed vaccine. Another vaccine type is live/attenuated. In this kind of vaccines, the virulence of the virus is still alive while given to the body. In this vaccine, the virulence or pathogenicity is reduced. Another technique is the subunit/conjugate technique. This technique is suitable for a weak antigen. Weak antigen (polysaccharide part) of the virus is combined with another antigen which is stronger than the first one. The other method is the mRNA technique, compared to the other techniques, this one relatively new. In this technique, a small part of the mRNA of the virus is introduced to the body (Schlake, T., et. al., 2012). The mRNA usually codes for a surface protein. Once the mRNA is introduced to the cells, the cells produce the viral

protein as a part of the normal transcription process. When the proteins are produced, immune cells recognize the protein as non-self and immune responses are produced. BNT162b2 vaccine produced by Pfizer/BioNTech, and mRNA-1273 vaccine produced by Moderna are mRNA vaccines against Covid-19. Beside these 4 main groups, vaccines can also be produced by virus like particle (VLP) technique and adenovirus technique as well (Pollard, A. J., et. al., 2021).

1.6 Vaccines produced against SARS-CoV-2 & Vaccination Strategies

Since the beginning of the pandemic, to eradicate the disease, many different vaccines were produced and started to be applied worldwide. Food and Drug Administration (FDA) approved the emergency use of 3 vaccines, mRNA-1273 (Moderna), Ad26.COV2.S (Johnson & Johnson–Janssen), and BNT162b2 (Pfizer-BioNTech) between December 2020-February 2021 (FDA,2021). Other vaccines are also currently in use in the healthcare system. Many other vaccine candidates are also in the phase trials. In Turkey the vaccination strategy started with inactivated vaccine CoronaVac (Sinovac). Healthcare workers and individuals 65 years of age received 2 doses of CoronaVac. Remaining of the population mostly vaccinated with mRNA vaccine, BNT62b2 vaccine. After this regimen, application of booster doses was started due to the waning immunity. Individuals received CoronaVac or BNT162b2 as the booster doses. In summary, for the Turkish population, some individuals received a homologous vaccination with 3 doses of CoronaVac and some individuals received heterologous vaccination 2 doses of CoronaVac and 1 dose of BNT162b2 vaccine (Ministry of health, 2020) Turkish National COVID-19 Vaccine Administration Strategy). Based on the ‘Our World in Data’ (4.5.22) in Turkey 62.36% (53.04 million) of the population is fully vaccinated (2 doses of vaccine) and 5.63% (4.78 million) of the population is partially vaccinated. Which means that 32.01% of the population is not vaccinated at all.

In different countries vaccination strategies were different than Turkey, as an example in some countries 2 doses of BNT162b2 was applied or in others 1 dose of Ad26.COV2.S vaccine was applied. There are also a small number of countries in which

CoronaVac was applied. Nowadays many countries have started the application of booster doses. With the booster doses, heterologous and homologous vaccination was started to be applied (Atmar, R. L., et. al., 2022).

In previous studies it was shown that BNT162b2 has high efficacy against wild-type virus and other variants and CoronaVac produces high levels of antibody responses shortly after vaccination, but the antibody responses decrease rapidly and because of this reason the protectivity of the vaccine decreases rapidly. However, the effect of booster doses on the immune responses are still under investigation, also there are some knowledge gaps in the literature about the immune responses after booster doses in the individuals primarily vaccinated with CoronaVac (Jara, A., et. al., 2022; Clemens, S. A. C., et. al., 2022).

The aim of this study is to investigate the effects of BNT162b2 or CoronaVac boosters on humoral immunity of individuals fully vaccinated with CoronaVac. This is a longitudinal study.

Chapter 2:
METHODOLOGY

2.1 Study Design and Participants

For this longitudinal study 52 adult participants (health care workers) who had no history of Covid-19 infection and immunized with two doses of inactivated vaccine CoronaVac were included to the cohort. The participants were selected from the healthcare workers in Koç University Hospital, Istanbul University Cerrahpaşa Hospital, and Istanbul University, Istanbul Medical School Hospital.

From all participants, venous blood samples were collected at baseline, three to five months after the second dose of CoronaVac. (n=19 for 3 months, n=26 for 4 months, n=7 for 5 months). Follow-up venous blood samples were collected one and three months after the administration of BNT162b2 or CoronaVac booster doses. During this study all participants were followed up in case of occurrence of Covid-19 infection. From all participants, age, gender, height, weight, current smoking status, presence of comorbidity and presence of immunosuppression information were obtained. From height and weight information, BMI of all participants was calculated. Informed consent was obtained from all participants.

From 52 participants, 42 of them received BNT162b2 booster and 10 of them received CoronaVac booster. After one month of administration, venous blood samples were collected from all participants in both groups (n=42 and n=10). After 3 months of administration only 6 participants from BNT162b2 and 4 participants from CoronaVac group were able to donate blood samples. The number of participants had to be decreased because some of the participants received the 4^{th} dose of vaccination before the third month. The timeline, number of participants for each time point and the sampling strategies were presented in Figure 2.1. This study was approved by The Institutional Review Board of Koç University by the number of 2021. 151.IRB1.055.

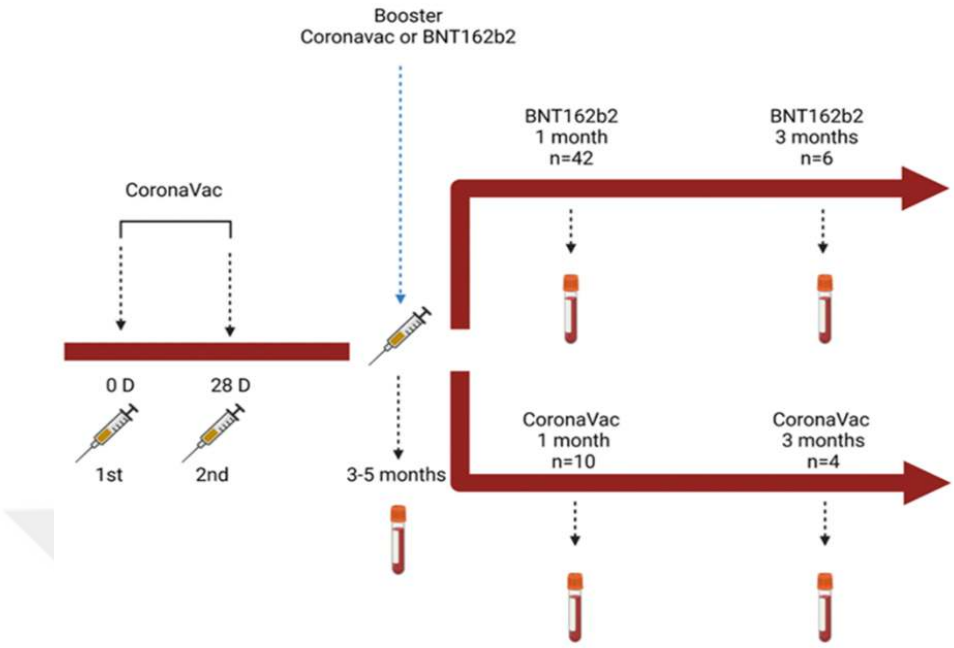


Figure 2.1: Methodological plan of the study. Baseline and follow-up blood sample collection time points and the number of participants which donated blood samples in that particular time point (This figure was created in Biorender.com).

2.2 Blood collection & PBMC and Serum Isolation

To investigate the antibody titers and B cell responses of the participants, venous blood samples were collected. PBMCs and serum samples were isolated. Sera were used in the Plaque Reduction Neutralization Assay after growth of the live Wuhan strain (B303) virus. PBMC samples were used in the Flow Cytometry to determine the percentage of naïve and memory B cells in addition IgG expressing and CD38 expressing B cells.

Blood samples from vaccinated individuals and healthy individuals (SARS-CoV-2 naïve individual which had no history of Covid-19 infection or Covid-19 vaccination in any type), were collected in green capped blood collection tubes in which the additive agent is heparin and yellow capped tubes which are also known as serum separator tube (SST).

For each time point, 3 green capped tubes (approximately 30 ml of blood sample) and 1 yellow capped tube (approximately 10 ml of blood sample) of venous blood was collected. Right after the collection blood samples were transferred to the laboratory to process.

From the whole blood samples, peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep density gradient method. In this step Lymphoprep was transferred to the Falcon tubes, the whole blood sample diluted with 1:1 1XDPBS was transferred onto the Lymphoprep. While transferring the whole blood sample on to the Lymphoprep, to create the density gradient, the tube was tilted to a 45-degree angle and blood sample was slowly added to the tube from the wall. The tubes were then centrifuged at 2000 rpm for 15 minutes with AC:5/DC: off settings. After centrifugation blood samples were separated into 3 distinct phases: the bottom layer containing the red blood cells (RBCs), the middle layer containing the PBMCs and the upper layer containing the serum. The three distinct layers after centrifugation were shown in Figure 2.2. The middle phase (buffy coat) was collected by Pastor pipettes without disturbing and mixing the layers. The collected PBMC layer was then transferred to a new Falcon tube and diluted with 1XDPBS. Tubes were centrifuged at 2000 rpm for 5 minutes with AC:max/DC:max settings. The cells were pelleted by the help of centrifugation, and the pellet was resuspended with 3 ml of freezing medium. (10% DMSO (Dimethyl sulfoxide) and 90% FBS (Fetal Bovine Serum)). Resuspended PBMCs were aliquoted to the cryovials in equal amounts. PBMC samples were stored at -80°C until use for Flow Cytometry. Sera were isolated with centrifugation; the tubes were centrifuged at 2000 rpm for 10 minutes with AC:max/DC:max settings. After centrifugation the blood sample was separated into 2 distinct layers: the upper layer containing the serum and the bottom layer which contains other components of the blood. The gel of the yellow capped tube separated the two layers. After centrifugation, the serum samples were aliquoted in equal amounts into the 1.5 ml Eppendorf tubes. The serum samples were stored at -20°C for a short amount of time until their use in plaque reduction neutralization tests.

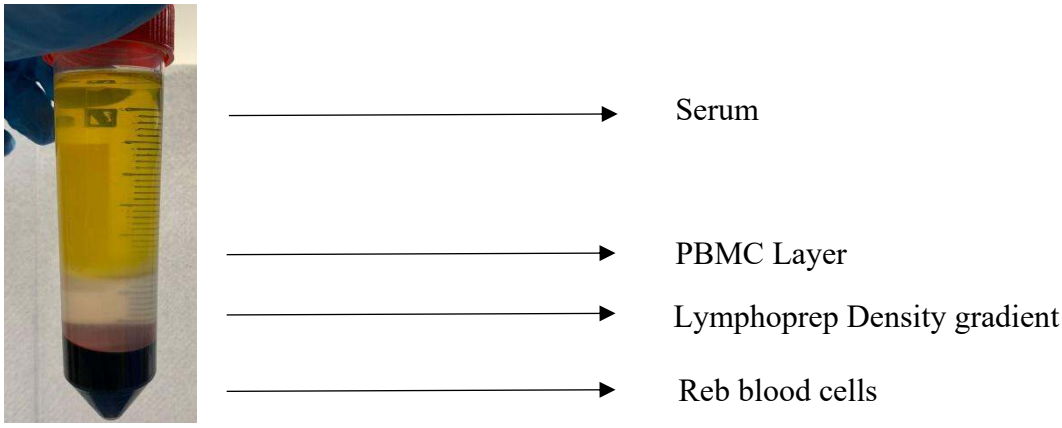


Figure 2.2: Three distinct layers formed in PBMC isolation, upper layer serum, middle layer (buffy coat) PBMCs and bottom layer red blood cells. Picture was taken after Lymphoprep density gradient centrifugation.

2.3 Flow Cytometry for B cell responses

For the investigation of the B cells responses, flow cytometry was performed with specific B cell marker antibodies. Frozen PBMCs were thawed in the 37°C water bath. Upon thawing, the cells were transferred into the 15 ml Falcon tubes in which contains warm RPMI (Roswell Park Memorial Institute)1640 supplemented with 5% AB Serum (AB Serum was obtained from a healthy individual which has AB blood type.). After that the tubes were centrifuged and the cells were pelleted. After the centrifugation, the supernatant was discarded, and the cell pellet was thawed by using RPMI1604 media. 1 µl from each PBMC suspension was taken and mixed with 9 µl of Trypan Blue. The mixture was loaded onto the hemocytometer and the cells were counted with the help of Cell Counter. After that 2 x 10^6 PBMCs were taken to flow tubes. The cells were stained with viability dye Zombie NIR. After staining, the flow tubes were incubated at room temperature in a dark environment for 20 minutes. After that the tubes were centrifuged at 2000 rpm for 5 minutes AC:max/DC:max settings. The supernatant was discarded and then the cell pellet was resuspended with 1% BSA (Bovine Serum Albumin). Then the tubes were centrifuged at 2000 rpm for 5 minutes and the cells were pelleted. The supernatant was discarded, and the pelleted cells were resuspended with specific antibodies for B cell markers for memory and effector B cells Anti-CD19, Anti-CD20,

Anti-IgG, Anti- CD27, Anti- CD14, Anti-CD38 and Anti-CD56. After staining, the samples were run by Attune NxT flow cytometer. First the percentage of naïve and memory B cells were determined. After that, the percentage of naïve B cells expressing CD38 expressing (naïve effector) and memory B cells expressing CD38 (effector memory) were determined. The other group of B cell subpopulations which were investigated, are naïve B cells expressing IgG and memory B cells expressing IgG. For the B cell response analysis 35 individuals were included. Respectively the sample sizes were 35 For baseline, 28 for BNT162b2 booster 1st month, 7 for CoronaVac booster 1st month, 6 for BNT162b2 booster 3rd month, and 4 for CoronaVac booster 3rd month groups. The percentages of the naïve and memory B cells, naïve cells expressing IgG and memory cells expressing IgG, naïve cells expressing CD38 and memory cells expressing CD38 were analyzed and estimated by the FlowJo software. The changes in these B cell subpopulations were investigated based on different time points: baseline 1 month and 3 months after the administration of booster dose. First memory B cell and naïve B cell percentages were investigated for each participant in the given time points.

2.4 Plaque Reduction Neutralization Test (PRNT50): Neutralizing antibody titers

2.4.1 Cell Culture and Cell Passaging

Vero E6 cell line (ATCC (American Type Culture Collection) CRL-1586) was obtained from another laboratory. Upon receiving, the cells were thawed at 37°C water bath and were transferred to a 15 ml Falcon tube which contains cell culture medium, DMEM high glucose supplemented with 10% FBS (Fetal Bovine Serum) and 1% Penicillin/Streptomycin and Amphotericin B. the Falcon tube was centrifuged at 2000 rpm for 5 minutes and the cells were pelleted. Supernatant was discarded and cell pellet was resuspended in 1 ml of fresh cell culture media. Resuspended cells were seeded to T75 tissue flasks containing 9 ml of fresh media. The tissue flask was monitored daily. After 100% fluency was achieved, cell culture was expanded. Confluent cells in T75

tissue flasks are shown in Figure 2.3. For the expansion first, the media in the T75 tissue flask was discarded. The cells were washed with 1XDPBS and DPBS was discarded. 3 ml of Trypsin-EDTA was added to the tissue flask and the flask was incubated at 37°C and 5% CO_2 incubator. After 5 minutes, detachment of the cells from the tissue flask was observed. The cell suspension was collected into a Falcon tube. The tube was centrifuged at 2000 rpm for 5 minutes. After centrifugation, the cells were pelleted. The supernatant was discarded, and the cell pellet was resuspended with fresh cell culture medium. The resuspended cells were passaged to 2 new T75 tissue flasks. The cells were passaged in every 2-3 days. While the cell culture was expanding, the main stocks were created by freezing the cells. After trypsinization and formation of the cell pellet by centrifugation, the pellet was resuspended with 1 ml of freezing medium, %90 FBS supplemented with 10% DMSO (Dimethyl sulfoxide). The resuspended cells were aliquoted in equal amounts into the cryovials. The cryovials were then transferred to -80°C. For long term storage, the cells were maintained in a liquid nitrogen tank. In the case of a need, the different batches of frozen cell lines were thawed and seeded to T75 tissue flasks. After cell culture expansion, for the plaque assay experiments, the cells were detached from the T75 tissue flasks with the help of trypsin. The cells in one 100% confluent T75 were seeded to two 6 well plates. For this purpose, after the cells were pelleted by centrifugation, the cells were resuspended in 12 ml of fresh cell culture medium. The 2 ml of resuspended cells were seeded to each well of the 6 well plates. The 6 well plates were monitored daily until 100% confluency was reached. The one well of 6 well plate with 100% confluency was shown in Figure 2.4. For each experimental set, the 100% was achieved in approximately 2-3 days. After that, the cells were transferred to BSL-3 laboratories.

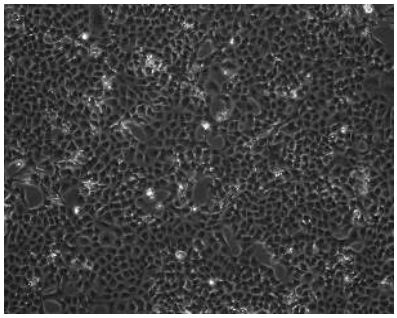


Figure 2.3: 100% Confluent Vero E6 cells grown in T75 tissue flasks. Picture was taken by Paula Leica microscope.

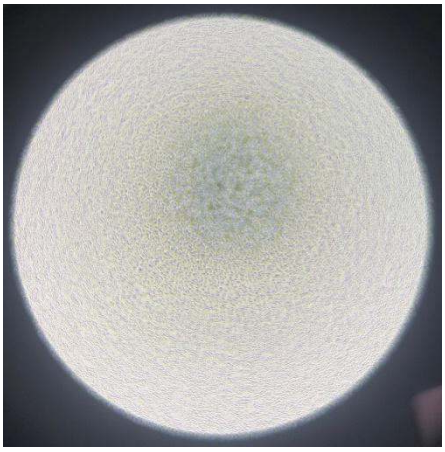


Figure 2.4: 100% Confluent Vero E6 cells grown in 6 well plates. Picture was taken with a Leica light microscope with a 20X objective.

2.4.2 SARS-CoV-2 viral Culture

Live SARS-CoV-2 Wuhan strain (B303) was used for the plaque assay experiments. The live virus was previously isolated from SARS-CoV-2 RdRp PCR positive nasopharyngeal specimen of a patient under the Biosafety Level-3 (BSL-3) conditions. After nasopharyngeal samples were transferred into the VTM (viral transport medium). The VTM was DMEM high glucose supplemented with 5% FBS (Fetal Bovine Serum) and 1% Penicillin/Streptomycin and Amphotericin B. The nasopharyngeal specimen was inoculated onto the Vero E6 cells grown in 96 well plates. For the inoculated samples cytopathic effect (CPE) was monitored. For the samples which CPE was present, viral culture was expanded. For the expansion, the supernatant of the wells in which CPE was observed was collected and the cells were pelleted by centrifugation at 2000 rpm for 5 minutes. The supernatant then was inoculated on to the Vero E6 cells grown in 6 well plates. The cell cultures were monitored for CPE. After the observation of CPE, the supernatants were collected, and the cells were pelleted by centrifugation at 2000 rpm for 5 minutes. The supernatant then was inoculated on to the Vero E6 cells grown in T25 tissue flasks. The cell cultures were monitored for CPE. After the observation of CPE, the supernatants were collected, and the cells were pelleted by centrifugation at 2000 rpm for 5 minutes. The supernatant then was inoculated on to the Vero E6 cells grown in T75

tissue flasks. The cell cultures were monitored for CPE. After the observation of CPE, the supernatants were collected, and the cells were pelleted by centrifugation at 2000 rpm for 5 minutes. The supernatant then was inoculated on to the Vero E6 cells grown in T175 tissue flasks. After the observation of CPE supernatants were collected, and the cells were pelleted by centrifugation at 2000 rpm for 5 minutes. The supernatant was aliquoted in equal amounts in to the cryovials and virus samples were stored at -80°C until use.

2.4.3 The Confirmation of the SARS-CoV-2 Growth

SARS-CoV-2 viral growth was confirmed by qRT-PCR. For the PCR, specific primers and TaqMan probes targeting the SARS-CoV-2 nucleocapsid (N1) were used. After the qPCR confirmation, TCID50 experiments were performed. For the TCID50 (Median Tissue Culture Infectious Dose), the stock virus was serially diluted (10^0 to 10^{-12}). Serially diluted virus samples were inoculated onto approximately 90% confluent Vero E6 cells grown in the 96 well plates. The plates were incubated for 7 days in 37 °C and 5%CO_2 environment. On the 7^{th} day, the plates were fixed with 4% PFA (paraformaldehyde). The CPE of different wells were compared with each other by light microscope. The TCID50 of the SARS-CoV-2 isolate was determined by the Spearman-Karber method. For the sequencing of the viral genome, first viral RNA was extracted with Viral RNA isolation kit (QIAamp viral RNA), DNA library preparation was performed by using the Illumina TruSeq stranded total RNA kit, and the viral RNA was sequenced using Illumina MiniSeq (GenBank: MT675956).

2.4.4 Plaque Assay

PRNT50 experiments were performed in KUISCID BSL-3 laboratories. Vero E6 cells were seeded to T75 tissue flasks by using DMEM High Glucose supplemented with 10% FBS and 1% Penicillin/Streptomycin and Amphotericin B. After 100% confluency was achieved, cells were tripsinized and then seeded to 6 well plates. The cultures were monitored for 100% confluency. For different vaccination status, different serial serum

dilutions were performed. For the baseline, three to five months after 2nd dose of CoronaVac, serum samples were serially diluted from 1/10 to 1/320. For the BNT162b2 group, the serum samples were serially diluted from 1/40 to 1/1280 and for the CoronaVac group, the serum samples were serially diluted from 1/10 to 1/320 in the 96 well reservoirs. Both for BNT162b2 and the CoronaVac group previously described serum dilutions were performed for both 1 month and three months after vaccination. The demonstration of serial serum dilutions was represented in Figure 2.5. 300 µl from serum dilutions of each participant and 300 µl SARS-CoV-2 at the multiplicity of infection (MOI) 0.01 were mixed and incubated for 1 hour at 37°C and 5% CO_2 incubator. After incubation 600 µl of mixture were transferred on to the 100% confluent Vero E6 cells which were seeded to 6 wells. Serum-virus mixture was incubated on the Vero E6 cells for 1 hour at 37°C and 5% CO_2 incubator. The mixture was discarded, and cell monolayers were coated with 2% methylcellulose and DMEM High glucose supplemented with 5% FBS and 1% Penicillin/Streptomycin and Amphotericin B. The plates were incubated for 4 days at 37°C and 5% CO_2 incubator. After 4 days, the methylcellulose was discarded, the wells were washed with 1XDPBS and then fixed with 4% paraformaldehyde (PFA). After fixation the wells were stained with Gram's crystal violet solution. For each experimental set, virus control was studied in duplicate as positive control, unexposed and unvaccinated individuals' serum samples were studied as negative control and a serum contains SARS-CoV-2 neutralizing antibody at the titer of 1:80 were studied as internal control. The healthy control, virus control, baseline, BNT162b2 1 month, BNT162b2 3 months, CoronaVac 1 month and CoronaVac 3 months plaque assay plates were shown in Figure 2.6.

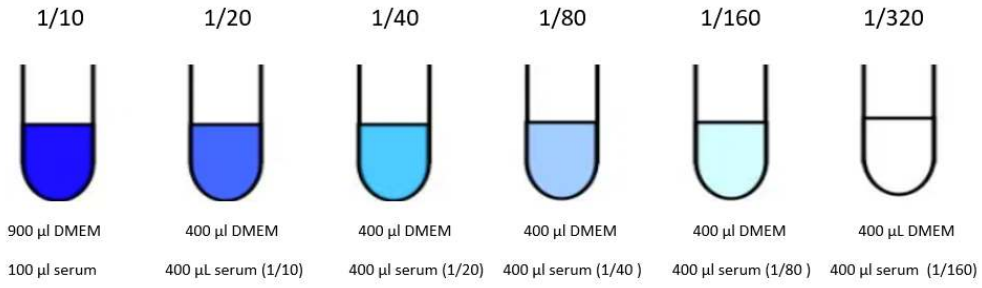
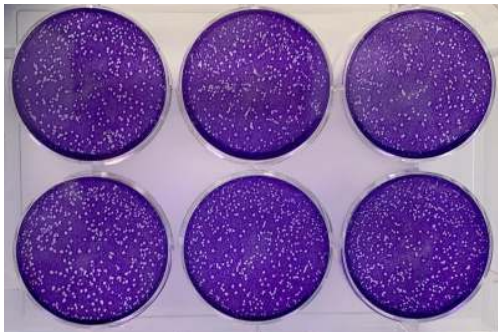
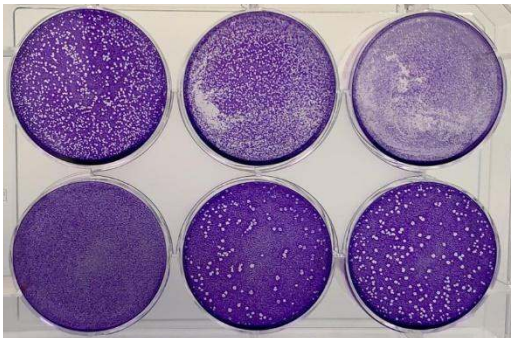


Figure 2.5: The demonstration of serial serum dilutions starting from 1/10 to 1/320.

A.



B.



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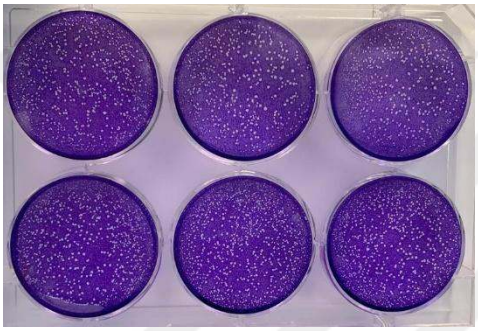

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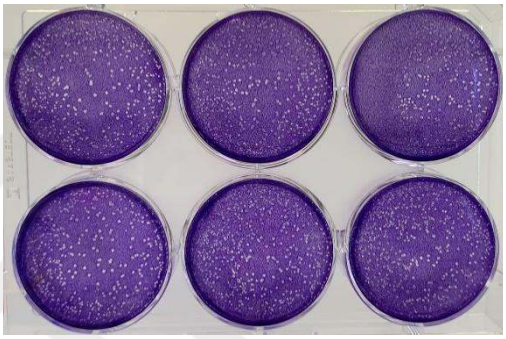
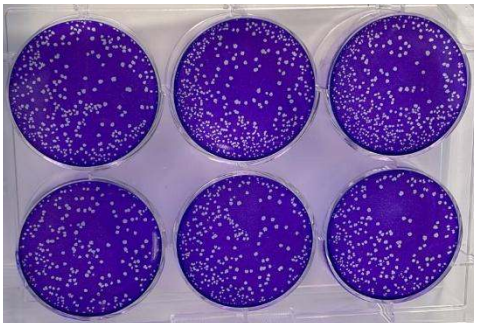
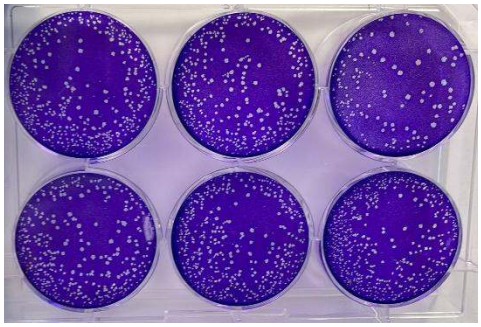


E.



F.



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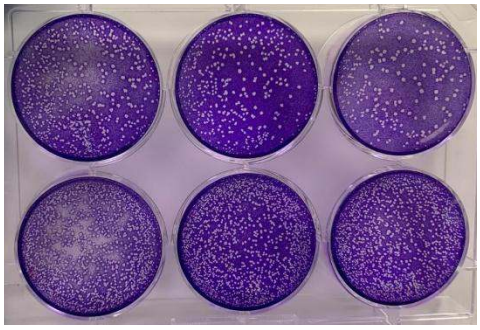

Figure 2.6: Plaque assay plates at the end of experimental procedure. **A**. Healthy control plate. **B.** Virus control plate. **C.** Baseline (3-5 months after 2 doses of CoronaVac) serum plate. **D.** BNT162b2 booster 1 month serum plate. **E**. BNT162b2 booster 3 months serum plate. **F.** CoronaVac booster 1 month serum plate. **G.** CoronaVac booster 3 months serum plate.

2.5 Plaque Counting and decision of PRNT50 Serum titer

Plaques in each well were counted by naked eye and Celigo Image Cytometer with colony counting settings. After counting, the serum titer which half of the 10^{-3} virus was inhibited were selected. For this purpose, the plaque number counted for each well was divided to the plaque number counted in the 10^{-3} virus plates. The first dilution in which the result of the division was lover than 0.5 was selected as the neutralizing antibody titer for that serum sample. For the samples which two serum dilutions gave close titers, the experiments were repeated.

2.6 Statistical Analysis

For the comparison of two different groups, statistical analysis was performed with non-parametric test, unpaired Mann-Whitney U. for the analysis, unpaired test was selected. The analysis and the visualization were performed by GraphPad Prism 8.0.2 Software. Statistical analysis was performed in STATA and significance levels were selected accordingly. The significance levels were selected as *$p<0.001$; **$p<0.05$.

Chapter 3:
RESULTS

3.1 Baseline demographics data of the participants

The baseline demographics of all participants, BNT162b2 group and CoronaVac group were presented in Table 3.1 separately.

Table 3.1: The baseline demographics of the participants.

	General Cohort (n=52) n (%)	BNT162b2 Group (n=42) n (%)	CoronaVac Group (n=10) n (%)
Mean age (Range)	34 (22-72)	30 (22-47)	49 (27-72)
Female gender	33 (63.5)	30 (71.4)	3 (30)
BMI			
<25	33 (63.4)	33 (78.6)	0 (0)
25-30	11(21.15)	7 (16.66%)	4 (40)
30≥	8 (15.38)	2 (4.76)	6 (60)
Smoking	17 (2.69)	13 (30.92)	4 (40)
Comorbidity	14 (26.92)	8 (21.42)	7 (70)
Immunosuppression	0 (0)	0 (0)	0 (0)

The majority of the cohort consist of female participants (63.46%). The mean age was 34, the mean age was higher for the CoronaVac group (49) than the BNT162b2 group (30). In the general cohort 14 (26.92%) participants had one or more comorbidity. The presence of one or more comorbidity was higher in the CoronaVac group. In this study, none of the participants were under immunosuppression.

3.2 Measurement of Neutralizing Antibody Titers from Serum Samples with PRNT50 Plaque Assay

The neutralizing antibody titers of each serum sample were estimated based on the virus control 10-3 virus dilution plaque count. The antibody titers of each participant were evaluated and compared with each other in baseline, 1 month after booster and 3 months after booster. The PRNT50 plaque assay well pictures which represent the geometric means (GM) were shown in Figure 3.1. The well representations were selected based on the cut-off neutralizing antibody titers which are estimated according to the plaque counts.

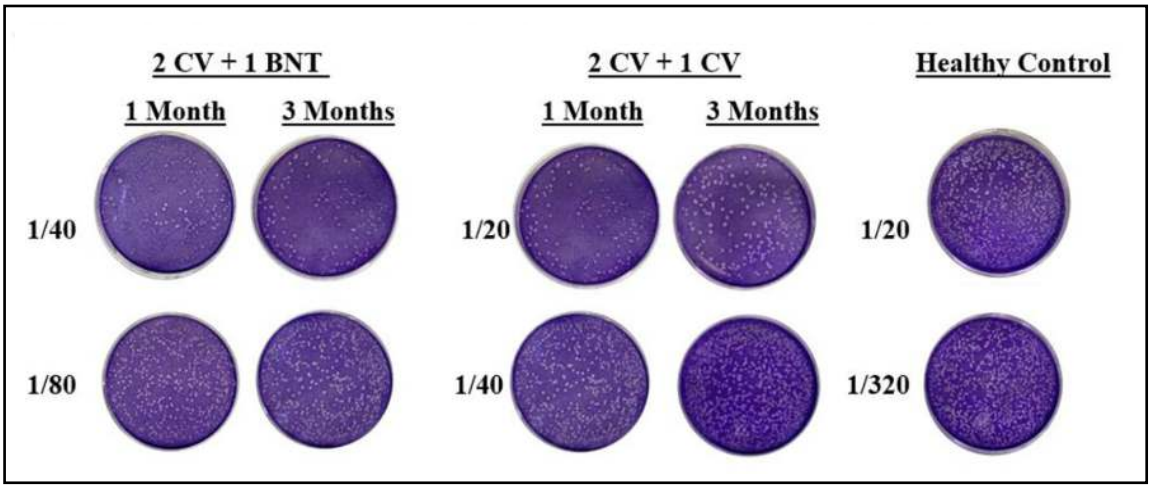

Figure 3.1: The PRNT50 plaque assay well pictures which represent the geometric means (GM). The wells were selected based on the geometric means. From left to right well pictures represent BNT162b2 booster, CoronaVac booster and healthy control.

The neutralizing antibody levels for 3 different time points, baseline, 1 month and 3 months were represented in the scatter dot plot graph shown in Figure 3.2. For this purpose, the neutralizing antibody levels observed after the booster doses were compared with each other in different time points. Each observed neutralizing antibody level was represented with a bullet.

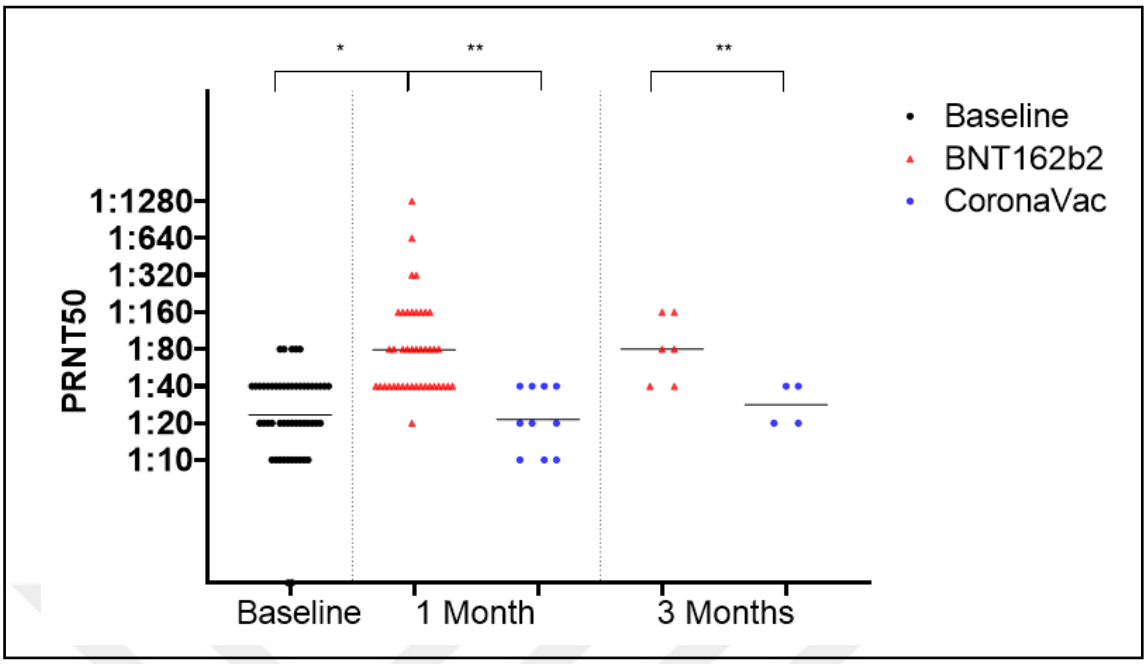

Figure 3.2: PRNT50 titers at baseline, one month and three months after boosters. Each neutralizing antibody titer was represented with one bullet. Data are presented in scatter dot plot format and line at geometric mean. Black lines are GMT=geometric mean titer. Baseline is presented as black, BNT162b2 booster is presented as red, CoronaVac booster is represented as blue. BNT162b2 is presented as triangle and CoronaVac is presented as circle (*P<0.001; **P<0.05).

For the baseline 40 participants out of 52 (76.92%) had neutralizing antibody levels higher than 1:20. The geometric mean of the baseline is determined as 23.27. The lowest neutralizing antibody level detected in this group was <1:10 and the highest neutralizing antibody level detected was 1:80 (Range <1:10 to 1:80). 1 month after administration of the BNT162b2 booster the geometric mean was detected as 78.69 (1:20 to 1:1280). Three months after administration of the BNT162b2 booster the geometric mean was detected as 80 (1:20 to 1:1280).

1 month after administration of the CoronaVac booster the geometric mean was detected as 21 (1:10 to 1:40). Three months after administration of the CoronaVac booster the geometric mean was detected as 28 (1:10 to 1:40).

In this period, all participants in the BNT162b2 group (100%) and 7 participants out of 10 (70%) of CoronaVac booster receivers had neutralizing antibody levels of >1/20. Meanwhile, 98% of the BNT162b2 and 40% of the CoronaVac participants had antibody

titers above the 1/30 threshold. As mentioned previously, the neutralizing antibody geometric means were obtained in similar levels 1 month and 3 months after the administration of the booster both for BNT162b2 and CoronaVac group.

The fold changes of neutralizing antibody titers were detected for BNT162b2 and CoronaVac group both in 1st and 3rd month after vaccination. The fold changes are respectively, 3.38-fold increase in 1st month BNT162b2 booster, 0.92-fold decrease in 1st month CoronaVac, 3.44-fold increase in 3rd month BNT162b2 booster, and 1.22-fold increase in 3rd month CoronaVac booster.

After the general evaluation, the neutralizing antibody titers of each participant were evaluated individually and the increase or decrease patterns were presented for BNT162b2 and CoronaVac booster separately. Each neutralizing antibody titer was represented with a single bullet. The individual evaluations for BNT162b2 were shown in Figure 3.3 and the evaluations for CoronaVac were shown in Figure 3.4.

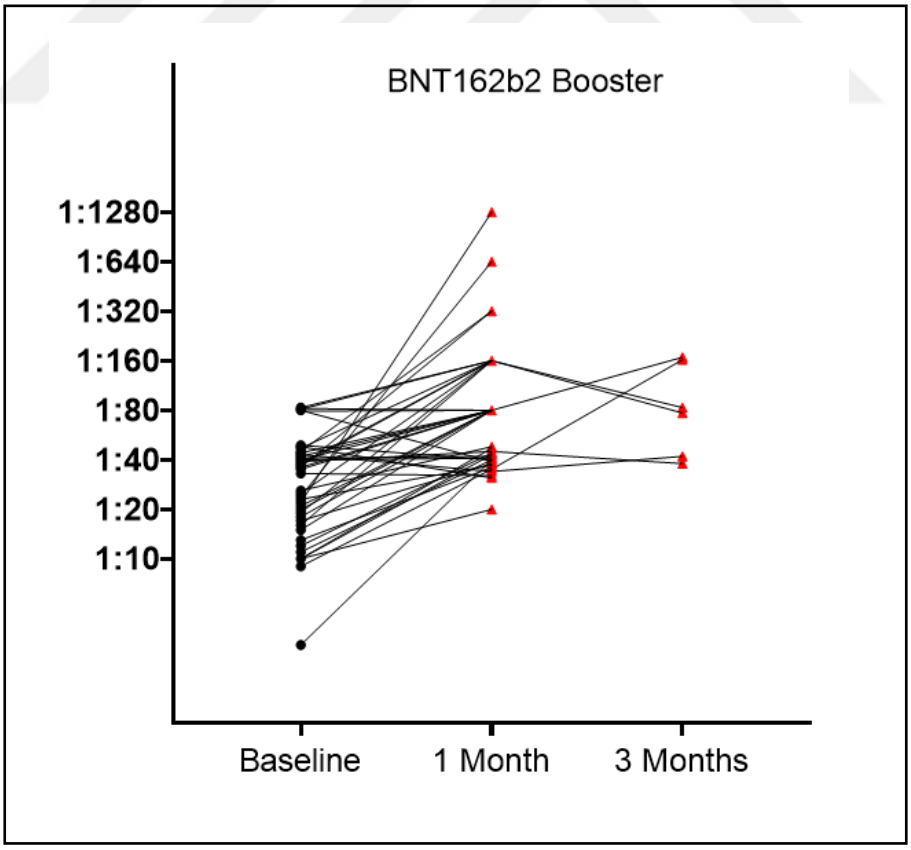

Figure 3.3: The individual values' pattern of BNT162b2 booster receivers. The figure represents the neutralizing antibody titers of the BNT162b2 booster receiving group in the baseline, 1 month and 3 months after the administration of the booster.

As can be interpreted from Figure 3.3, when the participants were vaccinated with BNT162b2 booster, almost all individuals (n=42) developed higher neutralizing antibody levels in 1st month than the baseline or maintained the same antibody levels as the baseline. In the 3rd month, the neutralizing antibody levels of 2 participants stayed the same, for 2 participants increased and for 2 participants the level decreased. The highest neutralizing antibody titer was observed 1 month after the BNT162b2 booster (1:1280).

Some of the participants were not able to donate blood samples 3 months after the administration of the BNT162b2 booster, either because those participants received their 4th doses as a part of the vaccination regimen or were simply not suitable for the blood donation at the point being. The analysis for 3 months was performed with the neutralizing antibody titers of only 6 participants.

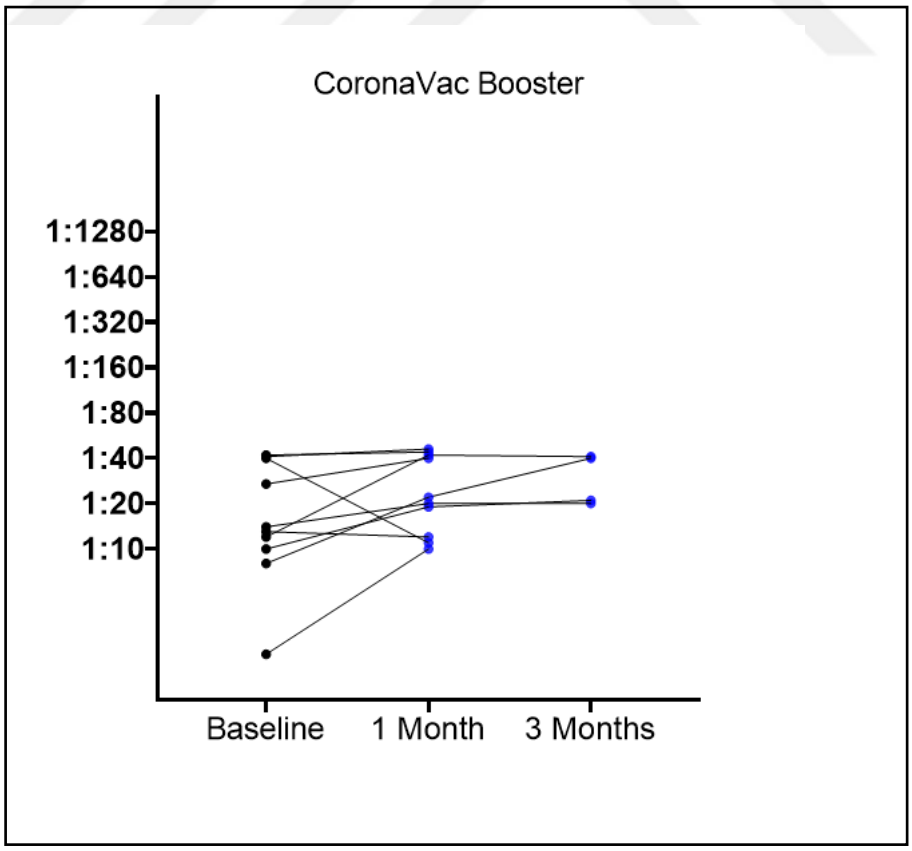

Figure 3.4: The individual values' pattern of CoronaVac booster receivers. The figure represents the neutralizing antibody titers of the CoronaVac booster receiving group in the baseline, 1 month and 3 months after the administration of the booster.

When the neutralizing antibody titers were investigated individually for the participants who received CoronaVac as the booster, the neutralizing antibody levels were steady during all studies. The neutralizing antibody levels stayed the same in baseline, 1 month after the administration of booster dose and 3 months after the administration of booster dose. All the neutralizing antibody titers were changing between <1:10 and 1:40 during the baseline, 1 month and 3 months.

3.3 Investigation of B cell responses by Flow Cytometry

After the evaluation of neutralizing antibody titers, to investigate the remaining humoral immune responses, B cells were examined. By flow cytometry the flowing B cell subpopulations were determined. The gating strategy used for the flow cytometry is shown in Figure 3.5.

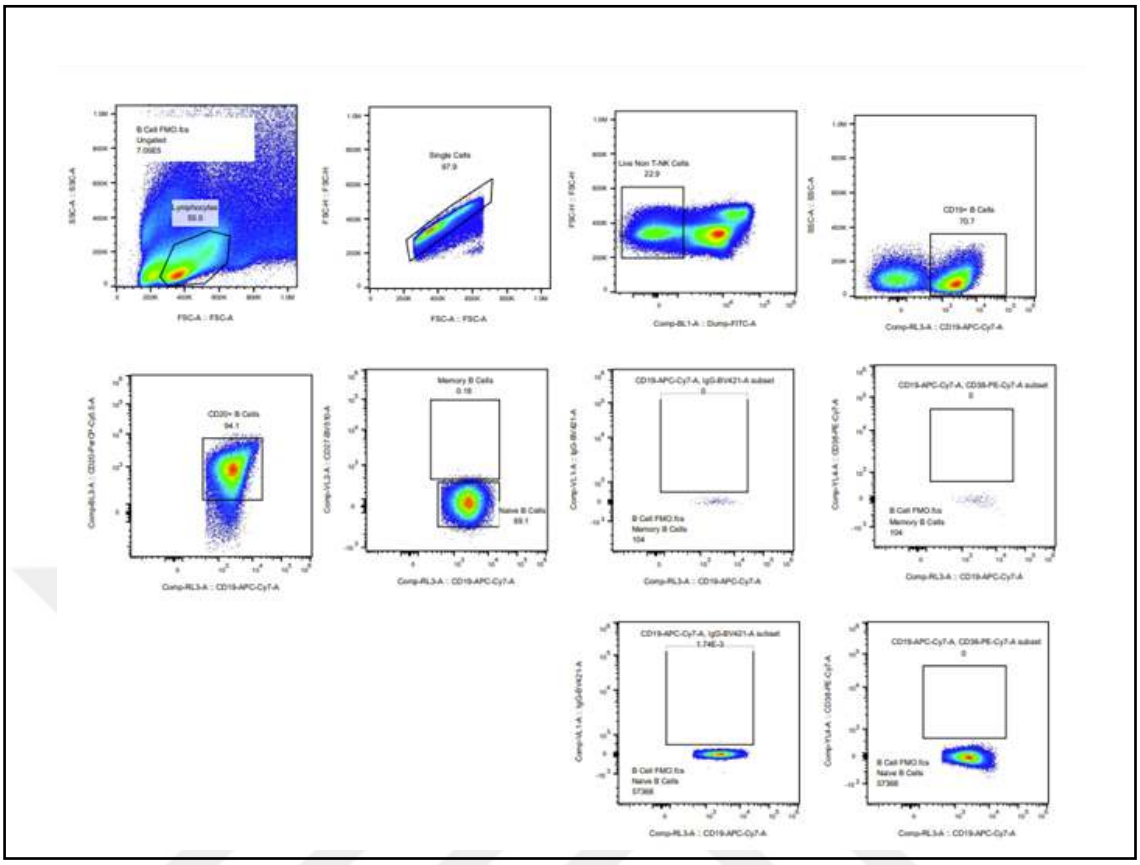

Figure 3.5: The gating strategy of the B cells in the flow cytometry.

The PBMCs were stained with the antibodies against the B cell surface markers as previously mentioned. For the gating, first lymphocytes were identified as SSC-A vs. FSC-A. Doublets were excluded with FSC-W vs. FSC-A gating. Live splenocytes were negative for the live/dead marker, CD56, CD3 and CD14 markers. B cells were identified with CD19 and CD20 markers. Among the B cells, memory B cells were identified with CD27 marker. From memory B cells, memory B cells expressing IgG were identified with IgG marker. From memory B cells, effector memory B cells were identified with CD38 marker.

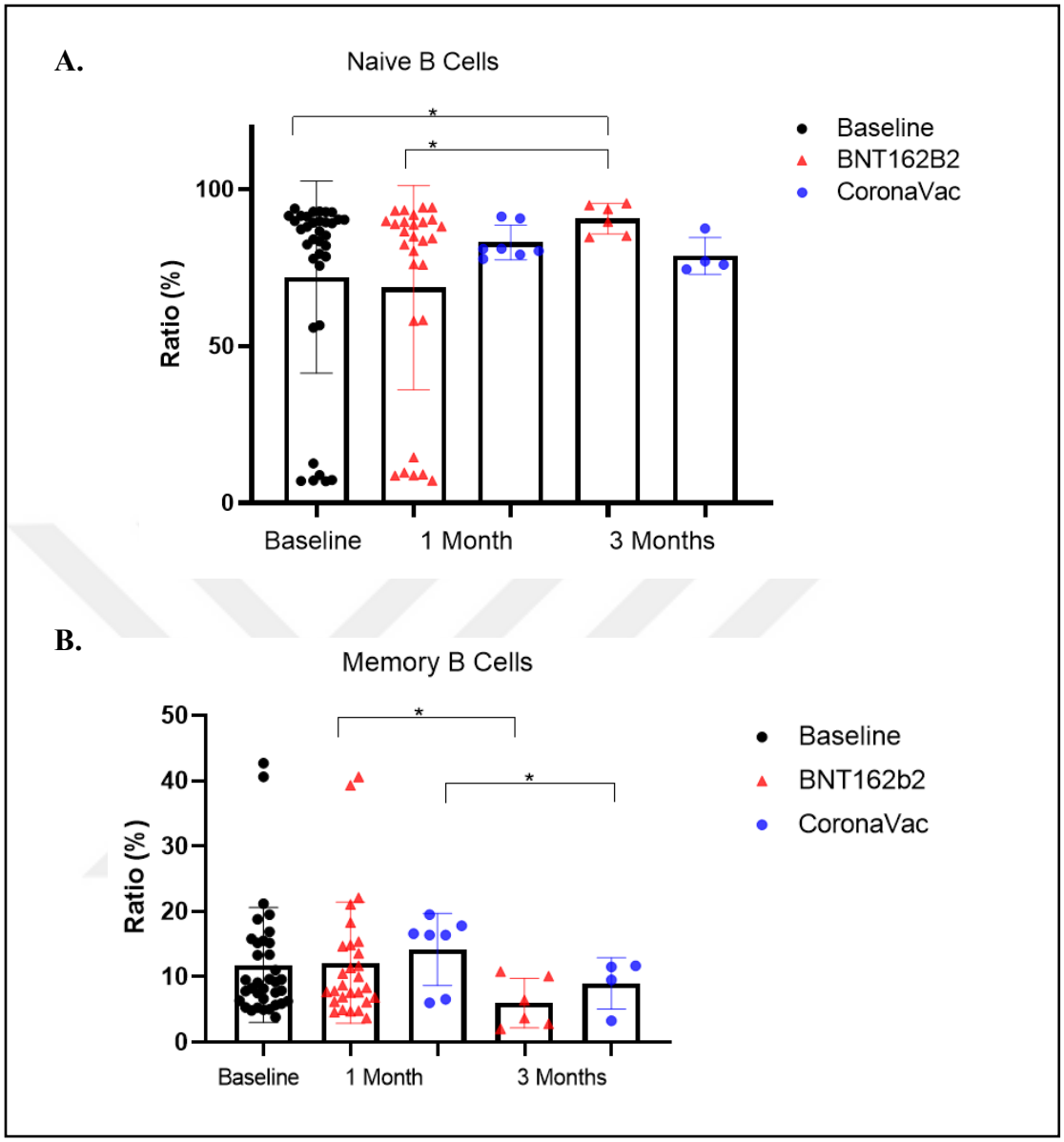

Figure 3.6: The ratio of naïve and memory B cells **A.** The ratio of naïve B cells **B.** The ratio of memory B cells (*P<0.001; **P<0.05)

The changes of in naïve and memory B cell populations were shown in Figure 3.6. In the baseline level of naïve B cells was 72.05%. After BNT162b2 booster in 1 month, the mean Naïve B cell ratio was 68.64% and it was significantly increased to 90.63% in three months. (p=0.0298) The mean ratio of the naïve B cells with CoronaVac booster were 83.07% and 78.78% after one and three months.

During the baseline stage, the mean memory B cell ratio was 11.82%. After one month, the mean memory B cell ratio was found to be 12.16% with BNT162b2 booster

and it was 14.18% with CoronaVac booster. After three months, the mean ratio declined to 5.98% (p=0.0475) and 9.00% (p=0.0198) with BNT162b2 and CoronaVac boosters, respectively. In the 3rd month, the ratio of the memory B cells was higher in the CoronaVac group compared to the BNT162b2 group, yet the increase was not significant (p=0.2571)

The individual trends were shown for naïve B cell and memory B cell subsets in Figure 3.7.

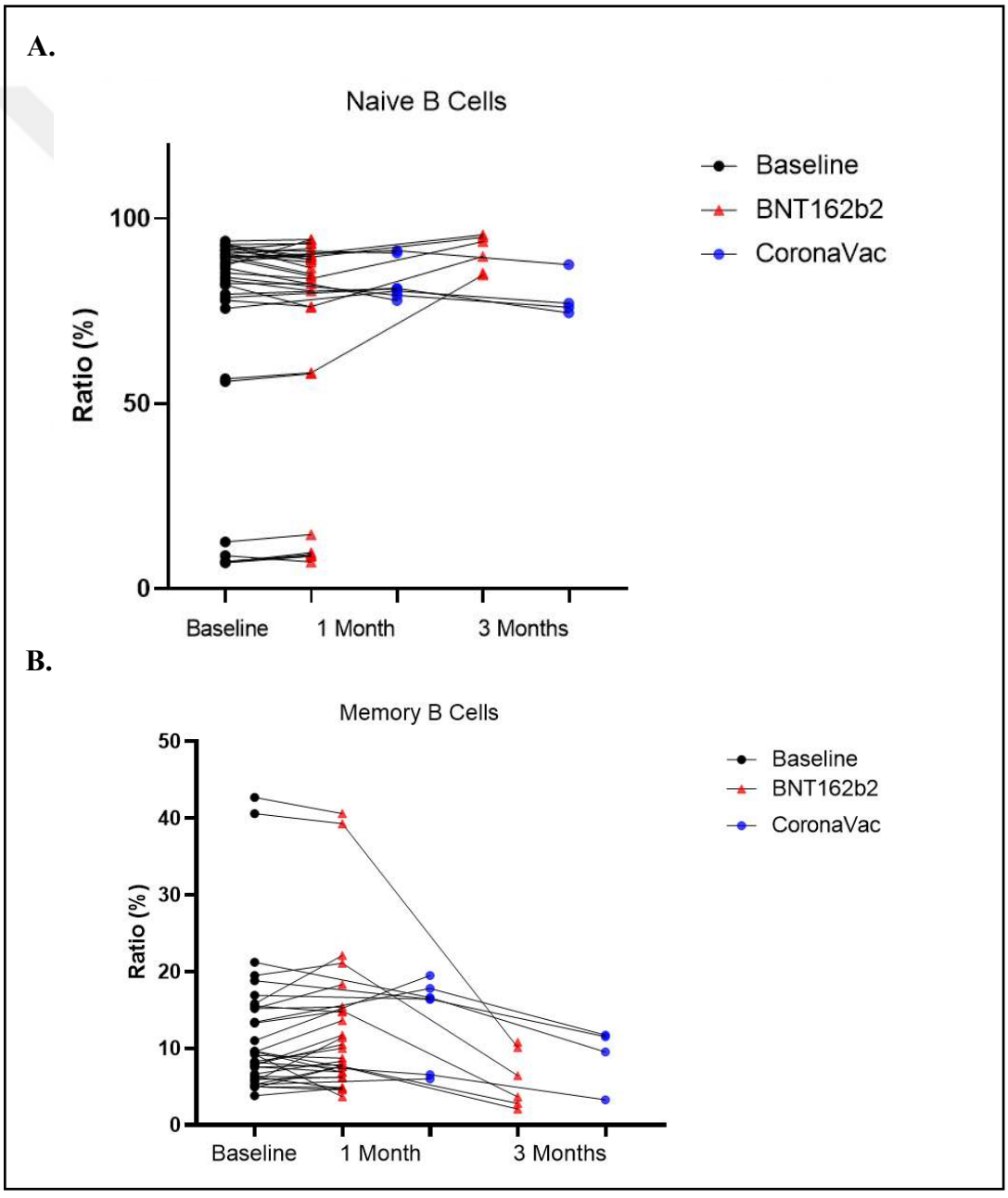

Figure 3.7: The ratio of naïve and memory B cells for each participant separately. **A.** The ratio of naïve memory B cells. **B.** The ratio of memory B cell.

For the naïve B cell subpopulation, an increase trend was observed with the BNT162b2 booster between 1 month and 3 months. During the baseline to 1 month, the trend was mostly stable for almost all participants. With CoronaVac booster, starting from the baseline the trend of naïve B cell population showed a decrease. After one month, the increasing trend in the memory B cell ratio was seen in almost all the participants and decline were observed after 3 months.

After the evaluation of the naïve and memory B cell populations, IgG expressing memory B cell and CD38 expressing (effector) B cell subpopulations were evaluated. The ratio of IgG expressing memory B cells and CD38 expressing memory B cells were shown in Figure 3.8.

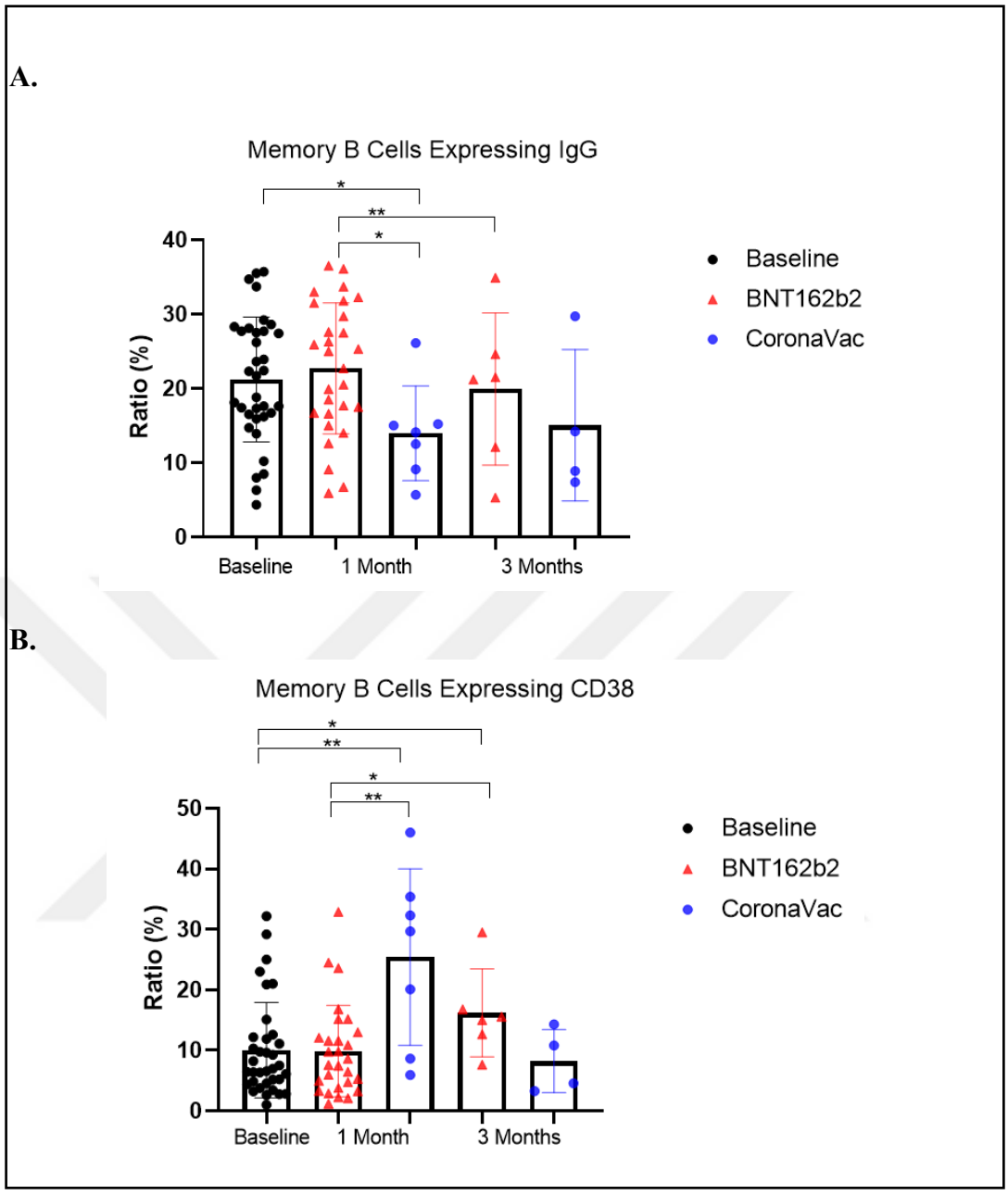

Figure 3.8: The ratio of IgG expressing memory B cell and CD38 expressing memory B cell subtypes **A.** The ratio of IgG expressing memory B cells **B.** The ratio of CD38 expressing memory B cells (*P<0.001; **P<0.05)

The changes of IgG expressing memory B cells and CD38 expressing memory B cells were shown in figure 3.8. In the baseline the level of memory B cells expressing IgG was 21.21%. With the CoronaVac booster 1 month after the administration the mean level decreased significantly to 13.96% (p=0.0154). When the mean levels of memory B cell expressing IgG were compared in the 1st month after the administration, the mean level

was 22.70% for the BNT162b2 booster and significantly higher than the CoronaVac booster. (p=0.0136)

For the memory B cells expressing CD38, baseline expression level was 10.05% and the expression levels was significantly higher with the CoronaVac booster in the first month (25.4%, p=0.0072) than the baseline. Similarly, the expression was significantly higher than baseline in the 3 months after CoronaVac as well (16.21%, p=0.0204). When the BNT162b2 booster 1st and 3rd month expression levels were compared, the expression for the 3rd month was also significantly higher in the 1st month. (16.21% vs 9.89%), respectively (p=0.0263). When the expression levels were compared in the 1st month between two vaccine types, it was observed that with CoronaVac booster the expression level was significantly higher than BNT162b2 booster (9.90% vs 25.44%, p=0.0099)

The individual trends were shown for memory B cell expressing IgG and memory B cell expressing CD38 subsets in Figure 3.7.

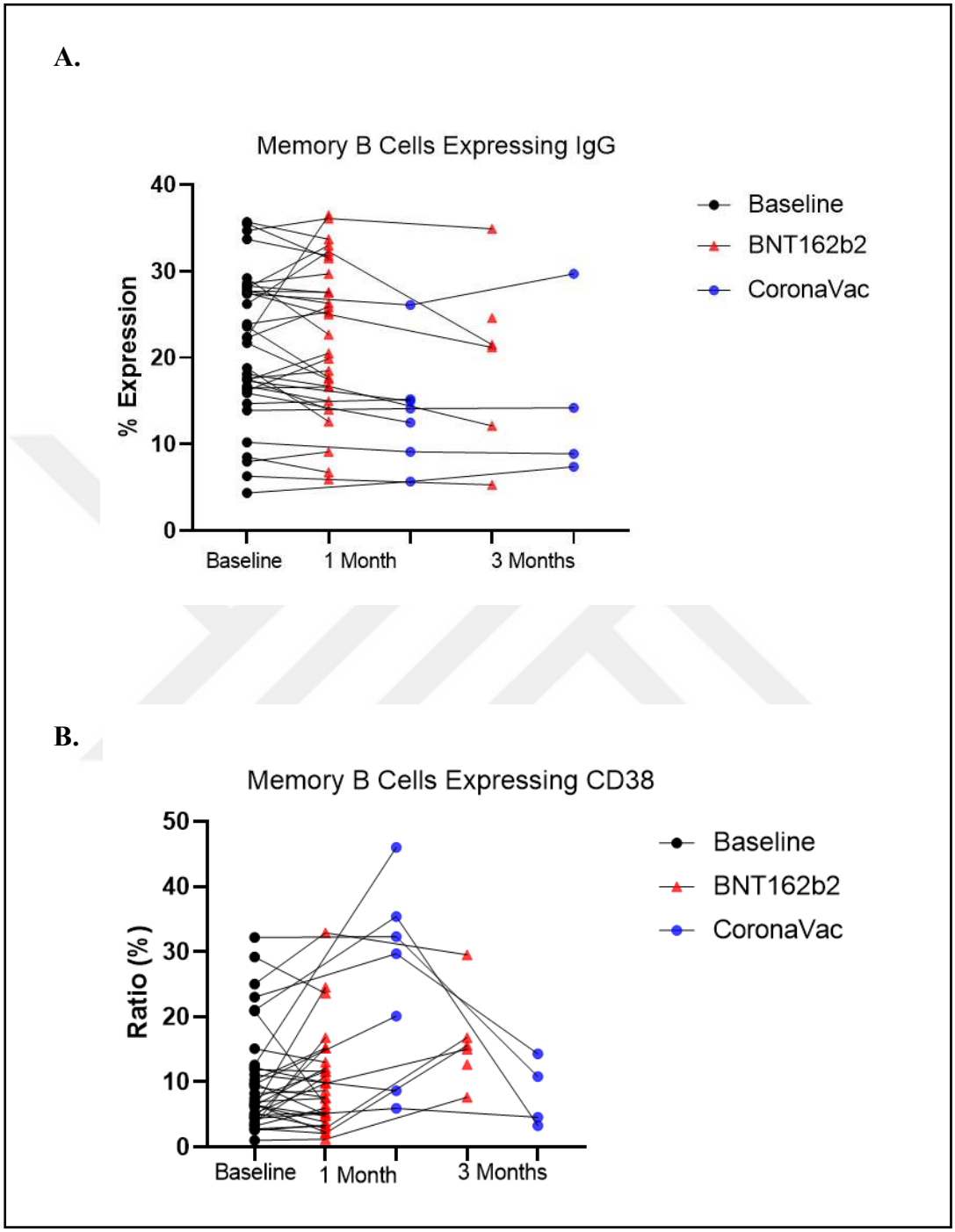

Figure 3.9: The ratio of naïve and memory B cells expressing IgG and memory B cells expressing CD38 for each participant separately. **A.** The ratio of naïve memory B cells expressing IgG. **B.** The ratio of memory B cells expressing CD38.

For the IgG expressing memory B cells, with the BNT162b2 booster the trend was relatively stable, for some of the individuals slight increase or slight decrease was

observed. After 3 months, mostly the trend was decrease in the ratio. Between the baseline and the 1 month the expression trend with CoronaVac booster declined and between the 1 to 3 months, the trend showed a slight increase. The CD38 expressing B cell subpopulation showed an increase trend with the CoronaVac booster in 1st month. However, the trend decreased between 1 month and 3 months. With the BNT162b2 booster the trend was stable for the 1st month when compared with the baseline. The trend was mostly increased in the 3rd month with the BNT162b2 booster. The expression pattern for each participant was different from each other.

Chapter 4:

DISCUSSION

The present study addresses the lack of knowledge on the immunogenicity of BNT162b2and CoronaVac boosters after 2 doses of CoronaVac administration. We focused on neutralizing antibody responses after 1 and 3 months elicited by BNT162b2 and CoronaVac boosters in fully vaccinated individuals with CoronaVac.

In this study a 3.38-fold increase in neutralizing antibody titers (GM 78.69) one month after the BNT162b2 booster, and antibody titers were found to be maintained after 3 months (GMT 80) were found. However, in the CoronaVac group, low GMs after 1 month and 3 months (21.44 and 28.44) indicated the weak immunogenicity of the CoronaVac booster. For the protection of 50% of the individuals, a neutralizing antibody titer of 1/19 for BNT162b2 and 1/30 for CoronaVac was suggested as cut-off levels of protection (Khoury, D. S., et. al., 2021). All BNT162b2 booster receivers had antibody levels above the protection threshold of 1/19 after 3 months, but only 40% of donors had antibody titers above the 1/30 threshold in the CoronaVac booster group. High neutralizing antibody levels following 14 days after CoronaVac booster dose (Cao, Y., et. al., 2022) and decreased neutralizing antibody levels after 28 days were reported (Wang, K., et. al., 2021). Moreover, a recent study from Turkey reported a marked increase in binding antibody response after BNT162b2 booster compared with a CoronaVac booster (104.8- vs. 8.7-fold, respectively). We found low neutralizing antibody levels at a prolonged time (1–3 months) after booster doses of CoronaVac. The concern about the weak immunogenicity would be increased by considering the reduced efficacy of vaccines against the Omicron variant (Lu, L., et. al., 2021; Pérez-Then, E., et. al., 2021)

In the expression of B cell subtypes, significantly higher expression was observed for memory B cells expressing IgG in the BNT162b2 booster group 1st month after booster than CoronaVac booster group ($p=0.0099$). When compared with the neutralizing antibody results, significant increase in those B cell subtypes was expected (Kotaki, R., et. al., 2022) The higher expression, also higher response of CD38 expressing effector memory B cells with BNT162b2 booster 1 month after the vaccination shows a positive correlation between higher neutralizing antibody responses. At the same time the expression and responses of CD38 effector memory B cell, decrease at the 3rd month.

This shows the short half-life of these cells. With the BNT162b2 booster, the ratio of IgG expressing memory B cells increases 1 month after the vaccination. Higher expression of IgG shows that when a primary antigen is detected in the human body, the generation of the antibody responses will be higher with the BNT162b2 booster. (Goel, R. R., et. al., 2021 & Chen, Y., et. al.,2021)

There are some studies in the literature that also suggest similar results. (Liu, Y., et. al., 2022). For the effector memory B cells 1 month after the CoronaVac booster the expression level was higher than BNT162b2 booster. In previous studies it was shown that the neutralizing antibody levels 1-2 months after 2 doses of CoronaVac vaccines were highly above the protectivity level. Also, according to the unpublished data obtained in our studies, the neutralizing antibody levels are also high 2 months after 2 doses of CoronaVac vaccine. The increase in the effector B cells with CoronaVac booster supports and correlates with these findings.

The strength of this study is being a longitudinal study and having a prominent advantage of being performed in Turkey, of where the primary vaccine schedule consisted of two CoronaVac doses. Main limitations of this study can be stated as having a low sample size to detect the statistical significance especially in the CoronaVac booster group. However, our results are in parallel with reported studies that evaluated anti-S antibody titers by ELISA in Turkey (Yalçın, Y. T., et. al., 2021). Some of the participants were excluded from the study since they received their fourth doses after 2 months. Moreover, we could not cover the newly emerged Omicron variant, and this will be done in future studies.

Our study has implications for the countries that have used a two-dose regimen of CoronaVac. The neutralizing antibody levels after 3 months of the BNT162b2 booster were higher than the antibody levels after the CoronaVac booster. Taking into consideration the waning immunity, we suggest a second booster dose with BNT162b2 for the individuals who already had BNT162b2 or CoronaVac boosters as their third doses following two doses of CoronaVac.

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