UNIVERSITY OF CAPE COAST

EVALUATION OF T-CELL RESPONSES TO SARS-COV-2 SPIKE ANTIGENS IN PFIZER/BIONTECH AND JANSSEN BOOSTER VACCINATIONS IN FULLY VACCINATED INDIVIDUALS: A RETROSPECTIVE LONGITUDINAL STUDY

MARU KIPLIMO ADAMS

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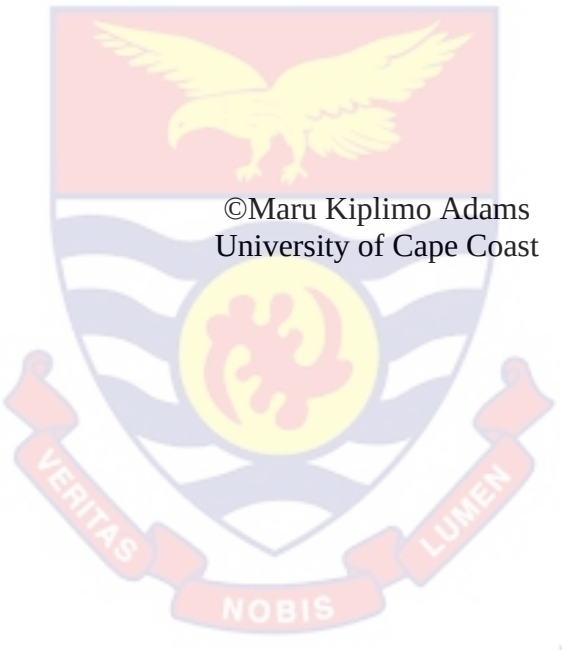

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BY

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Thesis Submitted to The Department of Microbiology and Immunology, School of Medical Sciences, College of Health and Allied Sciences, University of Cape Coast in Partial Fulfilment of The Requirements for The Award of Master of Philosophy Degree in Infection and Immunity

JUNE, 2024

DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original research and that no part of it has been presented for another degree in this university or elsewhere.

Candidate's Signature:..... Date:.....

Name:……

Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

Principal Supervisor's Signature:..... Date:.....

Name: Prof. Dorcas Obiri-Yeboah

Co-Supervisor's Signature:…….. Date:.....

Name: Prof. Kwadwo Asamoah Kusi

ABSTRACT

With the recent emergence of the deadly COVID-19 and its worldwide spread, vaccines of different formulations; the viral-vector, m-RNA and subunit vaccines among others were developed to curb the spread of the virus and reduce its disease burden. Very little is known about the durability of these vaccines and immune protection mechanisms from homologous and heterologous booster vaccines and their effectiveness remains largely understudied. Therefore, this study aimed to assess the longevity of cellular immune responses (interferon-gamma release) following the administration of Janssen or Pfizer booster doses. Archived PBMCs obtained across four time points; Pre-booster, Month 3, Month 6 and Month 9, isolated from subjects from the Legon community, Ghana, were used in this study. *In-silico* HLA restriction epitope prediction of SARS-CoV-2 spike protein was done to determine immunogenic peptides after which the Interferon-gamma release response ELISpot Assay was conducted. Briefly, 60% of all samples registered positive responses to at least one of the spike peptides with 55.5% from the Janssen booster vaccination and 44.4% Pfizer booster vaccination. Against all the peptides a comparison of responses between the two vaccine boosters was done across the four time points. Both Pfizer and Janssen boosters elicited durable spike-specific T-cell responses, with persistence observed for up to 6 months. The study found that homologous prime-boosting with a viral-vectored vaccine (Janssen) produced stronger T-cell responses compared to heterologous boosting. Heterologous m-RNA prime-boosting using Pfizer led to stronger T-cell responses than homologous m-RNA prime-boosting.

KEYWORDS

SARS-CoV-2

Vaccines

Homologous prime boosting

Heterologous prime boosting

T-cell immune responses

Interferon-gamma release responses

Enzyme-linked immunospot assay

Peptides

Cellular immunity

HLA supertypes

Epitope prediction

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DEDICATION

To my family

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LIST OF ABBREVIATIONS

WHO	World Health Organization
PBMCs	Peripheral Blood Mononuclear Cells
ELISPOT	Enzyme Linked Immunospot
SARS-CoV-2	Severe Acute Respiratory Syndrome Corona Virus-2
HLA	Human Leukocyte Antigen
SGP	Spike Glycoprotein
NTD	N-terminal Domain
CTD	C-terminal Domain

CHAPTER ONE

INTRODUCTION

This chapter discusses the contextual information on the study with references to related literature. It is organized into the background of the study, problem statement, significance of the study, objectives of the study, research questions, delimitations of the study, and limitations of the study.

The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) virus is a member of the coronavirus family that encompasses a varied group of enveloped, positive-sense single-stranded RNA viruses responsible for the recent global health emergency in history. With several vaccines having been authorized for use against SARS-CoV-2, very little is known concerning the durability and effectiveness of the immune protection provided by these vaccines.

Furthermore, with the emergence of SARS-CoV-2 variants and the need to administer booster vaccination, a lot more is not clear with regards to the cellular immune responses specifically the IFN- γ release responses following administration of these booster vaccines. This study sought to determine the longevity and specificity of T-cell responses specifically, the IFN- γ release responses targeting the SARS-CoV-2 spike protein in persons who were fully vaccinated and took a booster shot of either Janssen or Pfizer vaccine.

Background

One of the recent pandemics, COVID-19 disease is caused by the novel SARS-CoV-2 which has since become one of the most significant worldwide health emergencies in recent memory according to the World Health Organization

(WHO). The SARS-CoV-2 belongs to the coronavirus family comprised of encapsulated, positive-sense, single-stranded RNA viruses originating in various natural environments, that cause a wide range of diseases that affect the neurological, gastrointestinal, hepatic, and respiratory systems (Kung et al., 2022; Yang& Rao, 2021). The severity of the disease has been linked to more than a few risk factors, including inheritance, age, sex, and pre-existing medical disorders like diabetes, cancer, cardiovascular disease, obesity, and allergy conditions and these elements may have a role in organ failure, dyspnea, pneumonia, and acute respiratory distress syndrome (ARDS) development (Borczuk & Yantiss, 2022; Hu et al., 2021).

As of November 2023, over 772 million confirmed cases of COVID-19 and more than 6.9 million deaths have been reported to the World Health Organization (WHO) globally. According to data provided by WHO, there were 171,768 confirmed cases of COVID-19 in Ghana between 3 January 2020 and 30 November 2023, along with 1,462 fatalities (https://www.who.int/countries/gha).

Attempts have been made to develop and use vaccinations to stop the virus's spread and lessen its terrible effects together with the rise of several SARS-CoV-2 strains. Among others, some of the licensed COVID-19 vaccine formulations are; the COVID-19 mRNA vaccines from Pfizer-BioNTech and Moderna, Novavax which is a protein subunit vaccine and the viral-vector vaccines including the Janssen and AstraZeneca COVID-19 vaccines (Fiolet et al., 2022). By March 2024, over 13.5 billion COVID-19 vaccine doses have been administered worldwide. In

Ghana, more than 25.5 million doses of vaccines had been given as of July 2, 2023 (https://data.who.int/dashboards/covid19/vaccines).

The vaccine's main target is the viral spike protein that aims to generate humoral immune responses as well as induce cellular (T-cell-based) immune responses against it, which together play a pivotal role in promoting antiviral immune protection among vaccine recipients and offer robust protection against the infection (Fathizadeh et al., 2021; Fiolet et al., 2022; Goda et al., 2022; Mascellino et al., 2021; Moss, 2022; Yang & Rao, 2021). The immune system generates memory T and B-cells after vaccination, however, immune protection from the vaccines tends to decline over time approximately between 6 to 9 months from the time of vaccination, raising questions on the durability, effectiveness, and longevity of these vaccines and the possible use of booster vaccination (Bellamkonda et al., 2022; Dadras et al., 2022; Jordan et al., 2021).

Statement of the problem

Since the coronavirus disease became a pandemic, several vaccine formulations have been licensed to combat the disease among them Pfizer, Janssen, AstraZeneca, and others (https://www.cdc.gov/coronavirus/2019-ncov/vaccines/different-vaccines/overview-COVID-19-vaccines.html). Despite targeting the spike (S) protein, COVID-19 vaccines face challenges due to the high mutation rate of this protein. These mutations can diminish the durability and effectiveness of the vaccine's protection and as a result, vaccinated individuals can still contract infections from new variants, sometimes exhibiting high viral loads. Consequently, with the emergence of SARS-CoV-2 variants of concern (VOCs) and the gradual

decline of vaccine-induced immunity over time, there has been an increase in breakthrough infections leading to discussions about the necessity of booster vaccine doses. (Burckhardt et al., 2022; J. Li et al., 2021).

While considerations on using COVID-19 booster shots have been documented to prevent breakthrough infection, there is inadequate evidence to inform decisions on the timing and frequency of the booster vaccination to avert severe infection. Considerations to determine the frequency of COVID-19 booster vaccination therefore require evaluating the intensity and persistence of immune responses to aid in understanding how immune protection arises after booster vaccination, the dynamics and the usefulness of booster vaccination in disease prevention (Safont et al., 2022).

Cellular adaptive immune responses are key machineries of the immune system in SARS-CoV-2 vaccine-induced protection. To comprehensively evaluate vaccine effectiveness and to determine the potential necessity for booster doses, it is crucial to assess T-cell responses to specific SARS-CoV-2 antigens (Burckhardt et al., 2022; Dadras et al., 2022; Safont et al., 2022). Therefore, this study focused on the cellular adaptive immune responses, specifically interferon-gamma release responses, in trying to comprehend the immune responses to COVID-19 booster vaccines (Gilbert, 2012).

This study sought to explore the longevity and magnitude of T-cell immune responses, specifically IFN-γ release responses to SARS-CoV-2 spike antigen protein in fully vaccinated individuals who received a booster shot of Pfizer (mRNA vaccine) or Janssen (Viral-vector vaccine). By examining T-cell

interferon-gamma release responses to specific COVID-19 spike antigens in people who have previously received full COVID-19 vaccination, this study sought to close this important knowledge gap to inform the policy on the frequency of booster dose administration by establishing the duration of IFN-γ responses following the administration of booster shots.

Research questions

Primary Research Question

What is the magnitude, durability and specificity of T-cell responses (IFN-γ) to SARS-CoV-2 Spike antigens in fully vaccinated individuals who receive booster COVID-19 vaccination?

Secondary Research Questions

1. How do T-cell responses (IFN- γ release responses) differ among individuals who received different types of COVID-19 booster vaccines (e.g., mRNA vaccines, viral-vector vaccines)?
2. How do T-cell responses (IFN- γ release responses) vary based on the time elapsed since the last dose of COVID-19 full vaccination?
3. How do T-cell responses (IFN- γ release responses) compare in homologous and heterologous COVID-19 booster vaccination?

Objectives of the study

Aim

To determine the magnitude, durability and profile of T-cell responses (IFN-γ release) in fully vaccinated individuals who receive COVID-19 booster vaccination.

Specific objectives

1. To identify potential immunodominant peptides from the SARS-CoV-2 Spike protein by HLA restriction prediction using *In-silico* tools.
2. To measure predicted peptide-specific Interferon Gamma (IFN-γ) responses 3 months after administration of booster dose and assess the recognition of positive peptides by 6 and 9 months in the same individuals.
3. To compare IFN-γ responses between Janssen and Pfizer vaccine types used as boosters.

Significance of the study

The purpose of this study is to determine the effectiveness, durability, and specificity of T-cell immune (IFN-γ) responses in fully vaccinated individuals who receive a booster shot of the Pfizer or Janssen vaccine. The rationale of this study is to assess the longevity of T-cell IFN- γ responses following the administration of COVID-19 booster shots and ultimately, this study will serve to inform policy on the frequency of administering booster shots to sustain community-level immunity against COVID-19.

The study is relevant to research in infectious diseases immunology and vaccinology and understanding the T-cell responses to SARS-CoV-2 spike peptides in vaccinated individuals can provide vital insights into the vaccine protection mechanisms and help to develop vaccines that target the activation and induction of adaptive cellular immunity.

Delimitations of the study

This study was limited to participants from the Legon community and its environs, Accra, Ghana. We used archived samples from participants who were fully vaccinated before receiving a booster shot of either the Janssen or Pfizer vaccine.

The variables considered in this study included age, sex, vaccine type, time since last COVID-19 vaccination, history of COVID-19 infection and comorbidities of the participants.

Limitations of the study

There are a few limitations in this study. A small number of samples in each group was used which resulted in low statistical power and made it difficult to identify variations in T-cell responses amongst the groups.

Additionally, in this study SARS-CoV-2 N-antigen test was not performed to determine the infection within the four time points. The information on COVID-19 infection relied on subjects' responses by filling in a questionnaire hence asymptomatic cases could be easily overlooked and similar symptoms might have been misdiagnosed as not COVID-19.

CHAPTER TWO

LITERATURE REVIEW

Introduction

This chapter introduces and reviews the relevant literature on COVID-19 history, the epidemiology of Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-CoV-2), the biology of the virus, the pathogenesis of the disease, immune responses, and the role of vaccines.

Brief history and epidemiology of SARS-CoV-2

The initial cases of unidentified pneumonia were reported in Wuhan City, China, in December 2019 in the seafood market where aquatic and live animals were largely retailed. Samples from the individuals' lower respiratory tracts were shown to contain a previously unidentified beta-coronavirus by unbiased next-generation sequencing (Adhikari et al., 2020; Soliman et al., 2021). Phylogenetically, the novel coronavirus displayed greater similarities to strains of coronavirus that originated from bats than severe acute respiratory syndrome (SARS) and Middle East Respiratory Syndrome (MERS), the virus was formally called SARS-CoV-2 in 2022 in recognition of the genetic links, and the World Health Organization (WHO) named the ensuing ailment Coronavirus Disease 2019 (COVID-19) in response (Adhikari et al., 2020; Dhar Chowdhury & Oommen, 2020; Soliman et al., 2021).

Coronavirus Disease 2019 (COVID-19) is a contagious respiratory disease that caused havoc by its spread worldwide, with symptoms associated with this condition ranging from fever, chills, and sore throat (Adhikari et al., 2020). For

most people, the experience of COVID-19 is marked by a challenging but ultimately manageable illness where they typically recover without the need for hospital treatment, provided they adhere to self-isolation measures and receive appropriate care (Adhikari et al., 2020). Nonetheless, for those with severe symptoms, such as difficulty breathing or persistent chest pain, seeking immediate medical care is crucial to increase their chances of full recovery(Adhikari et al., 2020; Borczuk & Yantiss, 2022). The contagious nature of the virus led to a significant global effect, with over 760 million cases confirmed and approximately 6.9 million deaths recorded as of the last available data in December 2023 with Ghana reporting a total of 171,000 cases reported and slightly over 1,400 deaths (https://www.who.int/countries/gha). Nevertheless, there is a suspicion that the actual figures could be even higher due to underreporting in some regions (Lau et al., 2021; Thenon et al., 2022; P. Wang et al., 2022).

Transmission of SARS-CoV-2

Respiratory droplets are the primary means by which SARS-CoV-2 is transmitted either directly from one individual to another or indirectly via contact with fomites (Saulnier et al., 2023) and airborne transmission is now supported by current evidence (Klompas et al., 2020; Morawska & Cao, 2020). Even though the virus has been isolated in stool samples, the risk of fecal-oral transmission is uncertain (Meyerowitz & Richterman, 2022).

The period from exposure to the virus until the beginning of symptoms averages 5 to 6 days. A small percentage of patients about 2.5% may develop symptoms inside 2.2 days while the majority approximately 97.5% will show

symptoms within 11.5 days (Baselga et al., 2022). The time between symptom onset in the primary disease and the secondary case is estimated to be approximately 4 to 5 days (Meyerowitz & Richterman, 2022). There is a possibility of transmission during the asymptomatic phase of the illness where symptoms in secondary cases show before the primary case (Baselga et al., 2022).

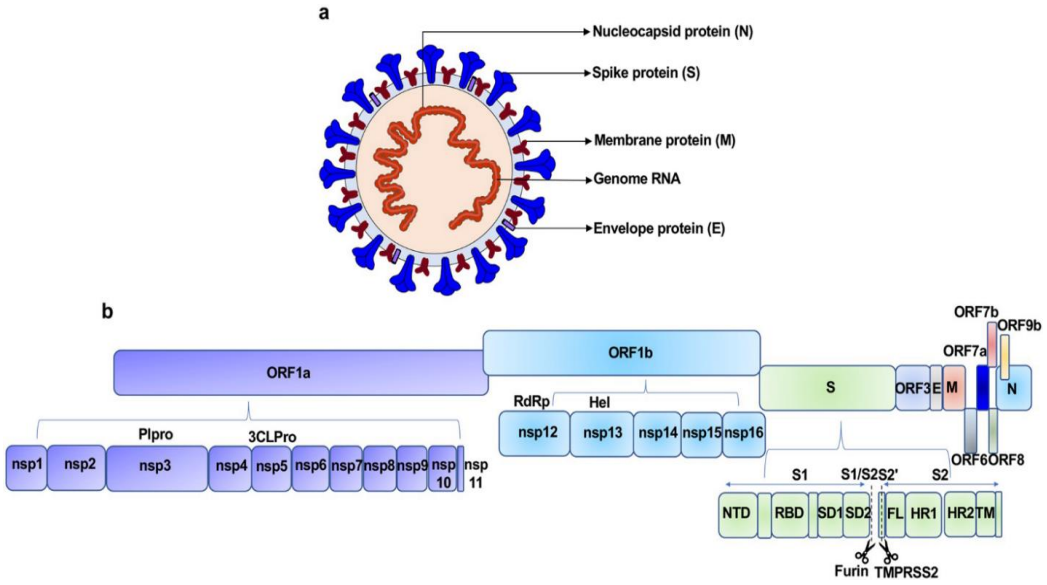
SARS-CoV-2 biology and viral properties

SARS-CoV-2 belongs to a family that constitutes a varied group of single-stranded RNA viruses that are enclosed with an envelope and have positive-sense genomes (Artika et al., 2020; D. X. Liu et al., 2020). This viral family is categorized into the subfamily Orthocoronavirinae and the order Nidovirales based on genetic and serological characteristics, and Orthocoronavirinae is further subdivided into four genera. Alphacoronaviruses as one of the genera comprise HCoV-NL63 and HCoV-229E while Betacoronaviruses include MERS-CoV, HCoV-HKU1, SARS-CoV, HCoV-OC43, and SARS-CoV-2, the latter, which is responsible for the COVID-19 (Adhikari et al., 2020; Artika et al., 2020; D. X. Liu et al., 2020; Lo et al., 2022).

The SARS-CoV-2 virus particle has a diameter of 60 to 140 nanometers and usually takes the form of an oval or sphere (Bar-On et al., 2020; Soliman et al., 2021). It is notably susceptible to different inactivation techniques and can be successfully deactivated by heating it for 30 minutes at 56°C or by subjecting it to ultraviolet (UV) light as well as a range of disinfectants, including diethyl ether, chlorine, 75% ethanol, peracetic acid and chloroform (Schuit et al., 2022; Soliman et al., 2021).

SARS-CoV-2 genome and proteins

The Coronavirus genome is the largest known with a positive-sense single-stranded RNA with a size between 27 and 32 kilobases (kb) and encodes for viral proteins; including structural (Spike, Membrane, Nucleocapsid, and Envelope), non-structural (nsp1–16), and eleven auxiliary proteins (ORF7a, ORF9c, ORF3a, ORF3c, ORF8, ORF3d, ORF6, ORF7b, ORF3b, ORF9b, and ORF10) (Artika et al., 2020; Mingaleeva et al., 2022).



(Q. Zhang et al., 2021)

Figure 1: Schematic representation of SARS CoV-2 structure.

A.) Nucleocapsid protein N, Spike Protein S, Membrane protein M, Envelope protein E, and Genome RNA. B.) SARS CoV-2 genomic organization. (Q. Zhang et al., 2021).

The genomic RNA of SARS-CoV-2 can function as a messenger RNA (mRNA) encoding the viral proteins directly since it has a 5'-cap end and a 3'-polyadenylate tail. The ORF organization is the same as that of MERS-CoV and

SARS-CoV which are arranged in the order replicase, protease (1a–1b), major proteins; spike protein, envelope protein, membrane protein, and nucleocapsid protein (Rajpal et al., 2022; V'kovski et al., 2021). These gene products are crucial for SARS CoV-2 entry, fusion, and host cell survival (Naqvi et al., 2020).

SARS-CoV-2 Structural Proteins

The SARS-CoV-2 Spike (S) protein is a trimer that is categorized as a type I membrane fusion protein with three identical subunits extending from the viral particle giving the virus a crown-like look that is essential in mediating the union of the viral membrane with the host cell membrane (Y. Huang et al., 2020; F. Li, 2016). The protein is large with over 1,200 amino acid residues and can be fragmented into two functional subunits, S1 and S2 where S1 mediates the virus's attachment to host cells, whilst S2 helps in the fusion of viral and host cell membranes (Mingaleeva et al., 2022; V'kovski et al., 2021).

The S1 subunit is divided into two vital regions; the N-terminal domain (NTD) and the receptor-binding domain (RBD) where RBD consists of small helical segments encircling a five-stranded antiparallel β-sheet at its core (Y. Huang et al., 2020). It also has a receptor-binding motif (RBM) that is crucial for binding to the host cell receptor, human angiotensin-converting enzyme 2 (ACE-2) (Y. Huang et al., 2020; Saputri et al., 2020). The NTD folds into a structure that binds sugar and is thought to induce conformational alterations in the S protein, hence playing a role in the virus's initial attachment to host cells (Saputri et al., 2020). The S2 subunit has the Fusion peptide (FP), two Heptad repeat domains (HR1 and

HR2), cytoplasmic (CTD) domains and a transmembrane domain (TMD) (Mingaleeva et al., 2022).

The Envelop (E) protein is the smallest SARS-CoV-2 structural protein with a molecular weight between 8 and 12 kDa. Its protein structure comprises a five-helix bundle with a tiny pore measuring approximately 35 angstroms in length and can act as an ion channel (Yadav et al., 2021; Zhou et al., 2023) and within the transmembrane region the channel it is filled with hydrophobic amino acid residues, except for the N-terminal region (Mingaleeva et al., 2022; Zhou et al., 2023). It controls the flexibility of the viral envelope ensuring that coronaviruses attain their distinctive round shape and structure (Soliman et al., 2021; Zhou et al., 2023). In both SARS-CoV-1 and SARS-CoV-2, the E protein plays a crucial role in the creation of vesicles containing viral proteins, and hence actively participates in the budding process of the virus (Gorkhali et al., 2021; Zhou et al., 2023).

The E protein is located in the cytoplasm, with its C-terminus in the cytoplasm and its N-terminus facing the lumen of the ER-Golgi intermediate compartment and controls how calcium ions (Ca2+) are taken out of the cellular endoplasmic reticulum (ER) (Zhou et al., 2023). This control over calcium ion levels can activate the cellular inflammasome, which strengthens the host's defense against the virus (Gorkhali et al., 2021; Zhou et al., 2023). Despite significant variation in the amino acid composition, the structural characteristics of E proteins are largely similar among different genera of β-coronaviruses, and the assemblage and release of new viral particles, as well as the pathogenicity of the virus, are significantly influenced by the E protein (Zhou et al., 2023).

The Membrane (M) Protein is an O-linked glycoprotein with a typical size range of 25–30 kDa with three different transmembrane domains that make up this most abundant structural protein in the virus (Dolan et al., 2022). The nucleocapsid and other viral structural proteins interact with the M protein in the form of homodimers which is necessary for the assemblage of virus particles hence the pathogenicity of the virus may also be influenced by this contact (Fehr & Perlman, 2015; Gorkhali et al., 2021). Its structural properties are mostly unaltered despite fluctuations in its amino acid makeup whereas the glycosylation via an O-link of M protein in coronaviruses such as β-CoVs and δ-CoVs can affect the virus's interferon signaling and organ tropism (Fehr & Perlman, 2015; Yadav et al., 2021).

The Nucleocapsid (N) Protein's primary function is to arrange the viral DNA into a nucleocapsid structure and differs from other proteins in that the C-terminal domain, the RNA-binding domain, and the N-terminal domain are highly conserved (J. L. Wu et al., 2023). These three domains promote RNA binding, and the N protein's ability to bind to viral RNA depends on its phosphorylation state (Cubuk et al., 2021). In addition, the N protein is essential for virion assembly, RNA packing, and improving virus transcription efficiency (J. L. Wu et al., 2023; W. Wu et al., 2023). The N protein is also a viable target for vaccine development because of its high immunogenicity (Feng et al., 2022; Rak et al., 2023).

SARS-CoV-2 Non-Structural Proteins (NSPs)

The 5′-region of the viral RNA genome of SARS-CoV-2 contains genes that encode sixteen non-structural (NSP1-16) that work together to establish a complex network of interactions and activities and that are important to the virus's ability to

replicate and interact with the host cell (Kakavandi et al., 2023). The complex machinery that controls the virus's RNA transcription and genome replication is an essential aspect of SARS-CoV-2's capacity to subvert host cellular machinery for its own gain (Mingaleeva et al., 2022).

Non-structural Protein 1 (NSP1) functions as a leader protein by becoming the viral replicase's N-terminal product that functions to prevent host cell proteins from being translated and aids in the mRNAs of host cells from being degraded (Yadav et al., 2021). It functions to mediate RNA processing and replication (M. Y. Wang et al., 2020). Non-structural protein 2 (NSP2) is an N-terminal product, 638 amino acids long, and interacts with PHB1 and PHB2, which are prohibitins 1 and 2, respectively, to facilitate several protein-protein interactions that are essential for different stages of the viral lifecycle hence modulate the survival-signaling pathway of the host cell (Kakavandi et al., 2023; M. Y. Wang et al., 2020; Yamkela et al., 2023).

Non-structural protein 3 (NSP3) is a proteinase containing 1945 amino acids and functions by separating the translated protein to release NSP1, 2, and 3 from the N-terminal region of polyprotein 1a and 1ab (Kakavandi et al., 2023; M. Y. Wang et al., 2020). NSP4 is a membrane-spanning protein that has a transmembrane domain and is a 500 amino acid molecule that works to alter the membranes of the endoplasmic reticulum (ER) and actively takes part in the viral replication-transcription complex (Low et al., 2022). Non-structural protein 5 (NSP5) with 306 amino acids, is the primary proteinase that can cleave proteins at several different places to produce mature and intermediate non-structural proteins

(Gorkhali et al., 2021). Non-structural protein 6 (NSP6) is a 290 amino acid putative transmembrane domain and is essential for the induction of Endoplasmic Reticulum (ER)-derived autophagosomes and double-membrane vesicles to facilitate viral multiplication and the disruption of host cell functions, (Low et al., 2022).

Non-structural protein 7 (NSP7) is an RNA-dependent RNA polymerase of 83 amino acids and works in tandem with Non-structural protein 8 (NSP8) and Non-structural protein 12 (NSP12) to support Non-structural protein 8's (NSP8's) RNA polymerase activity (Kakavandi et al., 2023; M. Y. Wang et al., 2020). NSP8 contributes significantly to RNA replication and is a multimeric 198-amino acid RNA polymerase/replicase and forms a heterodimer with NSP8 and NSP12 (Kakavandi et al., 2023; Low et al., 2022). NSP9 is a 198 amino acid viral protein that functions as a single-stranded RNA-binding protein during replication (Kakavandi et al., 2023; M. Y. Wang et al., 2020). NSP10 has two zinc-binding motifs within the 139 amino acid protein that resembles a growth factor and are presumably involved in controlling the processes involved in viral replication and for the cap methylation of viral mRNAs (Kung et al., 2022; M. Y. Wang et al., 2020). NSP11 is quite similar to the first segment of NSP12 but its exact function is still unknown (Gorkhali et al., 2021).

Non-structural protein 12 (NSP12) extends over 932 amino acids and is a significant RNA-dependent RNA polymerase that is essential for both methylation and reproduction of viral genomes (Mingaleeva et al., 2022). Non-structural protein 13 (NSP13) has a different zinc-binding domain of RNA-dependent RNA

polymerase of the same length, and is critical for transcription and replication, while the helicase core domain interacts and binds with ATP (Artika et al., 2020; Mingaleeva et al., 2022; M. Y. Wang et al., 2020).

Non-structural protein 14 (NSP14) has 527 amino acids and acts as a proofreading exoribonuclease domain (ExoN/nsp14) with 3′–5′ exoribonuclease activity and also has N7–guanine methyltransferase activity, which promotes RNA integrity (Gorkhali et al., 2021). Non-structural protein 15 (NSP15) has 346 amino acids and is an endoribonuclease that relies on Mn (2+) and is involved in the digestion of RNA (Low et al., 2022). NSP16 is a 2′-O-ribose methyltransferase that has 298 amino acids in its length and mediates 2′-O-ribose methylation at the 5′-cap structure of viral mRNAs, which is a crucial step in mRNA capping (Low et al., 2022; Mingaleeva et al., 2022; M. Y. Wang et al., 2020). Together, these NSPs are essential to the SARS-CoV-2 lifecycle because they coordinate the intricate processes of transcription, replication, and host defense evasion (Gorkhali et al., 2021).

Eleven auxiliary proteins are synthesized from various regions of the SARS-CoV-2 virus's genetic coding and these proteins work in tandem with non-structural proteins to support the virus's replication (Hassan et al., 2022; Mingaleeva et al., 2022; Yadav et al., 2021). Of these, ORF3a is a noteworthy accessory protein with 274 amino acids that is abundant in transmembrane domains and glycosylation as it forms ion channels in the host cell membrane, promoting the passage of specific ions, and plays a role in virus release, apoptosis, and the development of the disease (J. Zhang et al., 2022). ORF6 is a membrane-associated

protein with 61 amino acids that is present in patients' tissues and virus-infected cells where it is mostly found in the Golgi and endoplasmic reticulum (ER) of cells that express it (Yadav et al., 2021).

The auxiliary proteins ORF7a and ORF7b are derived from the identical SARS-CoV-2 RNA section, and while ORF7b is shorter and located in the Golgi compartment, ORF7a is a transmembrane protein with both a luminal and transmembrane domain (Yadav et al., 2021). ORF8 adopts a structure with 121 amino acid residues that are similar to the immunoglobulin fold and have a minimal resemblance to the SARS-CoV virus because of genetic differences. ORF8 has the greatest hypervariable sections after the S protein's RBD and has the greatest mutation density among the nonstructural proteins (Arduini et al., 2023). As a virokine, ORF8 induces the production of IL-17RA-mediated cytokine and promotes monocyte dysfunction in addition to performing other tasks within infected cells, including regulating epigenetics, viral spike expression, the interferon response, and CTL-mediated immunity (Arduini et al., 2023; Hassan et al., 2022; Vinjamuri et al., 2022).

ORF9b (97-amino acid protein) combines with the Mitochondrial Outer Membrane (TOM70) adaptor protein to prevent the host's interferon-I (IFN-I)-mediated antiviral responses (Chen et al., 2023). ORF14 is produced by leaky scanning of the N gene's RNA, its function in the viral replication process is yet unknown (Kakavandi et al., 2023). ORF10 has been detected in infected cells, even though it is rarely present in the matching RNA (Pancer et al., 2020). Understanding

the roles of these accessory proteins is critical to developing potential therapeutic strategies against SARS-CoV-2 (Hassan et al., 2022; Kakavandi et al., 2023).

SARS-CoV-2 Variants

The viral genome mutations can modify a virus's capacity for pathogenicity. Even a small change in an amino acid can significantly affect a virus's capacity to escape the immune system and hinder the development of vaccines to combat it (Carabelli et al., 2023). Similar to other RNA viruses, SARS-CoV-2 is predisposed to genetic modifications as it gradually creates changes in order to adapt to new human hosts. This might cause a variety of variants with unique characteristics from its initial strains to develop (Scovino et al., 2022).

In the early phases of the epidemic, SARS-CoV-2 experienced very little genetic alterations. But as time went on, more than a few variants emerged that showed variations in transmissibility and severity. The discovery of novel genetic variations of SARS-CoV-2 that are circulating in communities is made possible through the periodic sequencing of viral samples (Akkız, 2022).

There have been several identified variations of SARS-CoV-2, some of which are termed variants of concern (VOCs) because of the possible risk to public health that they pose when linked to increased transmissibility or virulence, resistance to detection, decreased antibody neutralization from vaccination or natural infection, or decreased effectiveness of treatments or vaccinations (Scovino et al., 2022).

SARS-CoV-2 VOCs

The VOCs include Alpha (B.1.1.7), Omicron (B.1.1.529), Delta (B.1.617.2), Gamma (P.1), and Beta (B.1.351). Each of these VOCs has mutations in both the RBD and the NTD whereas, some of these mutations, including N501Y, K417N, 69-70 deletion, E484K, N655Y, and P681H are known to enhance immune escape, infectivity, and transmissibility, leading to high concern of severe pandemic (Y. Liu et al., 2022). The RBD, in conjunction with NTD, is the key neutralization target and helps the generation of antibodies in response to antisera or vaccinations (Carabelli et al., 2023; Scovino et al., 2022). The RBD's N501Y mutation, for example, increases the spike protein's affinity for ACE 2 receptors and facilitates the attachment and subsequent entry of the virus into the host cell, is shared by all variants other than the Delta variant (Choi & Smith, 2021). The new deadly Omicron variant has more than thirty mutations in the spike protein, most of which are found in other variants of concern (Y. Liu et al., 2022).

Pathogenesis of SARS-CoV-2 infection

The spike glycoprotein is the main determinant of which cell the coronavirus infects by creating structures called trimers on viral particle’s surface which is vital for the virus to be able to infect host cells (Walls et al., 2020). The receptor-binding domain (RBD) of the spike protein S1 subunit attaches to the angiotensin-converting enzyme 2 (ACE2) entrance receptor on the host cell, and the S2 subunit facilitates the union of the virus and the cell membrane of the host cell (Lamers & Haagmans, 2022; Nguyen et al., 2021; Walls et al., 2020).

The spike protein upon attaching itself to ACE2 on the surface of the target cell it comes into contact with and after binding, it is broken down by transmembrane serine protease (TMPRSS2) (Lamers & Haagmans, 2022; Nguyen et al., 2021). The S2 subunit trimers are activated by this cleavage event, which allows them to connect the viral membrane with the lipid bilayer of the host cell and release the viral genome into the host cell as a result of this fusion, which promotes infection (Borczuk & Yantiss, 2022).

The virus can also enter the host cell through endosomes, a different route of entrance where cathepsins can break the spike protein inside the endosome, though this route is not very effective (Fehr & Perlman, 2015; Lamers & Haagmans, 2022). Other molecules proposed to take part in the entry of SARS-CoV-2 into host cells, in addition to ACE2 and TMPRSS2 comprise proteases such as cathepsin L, TMPRSS11D, and TMPRSS13 as well as co-receptors like neuropilin 1, although their precise function in the pathogenesis of SARS-CoV-2 is still unclear, these compounds may perform a role in the virus's capacity to infect host cells (Lamers & Haagmans, 2022; Yu et al., 2023).

When a patient is naturally infected with SARS-CoV-2, the virus first targets particular cell types, most likely the nasopharynx multi-ciliated cells (Chen et al., 2023). The positive-sense genome of the virus, once within these cells, initiates the synthesis of viral proteins, as well as replicase proteins by using the endoplasmic reticulum membranes to assemble into replication factories (Fehr & Perlman, 2015). Viral RNA transcription occurs in double-membrane vesicles found in these factories but the cytoplasmic pattern recognition receptors (PRRs)

are unable to detect the double-stranded RNA (dsRNA) transcription intermediates because of the double-membrane vesicles (Lamers & Haagmans, 2022).

MDA5 and RIG I are the major cytoplasmic PRRs thought to detect SARS-CoV-2 by identifying lengthy dsRNAs, starting a signaling cascade and transcriptional production of interferons of types I and III, which are crucial for the antiviral immune response to occur (Hatton et al., 2021; Lamers & Haagmans, 2022). Local immune cells including neutrophils and macrophages and the adjacent epithelial cells release interferons and chemokines in addition to infected cells by using endosomal Toll-like receptors (TLRs) where they react to the discovery of SARS-CoV-2 (Borczuk & Yantiss, 2022). Interferon-stimulated genes produce interferons which in turn induce an antiviral cellular state while they also stimulate the growth of adaptive immune responses including T-cells and B-cells which are essential for eliminating the virus (Hatton et al., 2021).

The virus can move from the upper to the lower respiratory tract by breathing virus particles or by gradually dispersing throughout the tracheobronchial tree if it can evade the immune system (Lamers & Haagmans, 2022). Sometimes the infection starts in the lower respiratory tract and spreads to the alveoli in the lungs, causing inflammation. Since type 2 alveolar (AT2) cells which release pulmonary surfactants necessary for lung lubrication and aid in the regeneration of alveolar type 1 (AT1) cells are the cells that are most infected by SARS-CoV-2 gaseous exchange is compromised in the alveoli (Borczuk & Yantiss, 2022; Fehr & Perlman, 2015; Lamers & Haagmans, 2022).

Innate immune responses to SARS-CoV-2

The primary line of protection against viral infections is the innate immune response. This line of defense relies more on general immune responses than on highly specialized mechanisms since it produces nonspecific immune reactions (Kaur & Secord, 2019). The innate immune responses contribute to the range of clinical symptoms seen in COVID-19 patients and are essential in determining the infection's overall course. When mediated by type I interferons and especially, the innate immune responses and early viral load are important factors that influence adaptive response (Diamond & Kanneganti, 2022) (Kombe Kombe et al., 2022).

SARS-CoV-2 being a single-stranded RNA virus, has a greater capacity to elicit innate immune responses because of particular sections of the viral genome that function as strong immunostimulants, activating receptors related to innate immunity (Borczuk & Yantiss, 2022; Y. Li et al., 2013; Schultze & Aschenbrenner, 2021a).

The interplay of macrophages, plasmacytoid dendritic cells, and conventional dendritic cells are an essential biological mechanism that regulates SARS-CoV-2 infection by recognizing Pathogen-Associated Molecular Patterns (PAMPs) and Damage-Associated Molecular Patterns (DAMPs) (Yamada & Takaoka, 2023). Viral RNA sensed by Toll-Like Receptors (TLRs) activate TLR3, TLR8, TLR7, and TLR9, and subsequently, the Nuclear Factor kappa B (NF-κB) and JAK-STAT pathways are activated leading to upregulating the transcription of hundreds of IFN stimulated genes (ISG) (Lowery et al., 2021). Interferons (IFN) are the mainstay of innate immunity's initial mechanism of defense against viral

infections and inhibit the development of viral proteins and avert their dire effects and the production of interferons many of which possess broad antiviral activities, have a crucial role in the initiation of inflammation caused by viruses, and which primarily attract monocytes and T lymphocytes to the site of infection rather than neutrophils (Mabrey et al., 2021).

The innate immune response is noticeably weakened when elements of the STAT1 signaling pathway are deleted, causing an individual to be more vulnerable to different infections and contributing to increased severity of SARS-CoV-2 infection (Tolomeo et al., 2022). SARS-CoV-2 viruses also encode proteins that inhibit interferon-stimulated gene (ISG) effector actions, thereby evading antiviral innate immune pathways hence severe COVID-19 instances are characterized by low generation of anti-SARS spike antibodies, sustained chemokine levels, and decreased expression of the ISG and immunoglobulin genes (Arish et al., 2023; García, 2020; Samuel, 2023; X. Sun et al., 2022).

The innate immune responses usually, successfully suppress viral infection in the pulmonary tissues in most COVID-19 cases, resulting in recovery but sometimes it causes dysregulated production of pro-inflammatory cytokines which brings about a cytokine storm which is a severe condition (Alcock & Masters, 2021). A cytokine storm is brought on by a subgroup of patients' evasion of innate immunity where Interferon (IFN) is unusually low whereas several cytokines including IP-10, MCP-1, IL-7, Granulocyte Colony-Stimulating Factor (G-CSF), IL-6, IL-2, Tumor Necrosis Factor-alpha (TNF-α), and IL-10 significantly increase during this storm (Lowery et al., 2021; Montazersaheb et al., 2022).

Precisely, it is unclear how SARS-CoV-2 circumvents innate immunity, which causes an exaggerated inflammatory response and an elevated viral burden but increased production of the Nucleocapsid protein represents a possible mechanism as this protein has been shown to hinder the IFN-β response in viral infected cells (Schultze & Aschenbrenner, 2021b). IFN production and signaling are also negatively impacted by SARS-CoV's ORF3b, NSP1, NSP13, and Orf6 among others (Schultze & Aschenbrenner, 2021b).

Deficiencies in innate immune components such as Mannose-binding lectin (MBL) protein increase susceptibility to infection and add to the severity of COVID-19. Complement activation mediated by the MBL pathway may lead to thrombosis and coagulation abnormalities in severe cases (Labarrere & Kassab, 2021). (Hurler et al., 2023; Queiroz et al., 2023)

The coagulation and complement pathways also have a critical role in SARS-CoV-2 infection (Afzali et al., 2022; Java et al., 2020; Kolb et al., 2023). Participation of immune complexes (ICs) in severe SARS-CoV-2 form of the infection has been specifically linked to endothelitis and disseminated microvascular thrombosis that impacts organs like the kidneys, bladder, and heart (Kolb et al., 2023). In this case, erythrocytes that have complement proteins attached to them transport ICs to phagocytes in the spleen and liver where prothrombotic and proinflammatory states are eventually responsible for the end organ damage (Afzali et al., 2022; Vandendriessche et al., 2021; Winberg et al., n.d.). (Afzali et al., 2022; Java et al., 2020; Zelek & Harrison, 2023). In addition,

the complement function influences and forecasts the clinical consequences, susceptibility, and immunity related to the infection (Java et al., 2020).

In as much as innate immune responses are efficient, the SARS-CoV-2 virus has developed tactics to subvert or avoid the innate immune reactions and this may exacerbate the illness by causing an overactive inflammatory response (Maison et al., 2023).

Adaptive immune responses to SARS-CoV-2

The consequence of COVID-19 on lymphocytes CD4+ and CD8+ T cells is vital because the pathogenic effects of the decline of these cell counts lead to pneumonitis and late removal of infections from the lungs as well as reduced neutralizing antibodies, production of cytokines, and lymphocyte recruitment to the lung (Silva et al., 2022; S. Zhang et al., 2021).

T-lymphocyte counts that are higher are linked to better survival rates and are essential in the fight against a continuing infectious process because when exposed to SARS-CoV-2, activated killer T-cells can stop the virus from spreading throughout the upper respiratory tract (Moss, 2022). A meta-analysis by I. Huang & Pranata (2020) note that reduced lymphocyte count is linked to higher mortality, ARDS, and severe disease (I. Huang & Pranata, 2020). Leukopenia and lymphocytopenia are also potential ways for the virus to elude the immune system whereas the intensity of the symptoms, the host's viral load, and the community's transmission rates are all influenced by how well this response works (Gerlach & Baig, 2023).

Low lymphocyte counts may be the result of the virus's direct cytotoxic impact, which prevents a cytokine storm and weakens innate immune responses whereas hypercytokinemia and lymphopenia are indicators of inadequate pathogen management, a pattern observed in severe infection and upregulation of apoptosis and P53 signaling-related genes may be a possible reason for the progress of lymphopenia (Alefishat et al., 2022). The lymphopenic state associated with severe SARS-CoV-2 infections is also influenced by host characteristics, including aging and comorbidities such as diabetes, hypertension, cardiovascular and cerebrovascular illnesses (Alefishat et al., 2022).

In cases of severe form of SARS-CoV-2 infection, B lymphocytes undergo a distinct transformation into cells resembling macrophages, impeding the immune system's capability to quickly neutralize the viral infection whereas, effector T-cell depletion compromises the cells' ability to defend against SARS-CoV-2 (Alefishat et al., 2022).

SARS-CoV-2-specific T-cell responses

T-cells mediate the cellular immune response through CD4+ and CD8+ T-cells where the CD8+ cells directly kill virus-infected cells whereas CD4+ cells aid in directing the immune response as a whole (Safont et al., 2022). CD4+ T-cells also support B-cells in their maturation into plasma cells and in the production of immunoglobulins that target the antigen, as part of the humoral immune response (Snyder et al., 2020).

In the early stages of the infection, prediction analyses *in silico* suggested potential immunogenicity for T-cell responses with laboratory studies

demonstrating the immunogenicity of the virus detecting CD4+ and CD8+ T-cells as well as IgG and IgA antibody responses (Grifoni et al., 2021).

Early, strong interferon and adaptive immune responses are linked with effective clinical regulation of primary infection, which in turn results in effective control of the viral load while inadequate or delayed interferon responses are linked to poor clinical outcomes and chronic inflammation (Bange et al., 2021). Virus-specific CD4+ T-cell responses lean towards Th1, minimizing concerns about Th2-related immunopathology (Howard et al., 2022) whereas CD8+ T-cells take part in limiting the amounts of virus, regulating disease severity, and interferon-gamma production, with interferon-gamma-producing CD8+ T lymphocytes showing a strong correlation with milder acute COVID-19 (Nowill et al., 2023). During the infection, cytotoxic CD8+ T-cell response develops quickly, within 7 to 14 days, and correlates with the resolution of the virus and a minor illness (Moss, 2022).

Helper and cytotoxic T-cell responses are robust to the spike proteins but they also recognize nonstructural and internal proteins. This suggests a potential for comprehensive immune responses through vaccine strategies focusing on spike protein and other viral proteins (Moss, 2022). Some studies have also described healthy individuals shown to have reactive CD4+ T-cells against SARS-CoV-2, indicating a possible cross-reactivity between circulating coronaviruses (CoV), hence T-cell cross-reactivity with other HCoVs may have some potential benefits for therapeutic protection (Moss, 2022). Comorbid conditions and aging are the major clinical factors of poor results that may suppress adaptive T-cell responses (García, 2020) (Files et al., 2021; García, 2020).

IFN-Gamma responses in T-cells

Interferon-gamma is a vital cytokine primarily produced by immune cells such as NK cells, T-cells, and macrophages and its main role lies in bolstering cellular immunity by promoting pathogen killing and activating macrophages. Upon its release, IFN-γ stimulates macrophages by boosting their microbicidal activity through the production of inflammatory mediators and reactive species, crucial for eliminating intracellular pathogens. (Bhat et al., 2017; Jorgovanovic et al., 2020; Ngai et al., 2007).

IFN-γ is involved in regulating immune responses by modulating antigen presentation, particularly through enhancing Major Histocompatibility Complex (MHC) antigen expression on macrophages, thereby facilitating efficient antigen presentation to T-cells (Alspach et al., 2019). Viral-specific cytolytic activity and IFN-γ or perforin expression in response to viral infections are highly correlated since interferon-gamma has antiviral effects on CD8+ T-cells where the ability of cytotoxic T-cells to move to the location of antigen-presenting cells is enhanced (Bhat et al., 2017; Ghanekar et al., 2001).

Humoral immune responses

The humoral/ antibody immune responses in SARS-CoV-2 happen in two phases; B-cells soon after infection develop into plasma cells outside of follicles, generating antibodies that are mostly of the IgM isotype with a brief half-life, capable of neutralizing the virus and with few somatic hypermutations (Elsner & Shlomchik, 2020). Phase two, the B-cells go through affinity-based selection and somatic hypermutation which lasts for several days to a week and produces

primarily isotype-switched, high-affinity plasma cells, producing antigen-specific memory B-cells in both stages (Assaid et al., 2023; Qi et al., 2022).

SARS-CoV-2-infected individuals within two weeks of the onset of symptoms, seroconvert to generate IgM and IgG antibodies that mostly detect the nucleocapsid and spike proteins (Assaid et al., 2023). Weeks after infection, neutralizing antibody levels reach their peak and can stay somewhat steady for a long time with IgG antibodies being steadier than IgM and IgA, and then start to decline which results in decreased protection and a higher chance of contracting the original strain or any developing variants (Emmerich et al., 2021; Goldblatt et al., 2022; Seow et al., 2020; Zamani et al., 2022).

Increased antibody titers, however, are related to more severe COVID-19 instances but do not always translate into improved outcomes (Goldblatt et al., 2022). Cross-reactive antibodies from prior exposure to different coronaviruses may also affect the formation of neutralizing antibodies specific to SARS-CoV-2 (Alturaiki, 2023; Chvatal-Medina et al., 2021; Goldblatt et al., 2022; Murray et al., 2023).

SARS-CoV-2 vaccines and their mechanisms

Extensive efforts have been put into the development and distribution of vaccines in response to the pandemic and the emergence of variants of concern. Globally, nearly 13.5 billion doses of the vaccines have been given out as of June 2023, marking a significant victory in the fight against the virus (https://data.who.int/dashboards/covid19/vaccines) and these vaccinations have

been essential in lessening the virus's capacity to propagate and lessening the severity of the illness (Fathizadeh et al., 2021).

These vaccines have different formulations eg. mRNA vaccines, Viral-vectored vaccines, and Subunit vaccines among others.

The m-RNA Vaccines

The Pfizer-BioNTech BNT 162b2 vaccine employs the mRNA-based technology which is synthetic and does not require cell culture or viral fermentation but utilizes lipid nanoparticles (LNPs) and formulated mRNA (A. Y. L. Wang, 2022). Because of their great efficacy, rapid creation and affordability, they were a viable treatment option in SARS CoV-2 (Al Fayez et al., 2023; A. Y. L. Wang, 2022; Wilson & Geetha, 2022).

The mRNA vaccines target the spike protein where a segment of mRNA that matches the spike protein is introduced into the cells which can create the viral protein utilizing this mRNA (Hogan & Pardi, 2021). The isolated mRNA is incorporated into a lipid nanoparticle and upon intramuscular injection, the nanoparticle attaches to host cells delivering its mRNA to the cytoplasm, moves to the ribosomes, and through translation, the production of viral spike proteins takes place (M. Li et al., 2022). These proteins move to the cellular membrane, evolving into either MHC-2 or MHC-1. MHC-2 proteins on B-cells, macrophages, and dendritic cells, trigger an immune response where T-helper cells produce cytokines, stimulating B-cells to develop into plasma cells that generate immunoglobulins targeting the viral spike proteins while interleukins stimulate the development of Th cells into memory cells (Al Fayez et al., 2023; Hogan & Pardi, 2021). MHC-1

proteins on cell membranes interact with cytotoxic T cells, producing cytotoxic proteins that can cause cell death when virally infected (Wilson & Geetha, 2022).

The vaccine induces a robust response, but the duration of immunity and antibody effectiveness against the virus remains uncertain and opinions on how long this immunity lasts vary, with suggestions ranging from six to nine months (Hogan & Pardi, 2021; A. Y. L. Wang, 2022).

Viral-vector Vaccines

AstraZeneca and Janssen are examples of viral-vectored vaccines that are crafted from a genetically modified virus that lacks pathogenic properties but carries genetic instructions for coronavirus proteins, triggering a safe immune response (H. Liu & Liu, 2023). The AstraZeneca vaccine triggers an immune response only to the viral proteins encoded in the host DNA by using an altered chimpanzee adenovirus that had not been exposed to humans before (H. Liu & Liu, 2023). Instructions for a protein that mimics the viral S-peptide and triggers an immune response are encoded in the DNA Adenovirus vector that binds to host cells when it enters humans and liberates the DNA which migrates to the cell nucleus through the cytoplasm and is not incorporated into the DNA of the cell, but it uses the enzymes of the host to transform into mRNA which then goes back to the cytoplasm and interacts with host cell ribosomes, either free or attached to the endoplasmic reticulum, to produce proteins (Travieso et al., 2022). These proteins are expressed on cell membranes, forming MHC-1 and MHC-2 complexes which leads to the stimulation of the adaptive immune cells (Vanaparthy et al., 2021).

Janssen Ad.26.COV2.S developed by Janssen Pharmaceuticals is administered through a single injection and can be conveniently stored in low temperatures for a long time as it does not rely on extremely low-temperature storage conditions typical of mRNA-based vaccines (Travieso et al., 2022; Vanaparthy et al., 2021). Similar to AstraZeneca, a benign cold Adenovirus 26 CoV2 is used in this vaccine as a carrier of a gene that codes for the spike protein present on the surface of the coronavirus and since it is a modified Adenovirus vector, it provides genetic information for the mRNA synthesis of the spike protein without having the capacity to replicate in human cells (H. Liu & Liu, 2023). After being injected, the vector adheres to human cells and moves the viral DNA into the nucleus without fitting in with the host DNA (Travieso et al., 2022).

The translated viral DNA strand produces mRNA in the cytoplasm, leading to spike protein synthesis which in turn triggers T cell and B cell immune responses. T-cells help in destroying infected cells, while antibodies protect uninfected cells by latching onto circulating free viral particles carrying spike proteins (H. Liu & Liu, 2023; Travieso et al., 2022; Vanaparthy et al., 2021).

Protective Role of Cellular Immunity in Vaccination

The central question surrounding cellular immunity against COVID-19 pertains to its significance in defense. It was believed, initially, that antibody responses provide defense against the first infection and that the development of neutralizing antibodies specific to the virus, especially in the airways, is a major indicator of future protection following vaccination or spontaneous infection.

However, new studies indicate that the prevention of the initial productive infection may potentially be significantly aided by cellular responses (Kumari et al., 2022).

A growing body of evidence suggests its potential consequences are in both limiting the infection and, more significantly, mitigating the severity of the disease post-infection (Z. Sun et al., 2022). While neutralizing antibodies are recognized as protective, the CD8+ T cell response has been found to contribute to the protection, especially in scenarios involving low or diminishing antibody levels, and the protective effects of CD4+ T cell adoptive transfer have been demonstrated in previous studies involving MERS and SARS-CoV-1 (Z. Sun et al., 2022).

Delivering the spike protein is the basis for the majority of COVID-19 vaccinations, and registration studies usually test participants' spike-specific cellular responses. Interestingly, a strong CD8+ T cell response is visible in the early stages, indicating its possible relevance to the protective clinical impact that is shown within 11 days of the initial immunization (Z. Sun et al., 2022). Dual vaccination with BNT162b2 has been shown to consistently generate virus-specific Th1 profile CD4+ T-cell responses which are essential for the generation and maintenance of antibodies and they become visible by day 8 following priming, reach their peak following the vaccine boost, and then drop to levels seen before the increase four months later (Z. Sun et al., 2022).

The activation of T-stem cell memory subsets following vaccination gives hope for a longer-lasting, more powerful cellular immunity, even though antibody levels are declining (Casado et al., 2022). One feature of vaccinations when compared to asymptomatic or moderate infection is their increased efficacy in

preventing serious disease, given the clinical protection they offer it raises the possibility that antibodies' capacity to fend off an initial infection may be limited, hence the possibility that cellular reactions are crucial in limiting significant tissue damage (Z. Sun et al., 2022). Many viral variations of concern (VOCs) strongly evade humoral immunity; yet, vaccine-induced cellular responses show significant cross-protection against VOCs, highlighting their important role in disease control (Jordan et al., 2021).

T-cell responses following dual vaccination resemble those following a natural infection in terms of size and given more worries about antibody fading following immunization, the question now centers on how long these responses will last (Bertoletti et al., 2021). Based on the vaccination subtype, the degree of spike-specific T-cell activation varies where in certain studies, adenovirus-based platforms provide relatively greater responses, whereas mRNA platforms result in larger antibody titers (Hyun et al., 2023; Sapkota et al., 2022; Takano et al., 2023). Though there are a few more short-term vaccination adverse effects with heterologous vaccination strategy, variation in vaccine efficacies has inspired investigations in heterologous vaccine platforms with reports of high-boosting effects compared to homologous vaccine strategy (Gerhards et al., 2023; Sapkota et al., 2022).

Vaccination and VOCs

Owing to the elevated frequency of mutations in the SARS-CoV-2 Spike protein, several recently surfaced variations saw a decrease in their vulnerability to neutralization by antibodies produced either through vaccination or spontaneous

infection (Wei et al., 2022). While some of these variations have managed to evade vaccination-induced immune protection and/or spontaneous infection, there is strong evidence that vaccinations reduce the risk of serious illness, as seen by decreased hospitalization and mortality rates (Y. Liu et al., 2022).

The continuing emergence of new variants poses a threat to reverse the incredible accomplishment made thus far in bringing to an end the transmission of the virus, despite the incredible pace at which vaccine development against COVID-19 has progressed and despite ongoing mass immunization campaigns (Gong et al., 2023). Insofar as the longevity of the protective immunity of booster immunization against the Omicron is yet unknown, booster vaccination has been demonstrated to elicit good neutralization against the Omicron (Wei et al., 2022). This supports the booster vaccination method. The most promising approach to putting an end to the COVID-19 plague in the future seems to be mass vaccination and additional booster shots using very potent and safe vaccinations (Y. Liu et al., 2022).

Booster Vaccination and Cellular Immune Responses

As the efficacy of two dosages of COVID-19 vaccines diminishes rapidly, numerous countries introduced booster doses to counter the danger posed by the Variants of Concern hence urgent and comprehensive studies are essential to understand the safety and effectiveness of boosters to ensure widespread vaccination coverage. Experiments on different boosters indicate that booster vaccinations generally offer robust protection(Chi et al., 2022; Deng et al., 2022; Munro et al., 2021).

Despite the VOC's increased immune escape ability due to numerous spike protein mutations, studies reveal that the third vaccine dose significantly boosts neutralizing antibody titers, particularly against Omicron (Paul et al., 2023). Current clinical data strongly support the success of booster vaccinations against the Omicron variant providing better defense than the principal series, with boosters exhibiting comparable or superior efficacy against symptomatic and severe infections compared to two doses, with sustained effectiveness against hospitalization (Paul et al., 2023).

Traditionally, vaccination methods rely on single-shot or repetitive shots of identical vaccines (homologous regimens) to induce immune responses. However, addressing present public health challenges may require a more robust immune response, both qualitatively and quantitatively, which can be challenging with traditional approaches. In contrast, a promising strategy involves heterologous booster vaccination regimens, aiming to stimulate collective humoral and cellular responses where the anticipated benefits include broader, stronger, and potentially longer-lasting immunity (Gerhards et al., 2023; Sapkota et al., 2022).

Although the precise mechanism behind the improved immunity resulting from heterologous vaccination is yet unknown, it could be because distinct immunological pathways are stimulated by diverse platforms, which in turn increases the robustness and scope of the immune response (X. Liu et al., 2023). Therefore, combining vaccines with other platform vaccines can modulate antibody responses, and cellular responses and increase neutralizing antibody levels (X. Liu et al., 2023; Takano et al., 2023). Other studies suggest that T-cell responses are

higher in groups receiving heterologous regimens, supported by vigorous cellular immune responses seen in animal research following such vaccination regimens with possible mechanisms for this phenomenon being the generation of a strong response from naive cells through epigenetic reprogramming when a heterologous vaccine is given (Wei et al., 2022).

Despite the paucity of information regarding the effectiveness and immunogenicity of homologous or heterologous booster vaccines, diverse booster vaccination regimens are necessary to improve flexibility during probable vaccine shortages as the demand for vaccine boosters develops globally, (X. Liu et al., 2023; Zhu et al., 2022). Although there are some inconsistent results, heterologous boosters generally show significant potential for boosting immune responses against COVID-19 while this finding may be linked to the different kinetics of immune responses induced by vaccines (Cheng et al., 2022; Hyun et al., 2023; Wei et al., 2022). To inform vaccination policies in the use of vaccines, it is imperative to conduct rapid assessments of the efficacy of various booster immunization regimens.

Assessment of T-cell responses: The ELISPOT Assay

ELISPOT, or enzyme-linked immunospot, is an immunological assay designed to identify the release of specific cytokines, such as gamma interferon (IFN-γ), upon exposure to an antigenic stimulus (Bercovici et al., 2000; Jeewandara et al., 2018).

In the assay, anti-IFN-γ antibodies are coated in wells where T-cells and antigen-presenting cells are cultured, and the released IFN-γ collected by these

antibodies are made visible by a second antibody that is attached to a chromogenic substrate (Leehan & Koelsch, 2015). Spots produced by cytokine molecules indicate a cell secreting IFN-γ, and the quantity of spots helps determine the rate of IFN-γ-secreting cells that are specific to a given antigen (Bercovici et al., 2000; Ranieri et al., 2014). Simultaneous detection of multiple cytokines has also been described and could be valuable for assessing immune deviation (Palzer et al., 2005).

The assay doesn't quantify the amounts of secreted cytokines but obtains rough estimates through computer-assisted image analysis to measure spot density and area (Bercovici et al., 2000; Slota et al., 2011). The assay's sensitivity, adaptableness, and capacity to be carried out directly *ex vivo* with a comparatively small number of T-cells make it an excellent choice for tracking immunological responses to vaccinations (Slota et al., 2011).

CHAPTER THREE

RESEARCH METHODS

Study site

This study was conducted in Legon, the Greater Accra Region of Ghana. Legon is a suburb of the Ghanaian city of Accra, situated about 12 kilometers (7.5 mi) northeast of the city center in the Ayawaso West Municipal District, a district in the Greater Accra Region of Ghana. Laboratory analysis was conducted at the Noguchi Memorial Institute for Medical Research, in Accra, Ghana.

Study Participants

In this study, we used archived samples from a population comprising individuals aged 18 years and older from the Legon community. All the study participants were fully vaccinated before taking a booster shot of either the Janssen or Pfizer vaccine. Pre-booster and 3-, 6- and 9-month post-booster blood samples were collected from the participants and were categorized into four groups, depending on the type of booster shot received; the Janssen and Pfizer vaccination and infection background: (1) individuals who received homologous vaccination; (2) individuals who underwent heterologous vaccination; (3) those who were vaccinated and later became infected with SARS-CoV-2; and (4) individuals who had contracted and recovered from SARS-CoV-2 and then received a subsequent vaccination.

Research design and Sampling

This study adopted a retrospective longitudinal design and is a subset of a larger project 'Immunocov' aimed at assessing immune responses in SARS-CoV-2 booster vaccination.

Archived samples from participants drawn from the Legon community; Madina New Road, Fadama, Dome Pillar 2, Kwabenya, Lashibi, Tema, Legon campus, East Legon, Okponglo, Korle-Bu, Takoradi Common Wealth area (Ablekuma), Pentecost University and Gbawe were used.

Peripheral blood mononuclear cells (PBMCs) from thirty (30) participants who received the Janssen and Pfizer vaccine boosters at four time points; Pre-vaccination/Day 0, Month 3, Month 6, and Month 9, were used in testing against the spike peptides and hence experimentally determined immunodominant peptides. Samples were from fifteen individuals that received Janssen as a booster shot and another fifteen took the Pfizer vaccine.

Inclusion And Exclusion Criteria

As a subset study, participants in the main study were included if they met the following criteria:

1. Aged 18 years and older.
2. No known medical conditions on screening; hemoglobin greater than 10 g/dL; no known immunodeficiency.
3. Females who were not expectant or nursing.
4. Willingness to consent and receive a booster dose of Janssen or Pfizer.

SARS-CoV-2 Stimulation Peptides

To determine the fragment peptide sequences for stimulation out of the entire spike protein sequence, we employed HLA restriction epitope prediction based on their binding to HLA supertypes A01, A02, A03, A24, B07, B44, and B58 which are the most dominant HLA supertypes among Ghanaian populations (Kusi et al., 2022). The bioinformatics analysis was conducted utilizing the NetMHCpan-4.1 tool (https://services.healthtech.dtu.dk/services/NetMHCpan-4.1/).

We achieved the predictions to human MHC class I molecules by uploading a full-length SARS-CoV-2 spike protein sequence and predictions made for 10 amino acids in length of peptides. We then select the peptides with strong binding values (below 0.5%) using the Eluted Ligand (EL) prediction score for the probability of a peptide being naturally presented by an MHC receptor to ensure a comprehensive and informative approach to our work. A selection from already synthesized SARS-CoV-2 spike antigens consisting of 15mer in span and overlapping by 10 amino acids, that contained the predicted 9-10mer epitopes for experimental testing was done.

Sample Processing

We used archived peripheral blood mononuclear cells (PBMCs) in our study. Briefly, the PBMCs were obtained from the participants by collecting 40 to 60mls of blood per participant into heparinized tubes, and separation of PBMCs from blood was done using Accuspin Histopaque-1077 cell separating tubes by gradient centrifugation. After washing, the PBMCs were cryopreserved which involved resuspending PBMCs in a cryoprotective solution e.g., FBS containing

10% DMSO, and aliquoting them into cryovials which were then placed in a controlled-rate freezer to gradually lower the temperature, typically to -80°C, before transferring them to a liquid nitrogen (-196°C) storage tank.

Recovery of Frozen PBMCs

The cryopreserved PBMCs were retrieved from liquid nitrogen and reconstitution of the cells in complete medium (10% FBS in RPMI) was done and rested in the incubator at 37ºC, 5% CO_2 for about 16 - 20 hours overnight. After 16-20 hours of incubation, the cells were centrifuged at 300 x g for 10 min at room temperature, the supernatant discarded, cells reconstituted in 1ml of the complete medium, and estimation for viability by Trypan blue. Cell concentration was adjusted to 3 x 10^6 cells/ml before adding to the wells.

The Ex-vivo ELISPOT Assay

The wells of sterile, clear 96-well Hydrophobic PVDF membrane plates (Millipore Corporation, USA) were pre-wet with 50 µl of 70% ethanol for 1 minute and washed 4 times with sterile phosphate-buffered saline (PBS). Anti-human IFN-γ mAb (1-D1K), unconjugated stock antibody (Mabtech AB, USA) was added to bicarbonate coating buffer (0.1 M bicarbonate buffer, pH 9.6) at the concentration of 10µg/ml and mixed and 100 µl/well dispensed into the PVDF membrane plates and incubated overnight at 4ºC.

The following day, pre-coated plates from the fridge were washed 6 times with 200 µl/well of sterile PBS using a multi-channel pipette and blotting of the plates to dry them after every wash. 200 µl/well of Blocking buffer was added into wells of a pre-coated plate and incubated for 2 hours at room temperature in the

biosafety cabinet. At the end of the incubation time, the blocking solution was discarded and 100 ul/well of appropriately diluted peptides and control stimulants were added to wells of ELISPOT plates according to the template design. Concanavalin A (Con A, Sigma Aldrich, USA) was used as the control stimulant. Briefly, the test peptides were reconstituted to a working concentration of 5µg/ml while Con A was reconstituted to a working concentration of 1.25µg/ml for plating. Subject PBMCs incubated with medium only were used as negative controls.

The 100 µ/l of recovered and rested PBMCS at 2 x 106 cells/ml to arrive at 200,000 cells/well was added to the test wells, while we 20,000 cells/well for the control stimulant. Cell suspensions were added in duplicate wells per test peptide according to the format on the plate template. The plated cells were then incubated in a CO_2 incubator at 37^0C, 5% CO_2 for 18-24 hours.

Non - sterile Procedure

After 18-24 hours of incubation the plates were retrieved from the incubator and washed 6 times with 200 ul of Washing Buffer for ELISPOT (1XPBS), blotted on dry lint-free tissue and 100ul of 1 µg/ml biotinylated anti-IFN-γ monoclonal antibody (Mabtech, USA) diluted in 0.5 % fetal calf serum (FCS) in PBS for 3 hours at room temperature solution was then added and incubated for 2hrs at room temperature. After incubation, the plate(s) are washed 6 times with 200 ul of washing buffer (1XPBS) then blotting the plates on dry lint-free tissues and 100ul of 1 µg/ml alkaline phosphatase-conjugated streptavidin (Mabtech, USA) added for 1 hour at room temperature.

Plates were subsequently washed six times as above and three times with plain PBS before incubation with an enzyme-specific chromogenic substrate (Bio-Rad, USA) for 15 min at room temperature. The reaction was stopped by manually flicking out the contents into the sink and immediately washing the plate with deionized water (DI) water or distilled water then air-dried overnight and counting of spots using the automated AID multispot Reader (AID GmbH, Germany).

Statistical Analyses

Activities were calculated as spot-forming cells per million (sfc/m) PBMCs. On assay performance, the ELISpot activity (sfc/m) for the unstimulated medium (negative control) was subtracted from the activities (sfc/m) for each test peptide. The assay was measured positive if there was at least a doubling up of spot-forming cells (sfc/m) in test wells comparative to control wells and a difference of at least ten spots between test and control wells and expressed as invalid if the negative control had more than 10 sfc/m as adopted from (Ganeshan et al., 2016).

We used descriptive statistics to summarize the demographic characteristics. Data management and statistical analysis were done using Excel and SPSS version 26. The levels of T-cell stimulation or responses in the ELISpot assay were compared between the groups using appropriate statistical tests, such as the Unpaired t-test or Mann-Whitney U test and ANOVA. A p-value of <0.05 was considered statistically significant. Graphics were obtained using Prism (Graph pad), SPSS version 26 and Microsoft Excel.

Ethics

As a subset of a larger study, the umbrella study was permitted with approval by the Institutional Review Board of Noguchi Memorial Institute for Medical Research (NMIMR); clearance Reference number: #010/22-23. The archived samples used were obtained from subjects 18 years or older who willingly accepted to be part of the study, met the inclusion criteria and gave written informed consent.

CHAPTER FOUR

RESULTS AND DISCUSSION

Demographic characteristics of the study

Peripheral blood mononuclear cells (PBMCs) from thirty healthy participants were used for this study, where the subjects were between 18 and 41 years (Mean 25 years, SD=5.89). All the participants had a normal medical history and physical examination with hemoglobin levels > 10g/dL. The time from the baseline vaccination to taking the booster shot ranged from 7 to 24 months (Mean 15 months, SD=3.88). All the subjects from which the PBMCs were taken had no comorbidities.

As part of the conditions for this study, all the participants were fully vaccinated and took a booster shot of either Janssen or Pfizer. Fifteen subjects comprising 10 males (66.6%) and 5 (33.3%) females took the Janssen vaccine and another fifteen comprising 9 males (60%) and 6 females (40%) took the Pfizer vaccine booster shot. Among the subjects that took Janssen as the booster shot, 9 individuals (60%) were previous recipients of a viral-vectored vaccine i.e., homologous vaccination, while 6 individuals (40%) received m-RNA vaccine i.e., heterologous vaccination. In the subjects that received Pfizer as a booster shot, 6 individuals (40%) were recipients of the m-RNA vaccine at baseline (homologous) while the other 9 (60%) had viral-vectored baseline vaccination (heterologous).

Table 1 is a summary comparison of the demographic characteristics of the study participants. No difference in age, sex, prime boosting type, or previous COVID-19 infection was demonstrated between the two groups ($p>0.05$).

Table 1: Demographic characteristics of the study

Variable	Total N (%)	Pfizer Booster (%)	Janssen Booster (%)	P-value
Age	**Mean 25 SD 5.89**			0.132
<25 years	12 (40)	8 (66.66)	4 (33.33)	
≥25 years	18 (60)	7 (38.88)	11 (61.11)	
Sex				0.5
Male	19 (63.33)	9 (47.36)	10 (52.63)	
Female	11 (36.66)	6 (54.54)	5 (45.45)	
Time since baseline vaccination	**Mean 15 SD 3.88**			0.223
7-12 months	6 (13.33)	3 (50)	3 (50)	
13-18 months	16 (63.33)	6 (37.5)	10 (63.5)	
>18 months	8 (16.66)	6 (75)	2 (25)	
Prime boosting type				0.233
Heterologous	15 (50)	9 (60)	6 (40)	
Homologous	15 (50)	6 (40)	9 (60)	
Previous COVID-19 infection				0.5
Infected	3 (10)	2 (66.66)	1 (33.33)	
Not Infected	27 (90)	13 (48.14)	14 (51.85)	

The p-values were obtained by chi-squared test, $p<0.05$ is considered statistically significant. Homologous prime boosting- booster vaccine formulation is the same as previously administered. Heterologous prime boosting- booster vaccine formulation different from the baseline. SD- standard deviation.

HLA restriction of SARS-CoV-2 epitopes

We sought to determine immunogenic epitopes from the entire SARS-CoV-2 spike protein that are targeted by CD8+ T cells. Bioinformatics prediction was done using the *in-silico* tool NetMHCpan-4.1 (https://services.healthtech.dtu.dk/services/NetMHCpan-4.1/**)** for T-cell epitopes utilizing the seven most common HLA class 1 supertypes among the Ghanaian population. We identified 22 immunogenic peptide sequences with strong binding values; Eluted Ligand (EL)

rank (EL_Rank) below 0.5%**.** We then selected the synthesized 15mer peptides overlapping by 10 amino acids that contained the predicted 9-10mer epitopes for experimental testing. (Table 2).

Table 2: Spike peptides used for stimulation

Peptide sequence	HLA haplotype	El_Rank (%)	Name of Synthesized Peptide
VYSSANNTF	HLA-A24:02	0.0481	SGP030
SSANNCTFEY	HLA-A01:01	0.1587	SGP032
YLQPRTFLLK	HLA-A03:01	0.153	SGP054
ATRFASVYAW	HLA-B58:01	0.2392	SGP069
LYNSASFTF	HLA-A24:02	0.051	SGP074
KLNDLCFTNV	HLAA-A02	0.3539	SGP077
RQIAPGQTGK	HLA-A03:01	0.0396	SGP082
KLPDDFTGCV	HLAA-A02	0.3198	SGP085
NLDSKVGGNY	HLA-A01:01	0.1564	SGP088
NTSNQVAVLY	HLA-A01:01	0.1315	SGP121
IHADQLTPTW	HLA-B58:01	0.1029	SGP125
TEILPVSMTK	HLA-A03:01	0.1532	SGP145
KQIYKTPPIK	HLA-A03:01	0.0347	SGP157
KRSFIEDLLF	HLA-B58:01	0.2545	SGP163
LADAGFIKQY	HLA-A01:01	0.0798	SGP166
LLTDEMIAQY	HLA-A01:01	0.0225	SGP173
VEAEVQIDRL	HLA-B40:01	0.1392	SGP198
HVTYVPAQEK	HLA-A03:01	0.1401	SGP213
SLIDLQELGK	HLA-A03:01	0.2339	SGP239
LIDLQELGKY	HLA-A01:01	0.1049	SGP240
FDEDDSEPVL	HLA-B40:01	0.2454	SGP251
SEPVLKGVKL	HLA-B07:02	0.2057	SGP252

EL_rank is the prediction score for the likelihood of a peptide being naturally presented by an MHC receptor. SGP- Spike Glycoprotein. The predictions were done based on the most common HLA supertypes among the Ghanaian populations; HLA A01, A02, A03, A24, B07, B44, and B58. The peptide sequences were obtained from the Wuhan strain of the SARS-CoV-2 spike protein sequence.

Characterization of responses at Baseline and Month 3

On performing the ELISpot assay on the subjects' PBMCs utilizing the synthesized peptides and applying the positivity criteria outlined, we performed the

analysis of Pre-booster and Month 3 responses. Baseline interferon-gamma responses were substantial in both groups with no significant intergroup differences. At Month 3, eighteen subject PBMCs (60%) registered positive responses to at least one of the spike peptides. Twelve samples out of the 30 (40%), did not register any responses to any of the spike antigen peptides at month 3. Ten subject PBMCs (55.5%) out of the positive 18 subject PBMCs were from the Janssen booster shot recipients while the 8 subject PBMCs (44.4%) were from the Pfizer booster shot recipients.

Peptide-specific Interferon-gamma responses in Janssen recipients

Ten subject PBMCs out of the 15 Janssen samples showed positive responses at Month 3. Peptides SGP125 and SGP163 were responsive to 5 subject PBMCs, and peptides SGP030, SGP054, SGP082, SGP166 were responsive in 4 subject PBMCs among the positive subject PBMCs. Peptide SGP252 did not register any positive responses. (Figure 2).

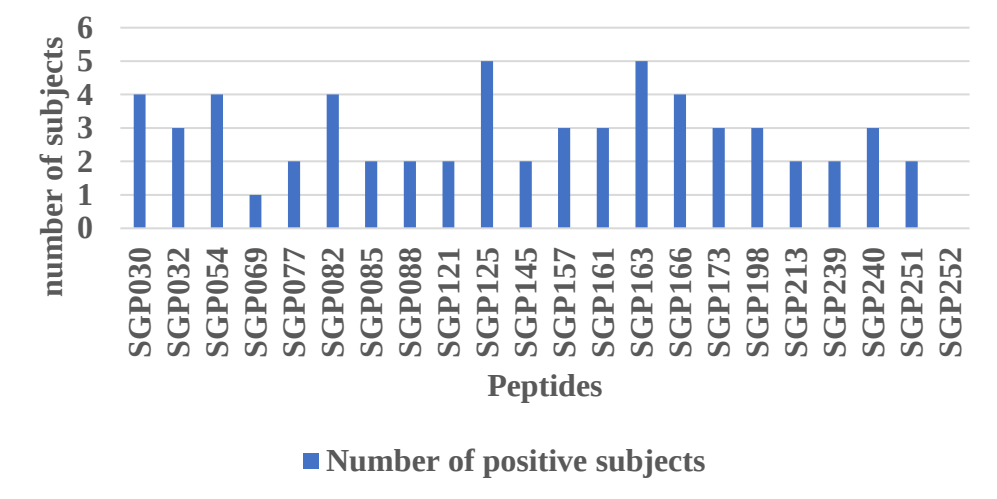


Figure 2: Proportion of peptide-specific positive responses in the Janssen group

The highest magnitude of interferon-gamma responses in the Janssen booster vaccination was against SGP240 (93 sfc/m PBMCs), SGP121 (66 sfc/m PBMCs) and SGP251 (54 sfc/m PBMCs). Peptide SGP252 showed the lowest magnitude of responses in this group. The average response in terms of sfc/m PBMCs against all the peptides was 39 sfc/m PBMCs. (Figure 3).

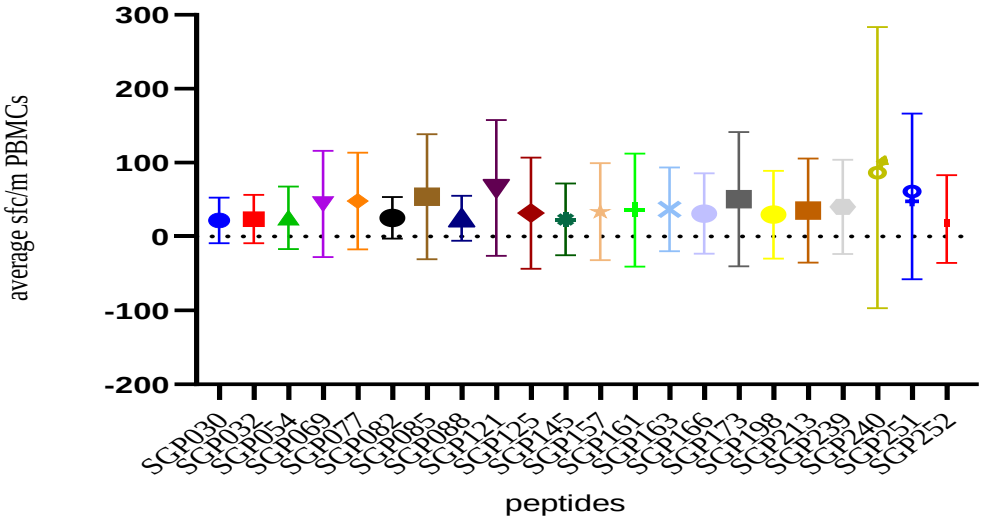


Figure 3: The average spike-specific interferon-gamma responses in terms of sfc/m PBMCs at Month 3 in the Janssen booster vaccination group.
The long bars are the CIs at 95% (confidence intervals) and the spots are the means. Peptides SGP240, SGP121 and SGP251 showed the highest responses while peptides SGP030, SGP032, SGP054, SGP082 SGP088 and SGP252 elicited low interferon-gamma responses.

We then compared the responses between the Pre-booster and Month 3, and there was a substantial increase in interferon-gamma responses at Month 3 relative to the Pre-booster in the majority of the spike peptides. Using the Related-samples Wilcoxon signed rank sum test (dependent t-test), there was a significant increase in the magnitude of responses in six spike peptides by month 3 in the Janssen booster vaccination. (Figure 4).

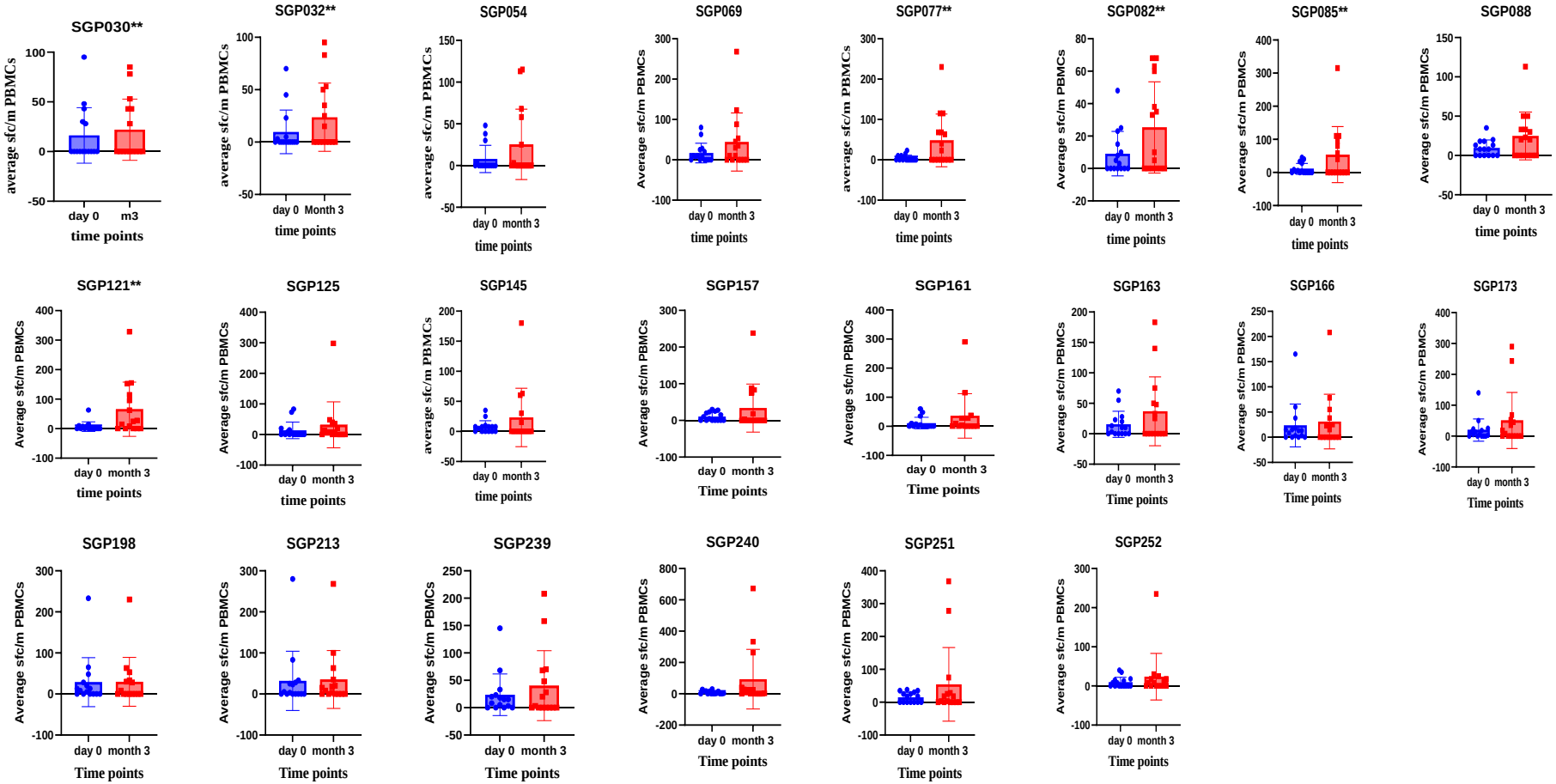


Figure 4: Comparison of spike-specific responses between Pre-booster and Month 3 in the Janssen booster samples.
Responses against SGP030, SGP032, SGP077, SGP082, SGP085 and SGP121 indicated ** showed statistically significant differences in the responses between Pre-booster and Month 3.

Interferon-gamma responses in Pfizer recipients

With regards to the Pfizer booster vaccination samples, eight subject PBMCs out of the 15 Pfizer samples showed positive responses at Month 3. Most of the subject's PBMCs were responsive to peptides SGP077 and SGP251; 3 subjects' PBMCs each, peptides SGP085, SGP173, SGP239 and SGP240 showed positive responses in 2 subject PBMCs each. Peptides SGP088, SGP057, SGP161, SGP163, SGP166 and SGP252 did not elicit positive responses in this group. (Figure 5).

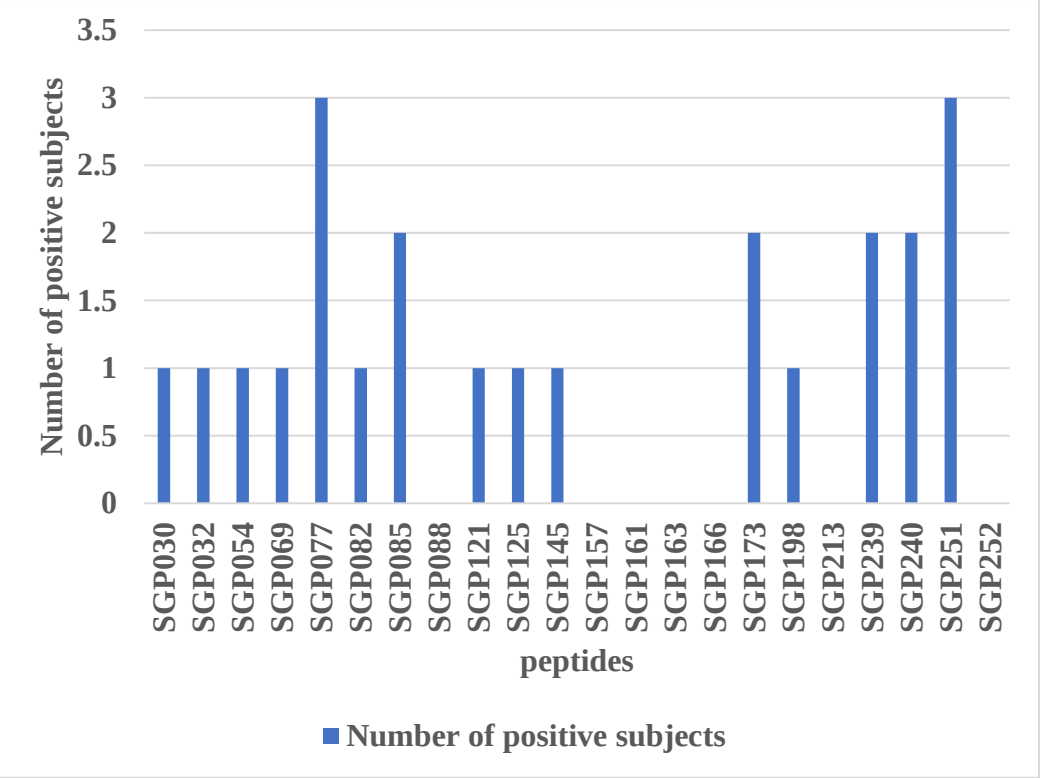


Figure 5: Proportion of peptide-specific positive responses in Pfizer vaccination recipients.

Against the subject PBMCs, peptides SGP088, SGP157, SGP161, SGP163, SGP166, SGP213 and SGP252 did not elicit positive interferon-gamma responses.

The highest responses in the Pfizer booster vaccination group were against peptide SGP054 (55 sfc/m PBMCs), SGP082 (52 sfc/m PBMCs) and SGP121 (52 sfc/m PBMCs), while the lowest amounts of responses were against SGP252 (15 sfc/m PBMCs). The average sfc/m PBMCs against all the peptides were 39sfc/m PBMCs. (Figure 6).

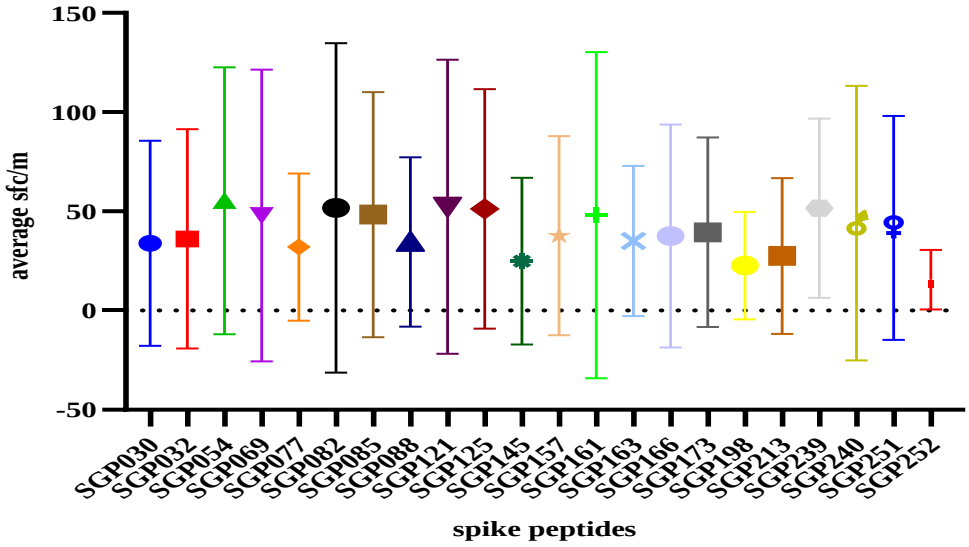


Figure 6: The average spot-forming cells per million PBMCs against specific spike peptides at month 3 in the Pfizer samples.
The long bars are the CIs at 95% (confidence intervals) and the spots are the means. Against the subject PBMCs, interferon-gamma responses against SGP054, SGP069, SGP082 and SGP121 showed the highest magnitude of responses. Peptide SGP252 registered the lowest amounts of interferon-gamma responses.

Using the related-samples Wilcoxon signed rank sum test (dependent t-test), we compared the magnitude of responses at Month 3 with the Pre-booster interferon-gamma responses for the Pfizer samples and found no significant differences in the magnitude of responses in many of the spike peptides. (Figure 7).

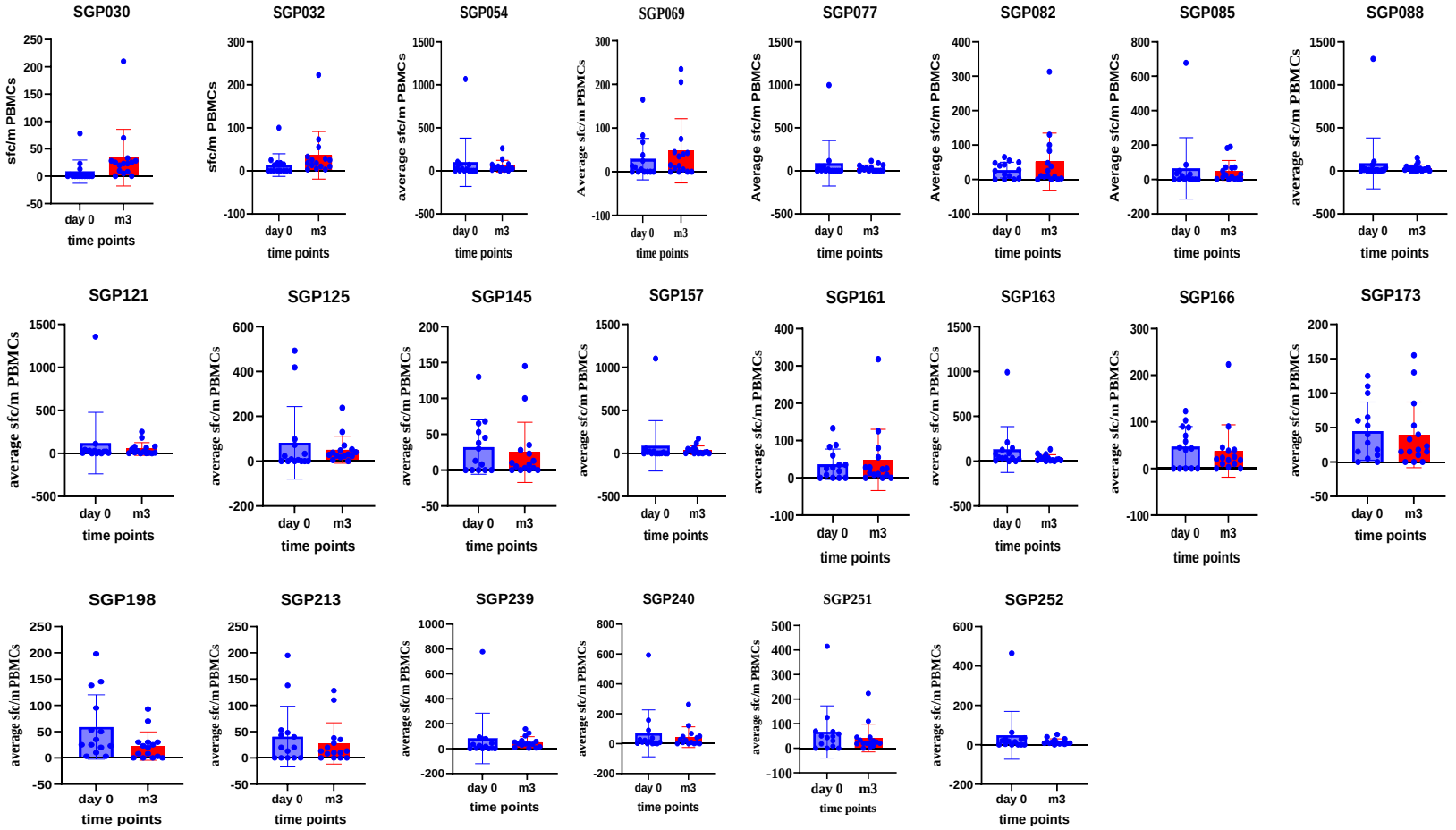


Figure 7: A comparison of spike-specific interferon-gamma responses for Pre- booster and Month 3 in Pfizer vaccination

Comparison of responses between the Pfizer and Janssen boosters at Month 3

A comparison of these responses' proportions is shown (Figure 8). The majority of positive peptide-reactive subject PBMCs by month 3 were seen in individuals who took the Janssen booster shot as compared to those who took the Pfizer shots (Figure 8).

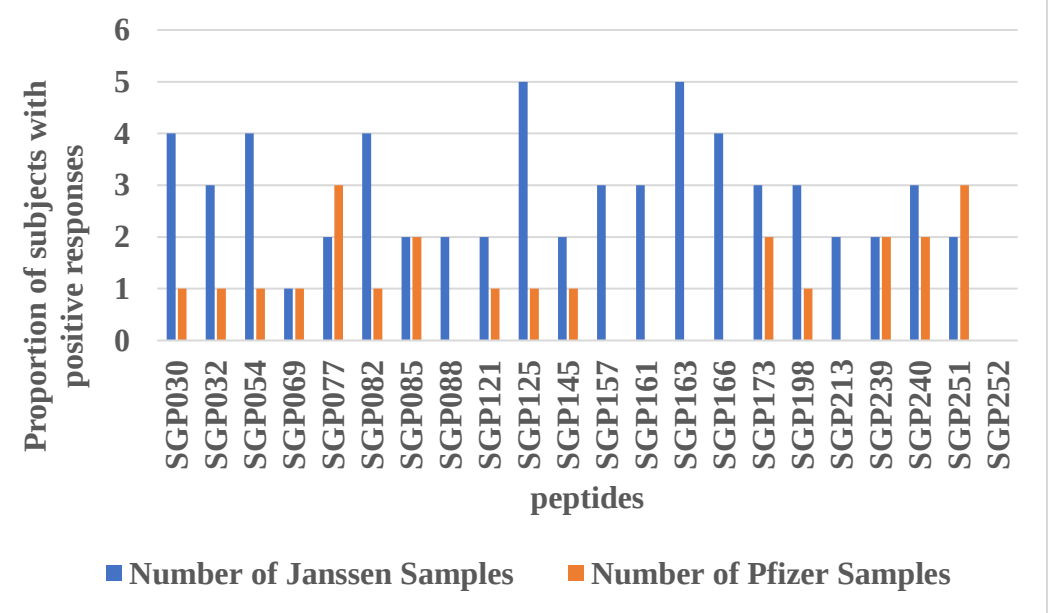


Figure 7: A comparison of peptide-positive responses at Month 3.
Janssen boosted samples showed a high proportion of positive responses against peptides SGP030, SGP032, SGP054, SGP082, SGP088, SGP121, SGP125, SGP145, SGP157, SGP161, SGP163, SGP166, SGP173, SGP198, SGP213 and SGP240. There were no registered positive responses in Pfizer-boosted samples against SGP088, SGP157, SGP161, SGP163, SGP166, SGP213 and SGP252, with the latter showing no positive responses to Janssen-boosted samples as well.

Using the Mann-Whitney U test (independent t-test), we compared the magnitude of interferon-gamma responses between the Janssen booster vaccination and Pfizer booster vaccination at Month 3 in terms of spot-forming cells per million (sfc/m) PBMCs and found no statistically significant difference in the magnitude of interferon-gamma responses between the recipients of Janssen and Pfizer vaccine boosters against all the spike peptides. (Figure 9).

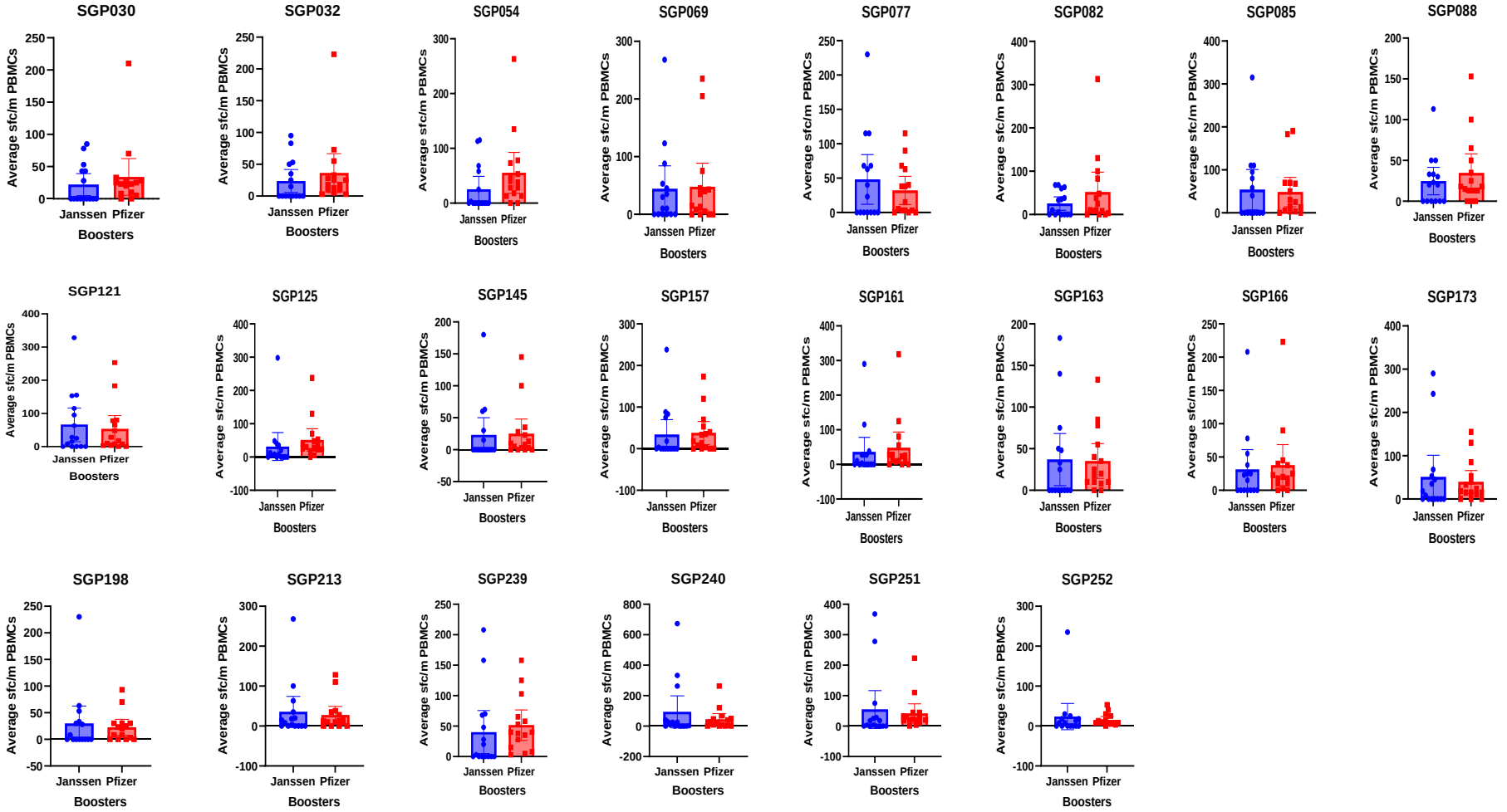


Figure 8: A comparison of interferon-gamma responses between the Pfizer and Janssen at Month 3.
Interferon-gamma responses against all the peptides showed no statistically significant difference between the vaccine boosters.

Peptide recognition at Months 6 and 9 and characterization of responses

For the peptides that showed positive responses at Month 3 relative to Pre-booster, we further assessed the recognition of antigen peptides using the sample subjects' PBMCs from Months 6 and 9. Seven samples (38.9%) of the 18 that showed positive responses at Month 3 showed positive responses to the spike protein peptides at Month 6. Eleven samples (61.1%) did not register any response to any of the spike peptides.

Out of the 7, the five subject PBMCs that registered positive responses were from individuals who received the Janssen booster vaccine while 2 were from the Pfizer booster recipients. On the Pfizer booster group, subject IDs P051 and P032 registered positive responses to 4 and 6 peptides respectively. Subject ID J171 of the Janssen booster group registered positive responses to 5 peptides, J114 and J031 to 2 peptides each, and J035 and J206 against 1 peptide each. The proportion of peptide-positive responses is shown in Figure 10.

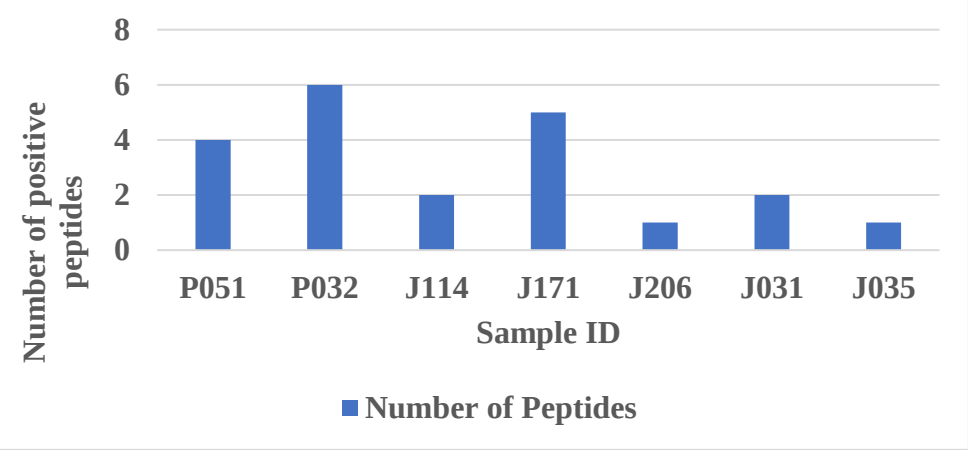


Figure 9: The proportion of positive peptides in the two vaccine boosters at Month 6.
Subject PBMCs P051 showed positive responses to 4 peptides, P032 to 6 peptides, J114 and J031 to 2 peptides each, J171 to 5 peptides, and J206 and J035 to 1 peptide each.

There was a significant decrease in the magnitude of responses at Month 6 in terms of sfc/m PBMCs as compared to Month 3. The average response at Month 6 was 33.3 sfc/m PBMCs and the highest responses were against SGP213 (125 sfc/m), SGP088 (48 sfc/m), SGP198 (47.5 sfc/m) and SGP121 (41.66 sfc/m). (Figure 11).

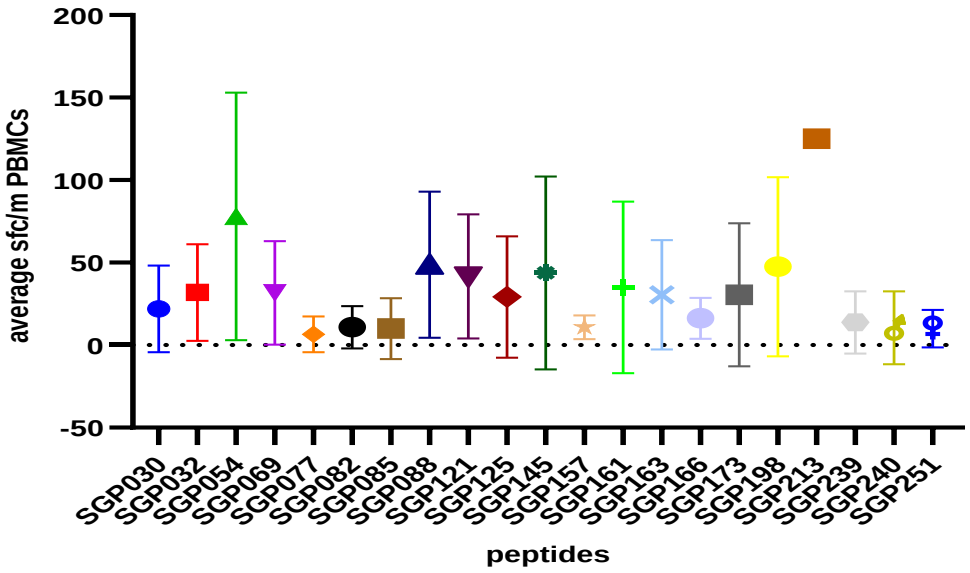


Figure 10: The average spot-forming cells per million against the specific peptides at Month 6.
The long bars are the CIs (confidence intervals) at 95% and the spots are the means. Against subject PBMCs peptides SGP054, SGP213 and SGP088 registered the highest responses. Peptides SSGP077, SGP082, SGP085, SGP157, SGP166, SGP239, SGP240 and SGP251 showed the lowest magnitude of interferon-gamma responses.

At Month 6, peptides SGP030, SGP166, SGP173 and SGP251 indicated positive responses in two samples each, while SGP032, SGP054, SGP069, SGP077, SGP082, SGP085, SGP12, SGP157, SGP163, SGP198, SGP213, SGP239 and SGP240 were positive in one sample each. Peptides SGP088, SGP121, SGP145, SGP161, and SGP252 exhibited no positive responses in any of the

subject PBMCs. (Figure 12). In total, 17 peptides were responsible for positive responses at Month 6.

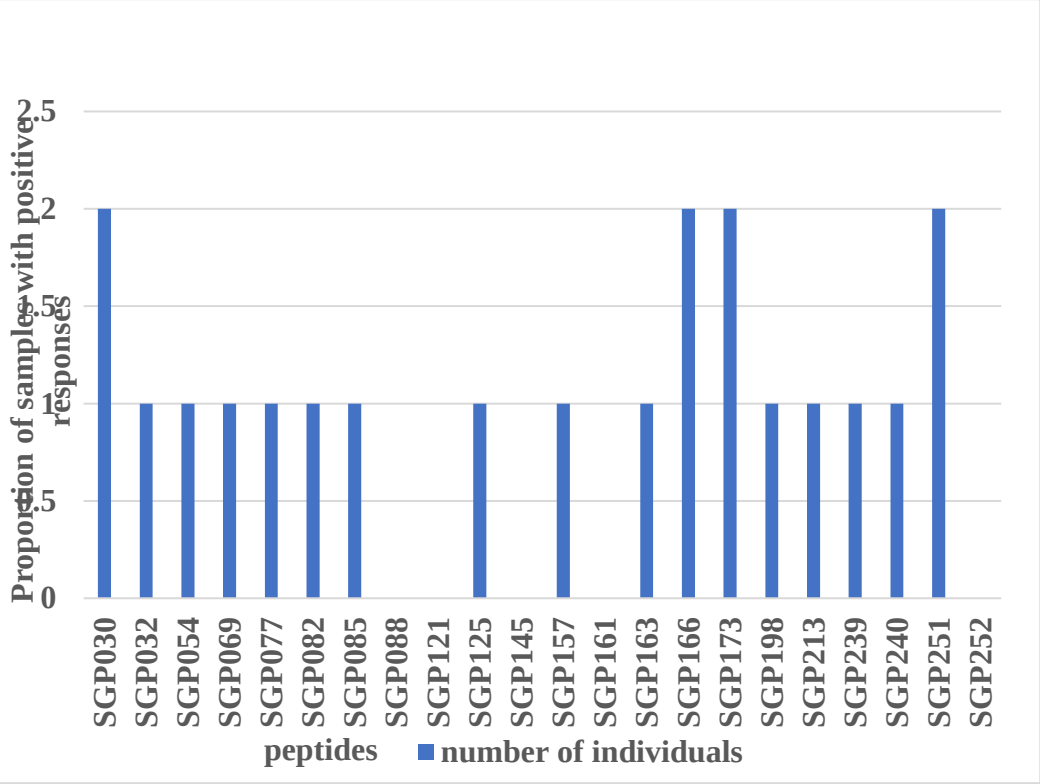


Figure 11: The proportion of samples with positive responses against the spike peptides at month 6.

Peptides SGP030, SGP166, SGP173 and SGP251 indicated positive responses in two subject PBMCs each, SGP032, SGP054, SGP069, SGP077, SGP082, SGP085, SGP12, SGP157, SGP163, SGP198, SGP213, SGP239 and SGP240 were positive in 1 subject PBMCs each. Peptides SGP088, SGP121, SGP145, SGP161, and SGP252 exhibited no positive responses

At Month 9, of the 7 subjects' PBMCs that showed positive responses at Month 6, only 1 sample from the Janssen booster group, ID (J171), registered positive responses specifically against the spike peptides SGP30, SGP32, SGP082, SGP157 and SGP173. There was a further significant decrease in the magnitude of responses at Month 9 and the average response was at 15.64 sfc/m PBMCs and the

highest responses were against SGP157 (89.1 sfc/m PBMCs), SGP082 (18.75 sfc/m PBMCs) and SGP121 (15.83 sfc/m PBMCs). (Figure 13).

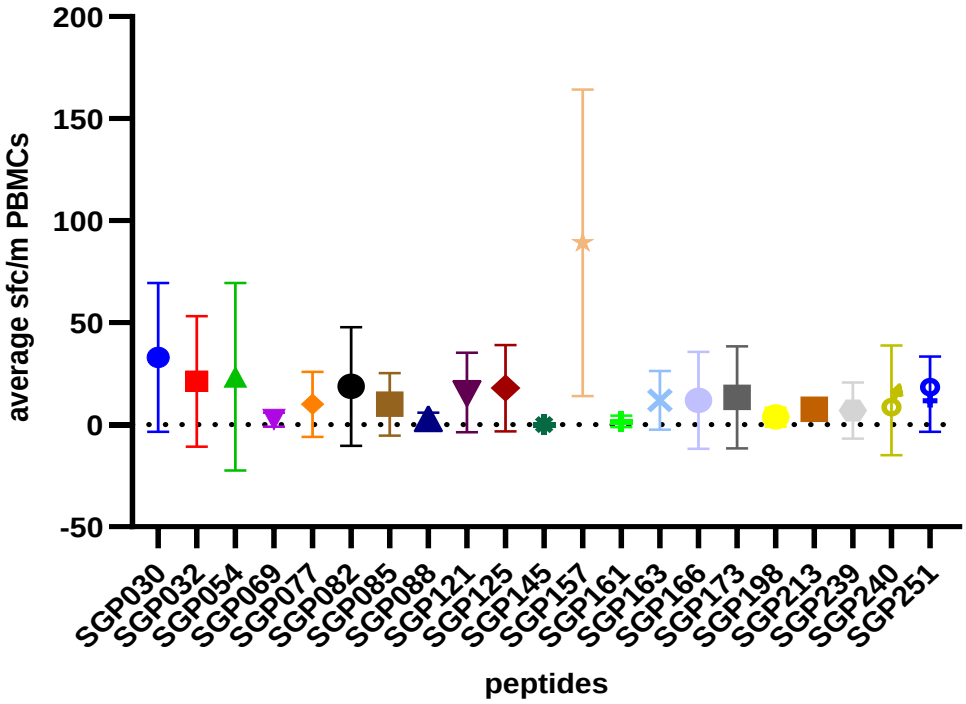


Figure 12: The average spot-forming cells per million against the specific peptides at Month 9.
The long bars are the CIs (confidence intervals) at 95% and the spots are the means. Against the subject PBMCs, peptides SGP030, SGPSGP032, SGP054 and SGP157 showed higher responses.

Comparison between homologous and heterologous boosters

At Month 3, 18 subject PBMCs were responsive against the spike peptides, predominantly the Janssen prime booster group; 10 from Janssen and 8 from the Pfizer group. Out of the ten Janssen-positive samples, 6 (60%) were from recipients of the viral-vectored vaccine pre-study (homologous boosting) while four samples (40%) were from the individuals who took an m-RNA baseline vaccine prior to joining this study (heterologous boosting). On the other hand, three Pfizer-positive

subject PBMCs had viral-vector pre-study vaccine prior to the study (heterologous boost) while five samples were from individuals who received the m-RNA vaccine pre-study (homologous booster). (Table 3).

Table 3: Pre-study and booster vaccinations by the subjects who showed positive responses at month 3.

Boosting Type: Homologous/ heterologous (%)	Booster Vaccine Type	Pre-study vaccines	Sample ID
Homologous (60%)	Janssen (viral-vectored)	Viral-vectored	J031, J035, J059, J171, J183, J206
Heterologous (40%)	Janssen (viral-vectored)	m-RNA vaccine	J113, J114, J176, J180
Homologous (62.5%)	Pfizer (mRNA)	m-RNA vaccine	P015, P051, P059, P066, P109
Heterologous (37.5%)	Pfizer (mRNA)	Viral-vectored	P018, P032, P056

In the viral-vector homologous booster vaccination, subject ID J059 registered responses against 17 peptides, while subject ID J035 registered responses against 18 of the 22 peptides used. In heterologous viral-vector booster vaccination, subject ID J114 registered positive responses to 6 peptides whereas the rest subjects' PBMCs showed responses to 1 peptide each. (Figure 14).

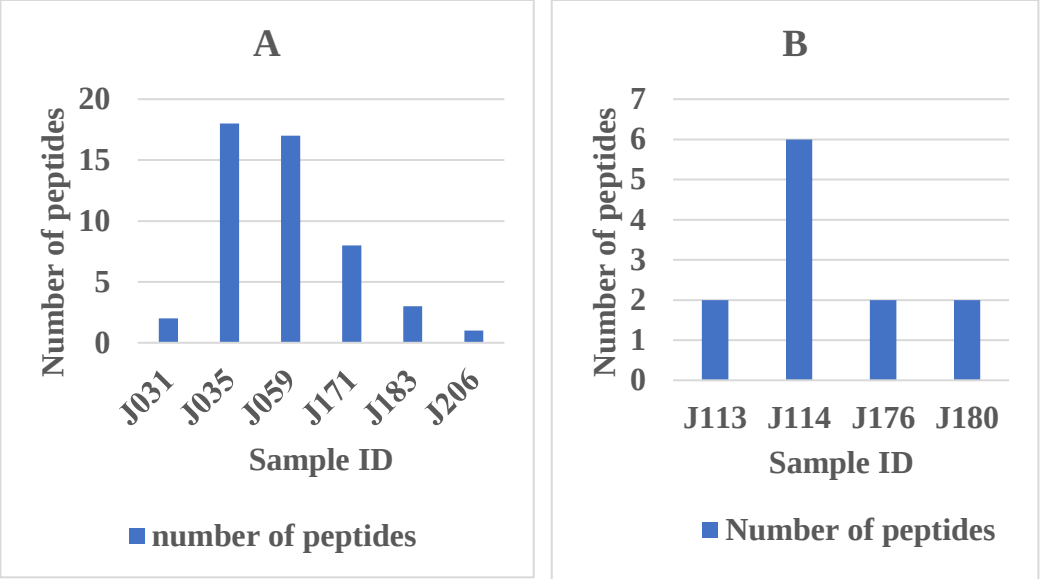


Figure 13: The proportion of peptide responses in the different prime-boosting types. (A) homologous Janssen (B) heterologous Janssen

For the homologous Janssen prime-boosting IDs J035 and J059 showed positive responses in 18 and 17 peptides respectively. IDs J114 of the heterologous prime-boosting showed positive responses to 6 peptides while J113, J176 and J180 showed positive responses to 2 peptides each.

In the Janssen group, we compared the magnitude of spike-specific interferon-gamma responses against all the peptides between the homologous prime-boosting and heterologous prime-boosting. Generally, most spike-specific responses showed increased magnitude in the homologous prime boosting compared to heterologous. However, there were no statistically significant differences in the peptide-specific interferon-gamma responses between the homologous and heterologous prime-boosting with Janssen. (Figure 15).

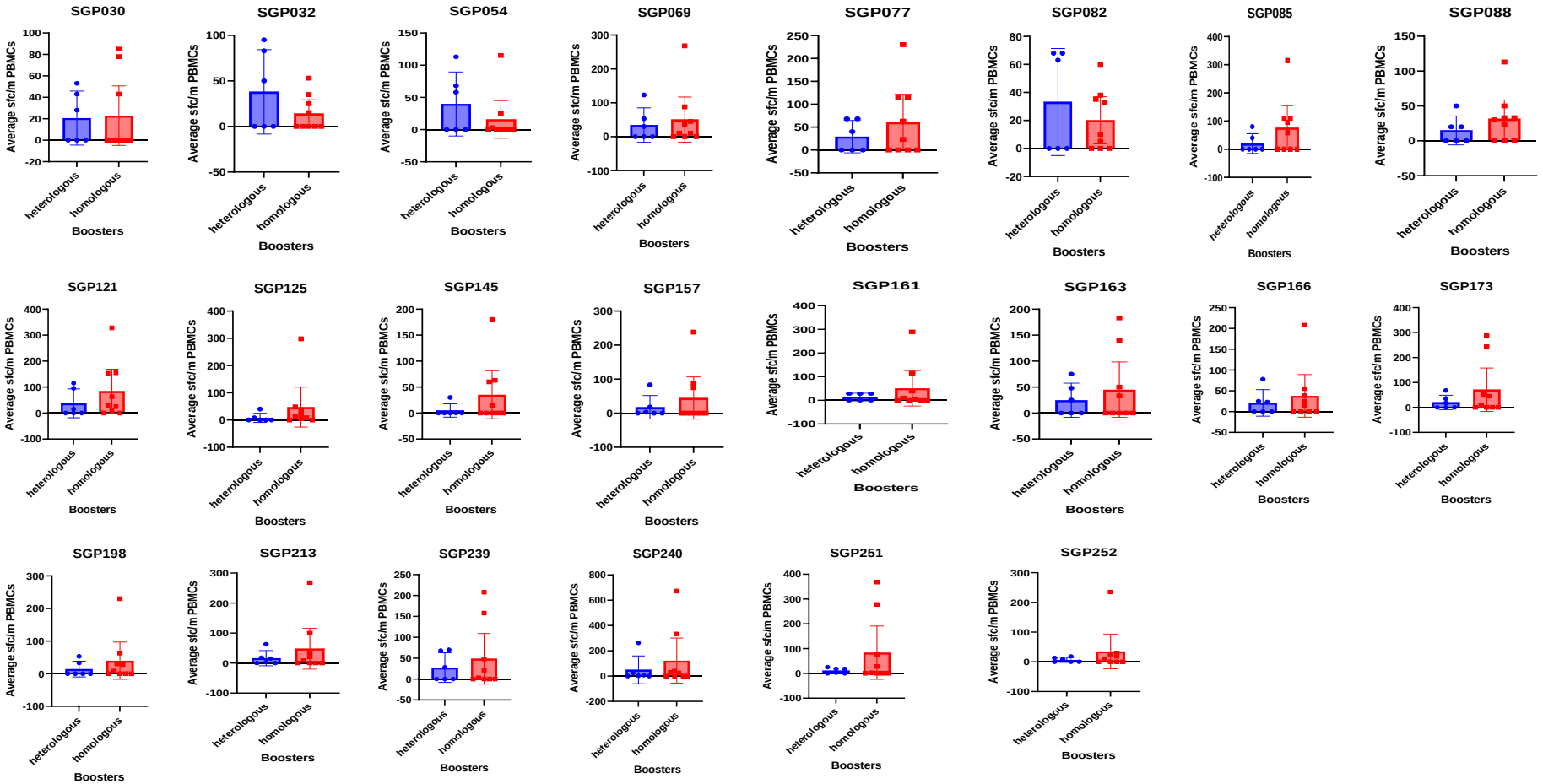


Figure 14: A comparison of Janssen booster vaccination between the homologous and heterologous.
Peptide specific responses against SGP030, SGP077, SGP085, SGP088, SGP121, SGP125, SGP145, SGP157, SGP161, SGP163, SGP166, SGP173, SGP198, SGP213, SGP239, SGP240, SGP251 and SGP252 were stronger in the homologous prime-boosting vaccination.

In homologous m-RNA booster vaccination, subject ID P051 showed the highest number of positive peptide responses (4) then P059 and P109 with 2 each. In heterologous m-RNA booster vaccination positive response rate, subject IDs P018 and P032 showed the highest responses; 5 and 6 peptides respectively. (Figure 16).

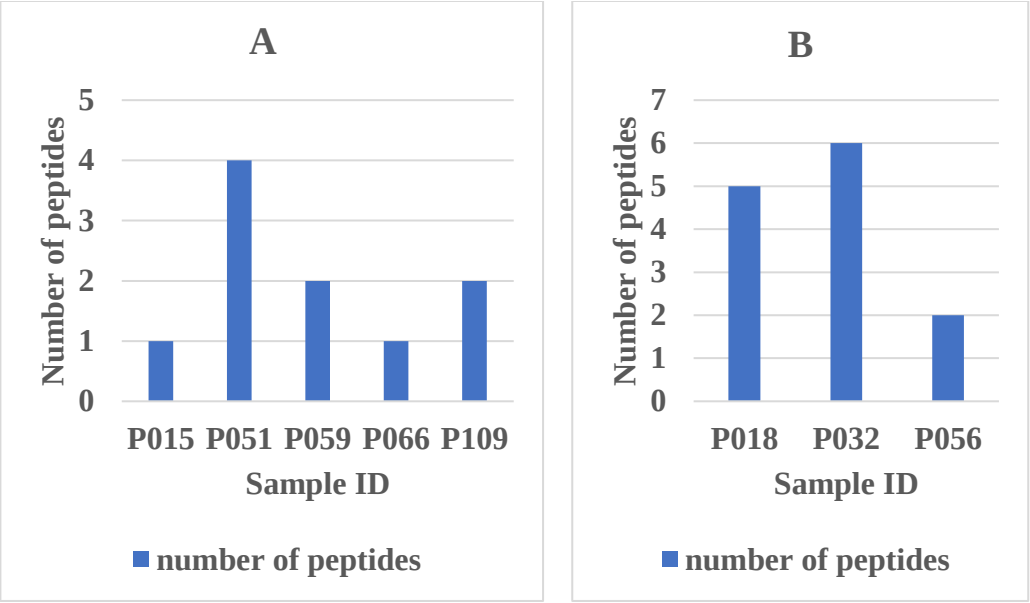


Figure 15: The proportion of peptide responses in the different prime-boosting types. A) homologous Pfizer (B) heterologous Pfizer.
For the homologous Pfizer prime-boosting, subject IDs P051 showed positive responses to 4 peptides, P059 and P109 to 2 peptides each, and P015 and P066 to 1 peptide each. Subject IDs P018 in the heterologous prime-boosting showed positive responses to 5 peptides, P032 to 6 peptides and P056 to 2 peptides.

A comparison of the magnitude of interferon-gamma responses at Month 3 against the peptides between the homologous and heterologous vaccination showed greater responses in the heterologous prime boosting compared to homologous prime boosting. However, there were no statistically significant differences in the peptide-specific interferon-gamma responses between homologous and heterologous booster vaccination. (Figure 17).

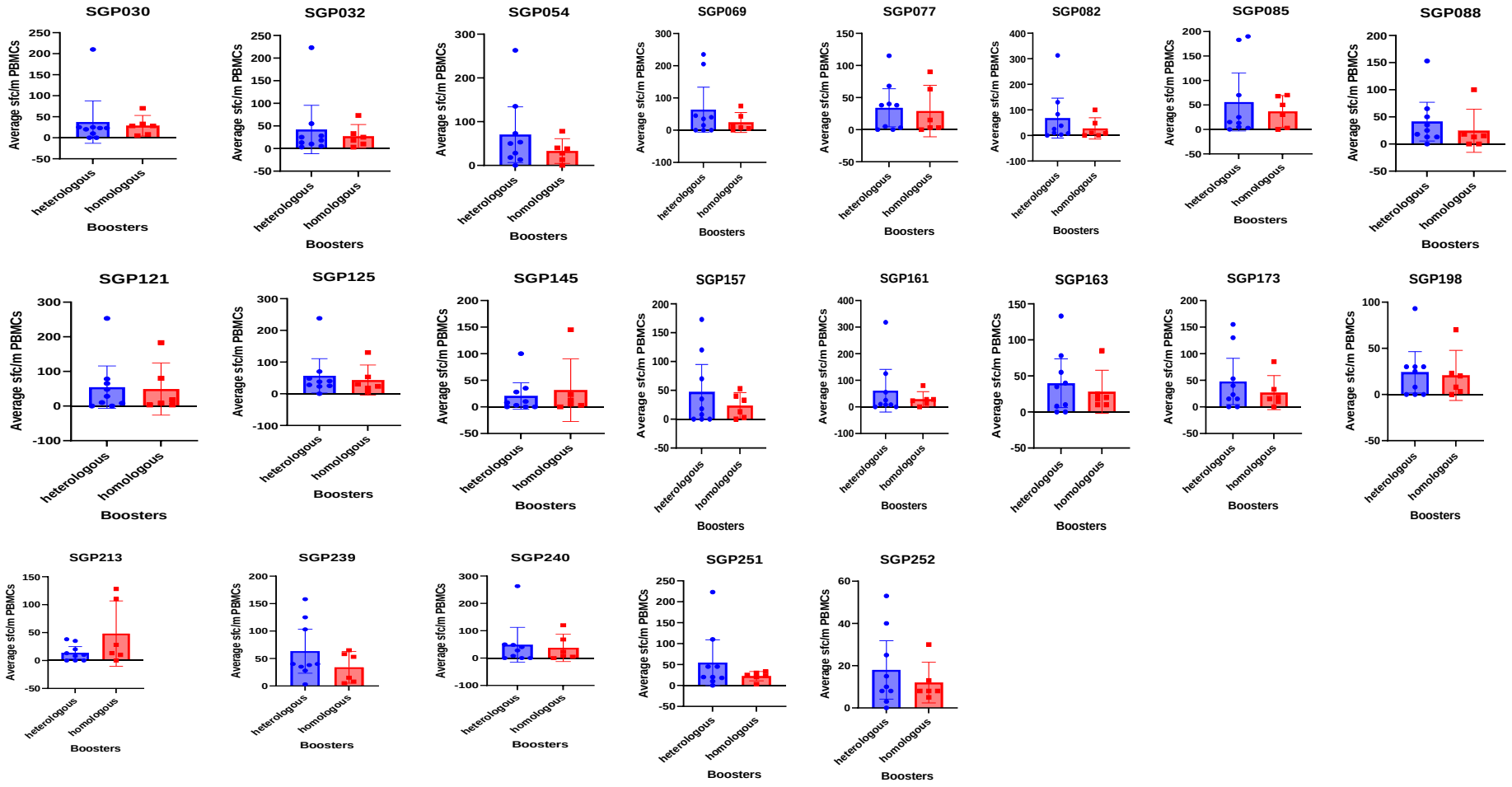


Figure 16: A Comparison of responses between homologous and heterologous Pfizer.

At Month 6, 7 subject PBMCs were positive, 5 from the Janssen group and 2 from the Pfizer. Out of the five in the Janssen booster vaccination, 4 subject PBMCs (IDs J171, J206, J031 and J035) were from viral-vectored homologous boosting type and 1 (ID J114) from the heterologous. With regards to the Pfizer booster vaccination, only 2 subject PBMCs registered positive responses at Month 6, subject ID P051 in the homologous booster type and P032 in the heterologous group.

Factors associated with specific interferon-gamma responses

To determine the possible interaction associations (confounding factors) between spike-specific interferon-gamma responses and independent variables age, sex and time since baseline vaccination to taking the pre-study booster shot, we performed the Univariate analysis. Age, sex and time from baseline vaccination to taking the pre-study booster vaccine did not affect the interferon-gamma responses between the two vaccine boosters in all the spike peptides used. (Table 5).

Table 4: Univariate analysis of independent variables by booster vaccine type.

Peptide-specific responses (sfc/m PBMCs)	Sex *p*-value	Age *p*-value	Time from baseline vaccination to taking booster shot *p*-value
SGP030	0.807	0.685	0.371
SGP032	0.514	0.493	0.958
SGP054	0.863	0.432	0.997
SGP069	0.611	0.876	0.827
SGP077	0.594	0.469	0.511
SGP082	0.212	0.333	0.778
SGP085	0.667	0.185	0.626
SGP088	0.482	0.244	0.642
SGP121	0.399	0.150	0.918
SGP125	0.718	0.637	0.497
SGP145	0.561	0.172	0.911
SGP157	0.529	0.703	0.436
SGP161	0.378	0.665	0.170
SGP163	0.609	0.789	0.515
SGP166	0.578	0.444	0.528
SGP173	0.745	0.331	0.968
SGP198	0.713	0.889	0.859
SGP213	0.275	0.190	0.737
SGP239	0.901	0.824	0.951
SGP240	0.429	0.356	0.203
SGP251	0.204	0.852	0.980
SGP252	0.658	0.903	0.287

P-values were computed using two-way ANOVA. (The cut-off significance was set at p=0.05). The analysis was done to determine if subjects' ages, time since vaccination, and sex as independent variables affect the interferon-gamma responses in Janssen and Pfizer booster vaccination. All the spike-specific interferon-gamma responses did not differ between the two groups of booster vaccinations with the influence of the independent variables i.e., p>0.5 in all the responses.

DISCUSSION

Vaccines against SARS-CoV-2 which include adenoviral-vector, inactivated virus, and m-RNA vaccine designs have been the most effective tools to combat COVID-19 where they induce the production of protective neutralizing antibodies (nAbs) and activation of cellular immunity (Dadras et al., 2022). The magnitude and duration of these responses however remain speculative (Bertoletti et al., 2021). Another problem yet to be answered satisfactorily is concerning the effectiveness of prime boosting using either heterologous or homologous vaccines. For this reason, we performed a detailed study to determine the longevity and magnitude of T-cell responses following Pfizer and Janssen COVID-19 boosters.

We sought to determine immunodominant epitopes on the SARS-CoV-2 spike protein recognized by human T-cells in response to SARS-CoV-2. We then did a comparison of interferon-gamma responses to the spike antigens between the two vaccine boosters across 9 months to assess the longevity of responses.

The first part of this study was to determine T-cell-specific immunodominant sections of the entire SARS-CoV-2 spike sequence which the immune system recognizes as foreign and against which an immune response is mounted (Awad et al., 2022). The diversity of HLA types in the human population is a potential obstacle to the study of immune responses since HLA molecules exhibit high polymorphism where hundreds of different alleles exist. Our prediction used the most common HLA supertypes among the Ghanaian population; HLA-A01:01, HLAA-A02, HLA-B07:02, HLA-B40:01, HLA-A03:01, HLA-B58:01, HLA-A24:02 according to a study done by Kusi et al. (2022).

Identifying these outputs of HLA restriction of SARS-CoV-2 T-cell epitopes is crucial for eliciting an effective immune response. Knowing the T-cell epitopes within these antigens allows the design of vaccines that specifically target these regions, ensuring robust T-cell activation and long-lasting immunity. Predicting T-cell epitopes in vaccine antigens also helps evaluate vaccine efficacy (Weingarten-Gabbay et al., 2021). Monitoring T-cell responses to these epitopes provides insights into the strength and duration of immune responses generated by the vaccine, informing decisions about booster doses or modifications to vaccine formulations (Mahajan et al., 2022).

Our predicted spike glycoproteins; SGP030, SGP032, SGP054 and SGP069 form part of the N-Terminal Domain (NTD), SGP074, SGP077, SGP082, SGP085, SGP088, SGP121 and SGP125 are drawn from the RBD of the S1 subunit of the spike glycoprotein. SGP145, SGP157, SGP163, SGP166, SGP173, SGP198, SGP213, SGP239, SGP240, SGP251 and SGP252 are part of the C-Terminal Domain (CTD) of S2 subunit. *In silico*, these peptide sequences show good interaction with MHC-I and are excellent predictors of immune responses in T-cells ex-vivo.

Experimentally, we demonstrated that most of the predicted peptides induced positive responses indicating excellent recognition by, and activation of T-cells. T-cell responses described in this study are focused on the measurement of IFN-γ production by T-cells as the effector function by activated T-cells and are associated with vaccine protection against SARS-CoV-2 (Natalini et al., 2022). Generally, the peptides from the NTD and RBD sections of the S1 subunit trigger

comparative responses to those from the S2 subunit indicating that there are relatively distributed T-cell immunodominant epitopes in the entire SARS-CoV-2 spike protein.

There were substantial interferon-gamma responses in both the Janssen and Pfizer with no significant intergroup differences at baseline measurements. These baseline responses suggest that the majority of individuals had been previously exposed to SARS-CoV-2 antigens, likely through earlier vaccination or natural infection. The absence of significant differences between groups indicates that participants shared a similar immunological background, possibly due to uniform vaccination schedules, similar rates of prior infection, or comparable public health interventions. Substantial baseline immunity is biologically significant, as it reflects the presence of memory T cells capable of mounting a rapid response upon re-exposure to the antigen, which is a critical factor in vaccine efficacy. Moreover, the variability in individual immune responses within the broader trend may be influenced by factors such as genetic diversity and timing of prior exposure.

There were differences in the proportion of positive responses as well as in the magnitude of responses between the Pfizer and Janssen groups. The differences in responses to specific peptides between the Pfizer and Janssen groups likely result from various factors, including variations in vaccine platforms and peptide-specific immunogenicity. Pfizer (mRNA-based) and Janssen (viral vectored) vaccines may differ in how they present antigens and activate immune pathways, which may influence T-cell responses to certain peptides. Additionally, HLA diversity among participants determines which peptides are effectively presented to T-cells, with

some peptides, like SGP252, being less immunogenic across both groups. Peptide-specific factors, such as sequence and binding affinity to MHC molecules may also play a role.

There was also a decrease in the number of peptide antigens being recognized beyond Month 3 and can be attributed to the natural waning of T-cell immunity over time. Following vaccination or infection, antigen-specific T-cells initially expand robustly but gradually contract as the immune system returns to a homeostatic state. This contraction phase often results in a decline in the frequency of memory T-cells capable of recognizing certain antigens, particularly subdominant or less immunogenic peptides (Hartley et al., 2022).

The principal finding from this study is that a booster dose of the Janssen and Pfizer elicits spike-specific T-cell responses that are durable 3 to 6 months after booster vaccination with Janssen booster showing extended persistence up to 9 months. There is a significant decline in SARS-CoV-2-specific T-cell responses 6–9 months post-booster vaccination in the two Janssen and Pfizer boosters. This is similar to recent findings in the US and other places where both homologous and heterologous booster vaccination regimens showed persistent T-cell responses beyond 6 months with significant decline beyond 9 months (Barros-Martins et al., 2021; Atmar et al., 2022; Munro et al., 2021; Schiavoni et al., 2023).

Positive interferon-gamma responses across the time points were predominant in the viral-vectored homologous prime-boosted samples across the time points. Homologous viral vectored booster vaccination shows greater interferon-gamma responses compared to heterologous viral-vector booster

vaccination. Similar findings have been reported where heterologous boosting with the viral-vector vaccine substantially increased spike-specific CD8+ T-cells in the m-RNA vaccine recipients (Atmar et al., 2022; Udiger Groß et al., 2022). In other recent findings, a higher T-cell-mediated immune response was reported in heterologous m-RNA followed by viral-vectored as compared to two doses of either of the two (Cheng et al., 2022; Gerhards et al., 2023; Hollstein et al., 2022; Sapkota et al., 2022). Possible explanations for these contradicting findings could be related to the genetic basis of participants, clinical impacts and small sample size.

Heterologous m-RNA booster vaccination showed a higher magnitude of interferon-gamma responses than homologous Pfizer booster vaccination. This finding is similar to findings from a study in the USA where they reported that heterologous prime-boosting using viral-vectored as the primary vaccine followed by m-RNA was more immunogenic than homologous of either viral-vectored or m-RNA, and also findings in other studies that show that heterologous prime-boost vaccinations outperform homologous vaccinations (Atmar et al., 2022; Cheng et al., 2022; Natalini et al., 2022; Udiger Groß et al., 2022).

The viral-vectored homologous prime-boost samples showed responses to a high frequencies/ proportion of peptides followed by Pfizer heterologous samples (viral-vector baseline followed by Pfizer booster) and Heterologous Janssen (m-RNA baseline followed by Janssen booster). Homologous Pfizer prime-boost produced the lowest proportion of positive peptides. Homologous viral vector booster vaccination thus offers better responses since recognizing multiple epitopes

increases the breadth of responses, hence the overall immune protection is enhanced.

We did not find any statistical evidence of an association between age, sex and time from baseline vaccination to taking the booster and interferon-gamma responses in the two vaccine boosters.

CHAPTER FIVE

SUMMARY, CONCLUSION AND RECOMMENDATION

Summary

The recent emergence of COVID-19 and it's spread to becoming a pandemic, saw the development of vaccines to curb the spread of the disease and reduce its burden. These vaccines are of different formulations from the viral-vector, m-RNA and subunit vaccines which mainly target the spike protein of the virus. Due to the dynamic nature of the genome, mutations especially in the spike protein led to the emergence of deadly variants of SARS-CoV-2 including the Omicron and Delta. This together with the waning of vaccine-induced immune protection with time necessitated for booster doses.

However, the intensity and persistence of immune responses from booster vaccine doses remain controversial. Immune protection mechanisms from homologous and heterologous booster vaccines remain understudied. There was a need therefore to look into the longevity of these responses following the administration of booster doses of Janssen or Pfizer by evaluating vaccine-induced cellular immune responses specifically the interferon-gamma release responses.

We, therefore, designed this research to determine the effectiveness and longevity of interferon-gamma responses following the administration of Janssen or Pfizer booster shots after full baseline vaccination. This study employed HLA restriction epitope prediction for MHC I to determine the immunogenic peptide sequences based on the most common HLA supertypes among the Ghanaian

population. We then performed the ELISpot Assay to determine the interferon-gamma release responses.

Conclusion

There are several immunodominant peptides within the N-terminal domain (NTD), receptor-binding domain (RBD) of the S1 subunit, and C-terminal domain (CTD) of the S2 subunit of the SARS-CoV-2 spike protein that are effectively recognized by T-cells. The Pfizer and Janssen boosters elicit durable spike-specific T-cell responses with persistence and durability for up to 6 months. The Janssen booster induced higher frequencies of T-cell responses after 3 months compared to the Pfizer booster by recognizing multiple SARS-CoV-2 spike peptides, translating to enhanced overall immune protection.

T-cell responses are more predominant in the Janssen booster vaccination than in the Pfizer booster vaccination. Homologous viral vector booster vaccination shows a greater magnitude of interferon-gamma responses compared to heterologous viral-vector booster vaccination whereas heterologous m-RNA booster vaccination shows a higher magnitude of interferon-gamma responses than homologous Pfizer booster vaccination.

Recommendation

We recommend the administration of booster shots after 6 months to guarantee durable and effective cellular responses. Our study suggests that homologous prime boosting using Janssen, and heterologous prime boosting with Pfizer vaccine guarantee broader anti-viral interferon-gamma responses up to 6 months after booster vaccine administration.

Suggestions for further research

A comprehensive assessment of immune responses including humoral responses should done to better inform the significance and effectiveness of booster vaccination against COVID-19. The mechanisms and dynamics of homologous and heterologous prime boosting in the activation of cellular immune responses should be investigated.

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APPENDIX

Appendix A: Chemical reagents

Chemical/reagents	Manufacturer/Supplier
Ethanol	BDH
Tween 20	Sigma
Sodium Hypochlorite	Aldrich
Phosphate buffered saline tablets	Gibco
RPMI-1640	Sigma
Foetal Calf Serum	Hyclone
Normal Human Serum	Promocell
Carbonate- bicarbonate	Sigma
Penicillin-streptomycin solution	Sigma
L-glutamine	Gibco
Trypan Blue	Sigma
Ficoll-Paque	GE Healthcare
Chromogenic alkaline phosphatase substrate kit	BioRad

Appendix B: Antigens, antibodies and stimulants

Anti-human IFN-gamma mAb 1D1k	Mabtech
Biotinylated anti-human IFN-gamma mAb 7-B6-1 mAb	Mabtech
Streptavidin Alkaline Phosphate Conjugate	Mabtech
Concanavalin A (Con A)	Sigma
Synthetic peptides	Via NMRC, or directly from Alpha Diagnostics Inc., USA

Appendix C: Ethanol Solution for Sterile Work

70 % Ethanol Solution

PROCEDURE:

1. Add 700 ml of ethanol and 300ml of deionized water

2. Shake well

Appendix D: Dilution medium For PBMC Separation

RPMI -1640 medium

L-glutamine (stock concentration = 100 mg/ml)

Penicillin-streptomycin solution (stock concentration = 100 mg/ml)

PROCEDURE:

1. Take 500 ml of RPMI-1640 medium, take 10 ml off and discard.

2. Add 5 ml of L-glutamine solution to a final concentration of 2 µg/ml and 5 ml of Penicillin-streptomycin solution to a final concentration of 10 µg/ml; Swirl to mix well before use.

Appendix E: Fresh Cell Wash for PBMC Separation

5% Foetal Calf Serum (FCS) in RPMI-1640 with L-glutamine and Penicillin-Streptomycin.

PROCEDURE:

1. Take 500 ml of RPMI-1640 medium and take 35 ml off.

2. Add 5 ml of L-glutamine solution at a concentration of 2 µg/ml, 5 ml of Penicillin-streptomycin solution at a concentration of 10 µg/ml and swirl to well.

3. Add 25 ml of Heat Inactivated Foetal Calf Serum and shake well.

4. Filter sterile with 0.22 µm Millipore filter.

Appendix F: Coating Buffer for ELISPOT

Sterile 0.1 M $NaHCO_3$

PROCEDURE

1. Dissolve 5 capsules of $NaHCO_3$ in 500 ml of deionized water.

2. Stir on a magnetic stirrer until it fully dissolves without heating

3. Adjust pH to 9.6 by adding concentrated NaOH drop-wise

4. Sterile filter with 0.22 μm Millipore filter.

Appendix G: Blocking buffer for ELISPOT

10% normal human serum in RPMI-1640 medium with L-glutamine and penicillin-streptomycin

PROCEDURE:

1. Take 500 ml of RPMI-1640 medium and pipette off 60 ml

2. Add 5 ml of L-glutamine solution, 5ml of streptomycin/penicillin and shake well.

4. Add and mix to 50 ml of heat-inactivated Normal Human Serum.

5. Sterile filter with 0.22 μm Millipore filter.

Appendix H: Cryo-freezing medium

90 % Foetal Calf Serum with 10 % DMSO

PROCEDURE:

1. Add 10 ml of DMSO to 90 ml of Foetal Calf Serum.

2. Shake well and keep at room temperature until use.

Appendix I: Washing Buffer for ELISPOT

Phosphate buffered saline (PBS) with 0.05 % Tween 20

PROCEDURE

1. Add 10 tablets of PBS to a beaker containing 5000 ml deionised water (ie 1 tablet/500 ml or 1X PBS) and stir using a magnetic stirrer until all is dissolved

2. Add 2.5 ml of Tween 20 and stir continuously till in solution.

Appendix J: Preparation of 1X (plain) phosphate-buffered saline

Phosphate-buffered saline (PBS)

PROCEDURE

1. Add 2 PBS tablets to 1000 mls of deionized water.

2. Stir the contents using a magnetic stirrer until the it is fully dissolved.

Appendix K: Diluent Buffer for ELISPOT

1 x PBS with 0.5 % heat inactivated (HI) Foetal Calf Serum (FCS)

PROCEDURE

1. Add 2.5 ml of HI FCS to 497.5 ml of PBS (Appendix I).
2. Mix by stirring for a uniform solution.