EVALUATION OF INTRACELLULAR CYTOKINES IN COVID-19 VACCINATED INDIVIDUALS

OFELIA BINTI YAHCOB

UNIVERSITI SAINS MALAYSIA

2022

EVALUATION OF INTRACELLULAR CYTOKINES IN COVID-19 VACCINATED INDIVIDUALS

by

OFELIA BINTI YAHCOB

Thesis submitted in partial fulfilment of the requirements for the degree of Master of Science (Biomedicine) Mixed Mode

September 2022

ACKNOWLEDGEMENT

In the name of Allah, the most gracious and merciful. First and foremost, I am thankful to Almighty Allah for letting me through all the difficulties, and giving me the strength, knowledge, and ability to undertake this study and complete it satisfactorily.

I would like to acknowledge and give my warmest thanks to my supervisor, Dr. Maryam Azlan, whose worthy guidance and professional attitude are appreciable in completing this thesis. I am also thankful to my co-supervisor, Assoc. Prof. Dr. Rapeah Suppian, Coordinator MSc Mixed Mode Biomedicine program, Assoc. Prof. Dr. See Too, Coordinator Course GTB540, Dr. Wong Weng Kin, and all lecturers in the Biomedicine program for their immense knowledge, insightful comments, and encouragement that made this work possible.

A very special thanks to Prof Dr. Norazmi Mohd Nor, all in the COVID project team at Universiti Sains Malaysia (USM) Health Campus, and my colleagues for letting my thesis be an enjoyable moment and for your brilliant comments and suggestions. Thank you.

Last but not least, special thanks to my husband, my parents, my kids, and my family as a whole for their continuous support and understanding when undertaking my research and writing my thesis. Your prayers for me were what sustained me this far.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

µg	Microgram
µm	Micron
x g	Times the gravitational force

LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
CMV	Cytomegalovirus
FSC	FURIN cleavage site
FSC-A	Forward scatter area
FSC-H	Forward scatter height
ICTV	International committee of taxonomy and viruses
ICU	Intensive care unit
IFN	Interferon
IL	Interleukin
PHA	Phytohemagglutinin
RPMI	Rosewell Park Memorial Institute
TNF	Tumor necrosis factor
USM	Universiti Sains Malaysia
WHO	World Health Organization

LIST OF APPENDIX

PENILAIAN TERHADAP INTRASELULAR SITOKIN INDIVIDU YANG MENDAPAT SUNTIKAN VAKSIN COVID-19

ABSTRAK

Penyakit Coronavirus 2019 (COVID-19) telah menjangkiti lebih 500 juta orang dan telah menyebabkan 6 juta kematian di seluruh dunia. Banyak kajian telah melaporkan bahawa "badai sitokin" dikaitkan dengan komplikasi teruk dalam jangkitan ini. Vaksin sepatutnya dapat menyekat dan mengawal badai sitokin atau sekurang-kurangnya menghalang kesan buruk akibat badai sitokin dengan merangsang sistem imun. Kajian ini penting untuk menyediakan data awal tentang sitokin intraselular dalam kalangan individu yang divaksin untuk kajian masa depan yang berkaitan dengan vaksinasi COVID-19. Kajian ini bertujuan untuk menentukan tahap IL-2, IL-4, IFN-γ dan TNF-α dalam individu yang diberi vaksin COVID-19 serta ekspresi antigen permukaan CD3/CD4, CD3/CD8. Dalam kajian ini, sel darah mononuklear periferal (PBMC) telah dirangsang menggunakan peptida PepTivator SAR-CoV-2 Prot_S1. PBMC yang dirangsang telah diwarnai dan sel-sel telah diperoleh dianalisa menggunakan flow sitometer. Keputusan menunjukkan bahawa IL-2, IL-4, IFN-γ dan TNF-α dirembeskan oleh $CD4^+$ dan $CD8^+$ dalam semua individu selepas vaksinasi. Dalam kajian ini, sitokin intraselular ini dihasilkan secara konsisten selepas dos kedua dan vaksin penggalak. Kesimpulannya, saiz sampel dalam kajian ini adalah kecil dan tidak mewakili keseluruhan populasi. Akibatnya, lebih banyak penyelidikan diperlukan untuk menentukan tahap ekspresi IL-2, IL-4, IFN-γ dan TNF-α pada sel T $CD4^+$ atau $CD8^+$ selepas vaksinasi dan peranannya dalam pencegahan badai sitokin dalam jangkitan COVID-19 dan perlindungan terhadap jangkitan.

EVALUTION OF INTRACELLULAR CYTOKINES IN COVID-19 VACCINATED INDIVIDUALS

ABSTRACT

Coronavirus disease 2019 (COVID-19) has infected over 500 million people and has caused 6 million deaths worldwide. Many studies have reported that the "cytokine storm" is associated with poor outcomes and severe complications in this infection. COVID-19 vaccine should be suppressed cytokine storm at least hamper its deleterious impact by stimulation of the immune system. This study is important to provide preliminary data on the intracellular cytokines among vaccinated individual for the future study related to COVID-19 vaccination. The study aimed to determine the level of IL-2, IL-4, IFN-γ and TNF-α in COVID-19 vaccinated individuals as well as expression of surface antigens CD3/CD4, CD3/CD8. In this study, peripheral mononuclear blood cell (PBMC) was stimulated using PepTivator SAR-CoV-2 Pot S1 peptide. Stimulated PBMC were stained, and cells were acquired by a flow cytometer. The results showed that IL-2, IL-4, IFN-γ and TNF-α were secreted by $CD4^+$ and $CD8^+$ in all individuals following vaccination. In this study, these intracellular cytokines were consistently produced after second dose and booster vaccination. In conclusion, the sample size in this study is small and does not represent the entire population. As a result, more research is needed to determine the level of IL-2, IL-4, IFN-γ and TNF-α expression on $CD4^+$ or $CD8^+$ T cells after vaccination and their role in the prevention of cytokine storm in COVID-19 infections and protection against infection.

CHAPTER 1

INTRODUCTION

1.1 Background

Coronavirus disease 2019 (COVID-19) is caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). The novel virus was discovered in December 2019 during an outbreak in Wuhan, China (Zhu et al., 2020). Currently, SARS-CoV-2 has infected over 500 million people and has caused 6 million deaths (Wang et al., 2020). From 25th January 2020 until 21st May 2022, there were 4,489,503 confirmed cases of COVID-19 in Malaysia, with 35,641 deaths (COVIDNOW, 2020). The incubation period for COVID-19 is typically 14 days after exposure, with most symptom appearing four to five days later (Guan et al., 2020). The infection can present in a variety of clinical manifestations, from mild to severe. The most common symptoms are cough, fever, and myalgia, with moderate forms requiring hospitalization to severe forms such as pneumonia, disseminated intravascular coagulation (DIC), acute respiratory distress syndrome (ARDS) and multiorgan failure resulting in death.

To date, no specific therapies against the SARS-CoV-2. Antiviral treatment and monoclonal antibodies are treatment options to overcome the symptoms. However, mass vaccination is the most available option to curb out the pandemic. The first COVID-19 vaccines were developed in 2020 and licensed for emergency use (Rogers, 2020). A COVID-19 vaccine is being developed using at least eight different technology platforms including inactivated viruses, non-replicating viral vectors, nucleoside-modified messenger RNA and DNA, peptides, recombinant proteins, and live attenuated viruses (Thanh Le et al., 2020). The vaccination program in Malaysia is currently being conducted in phases from 24 February 2021 to February 2022. Phase

one involved healthcare workers and frontline personnel who had received the COVID-19 vaccination, BNT162b2, also known as Comirnaty (Pfizer/BioNTech) (Wen Lau et al., 2021). The three most administered vaccines in Malaysia are BNT162b2, AZD1222 (AstraZeneca), and CoronaVac. The Ministry of Health (MOH) has determined that these three vaccines met safety and efficacy standards (Suah et al., 2021). Efficacy for the BNT162b2 vaccine was 91.3% after six months of follow-up, whereas the overall efficacy of the AZD1222 (AstraZeneca) vaccine against symptomatic COVID-19 is reported to be 74.0% (Falsey et al., 2021; Thomas et al., 2021). The efficacy estimates for the CoronaVac (Sinovac) vaccine are varied; 83.5% in a phase 3 trial in Turkey, 65% in Indonesia and 50.4% in Brazil (Baraniuk, 2021; Thomas et al., 2021).

Several studies have shown the deterioration of COVID-19 is linked to immunopathological damage (Azkur et al., 2020). Most COVID-19 patients have lymphocytopenia, and those with severe infection have shown a continuing decrease in lymphocytes, which play a protective role in our immune system. $CD4^+$ and $CD8^+$ T cell responses are crucial to the resolution of COVID-19, including regulating the severity of the disease (Tarke et al., 2021). After being activated and differentiated into discrete effector subtypes, $CD4^+$ and $CD8^+$ T lymphocytes play an important role in modulating immunological response via the secretion of specific cytokines (Luckheeram et al., 2012). Certain cytokines found to be higher in COVID-19 patients (Huang et al., 2020). The release of a large amount of pro-inflammatory cytokines will lead to cytokine storm. Particularly, multiple studies have reported that the cytokine storm is associated with poor outcomes and severe complications in this infection. High level of proinflammatory cytokines produced during the crosstalk between immune cells and epithelial cells in COVID-19 patients (Ragab et al., 2020). However,

the vaccines should be able to suppress cytokine storm or at least hamper its deleterious impact (Costela-Ruiz et al., 2020). Tumour necrosis factor-α (TNF-α) has been previously shown to be critical in the development of an immune response to vaccinations (Corbett et al., 2020). The COVID-19 vaccines were also successful in eliciting an effective cell immune response, particularly the production of Interferon gamma (IFN-γ) by SARS-CoV-2-specific T-helper 1 and cytotoxic T cells. According to Dayam et al., (2022) , T cell release cytokine of IL-2 and IL-4 increased from 1 to 2 doses of vaccine and correlated with humoral responses. This study is important to provide preliminary data on the IL-2, IL-4, IFN-γ and TNF-α intracellular cytokines among vaccinated individual for the future study related to COVID-19 vaccination. These findings also highlight the changes in intracellular cytokine levels and the significance of such a response following vaccination in terms of prevention of cytokine storm, which can lead to multiorgan failure in COVID-19 patients. If this turns out to be proven in a larger study, then this information might be used to target strategies for enhancing vaccine responses.

1.2 Rationale of the study

Several studies examined differences in vaccine response in convalescent and uninfected vaccine recipients (Zollner et al., 2021). However, there has been limited study on the level of intracellular cytokines in COVID-19 vaccinated individuals. This study will provide preliminary data on the levels of intracellular cytokines in vaccinated individuals, which may be a target approach for enhancing vaccine response in the future and for preventing cytokine storm.

1.3 Problem statement

COVID-19 vaccines were successful measured in reducing infection and fatality in the global pandemic. Researchers from all over the world conducted massive studies to determine how well the vaccines worked. So far, however, no sufficient studies have been conducted on the level of intracellular cytokines in vaccinated individuals. It is important to understand the level of intracellular cytokines secreted by $CD4^+/CD8^+$ cells after vaccination to understand the protective immunity given by vaccines. This study provided preliminary data on the level $CD4^+/CD8^+$ T cell expressing intracellular cytokines in BNT162b2 and CoronaVac vaccines.

1.4 Objectives of the study

1.4.1 Main objective

The main objective of the study was to determine the level of intracellular cytokines in COVID-19 vaccinated individuals receiving either BNT162b2 (Pfizer/BioNTech) or CoronaVac (Sinovac) vaccine.

1.4.2 Specific objectives

a) To determine the expression of CD3/CD4 and CD3/CD8 surface antigens in BNT162b2 and CoronaVac vaccinated individuals.
b) To determine the level of IL-2, IL-4, IFN-γ and TNF-α intracellular cytokines in BNT162b2 and CoronaVac vaccinated individuals.

1.5 Hypothesis

The study hypothesized that proinflammatory cytokines such as of IL-2, IL-4, IFN-γ and TNF-α intracellular cytokines will be secreted by $CD3^+CD4^+$ and $CD3^+CD8^+$ in individuals receiving COVID-19 vaccinations and booster immunization does not increase the level of cytokines production.

1.6 Overview of the study

The methodology of the study is as shown below.

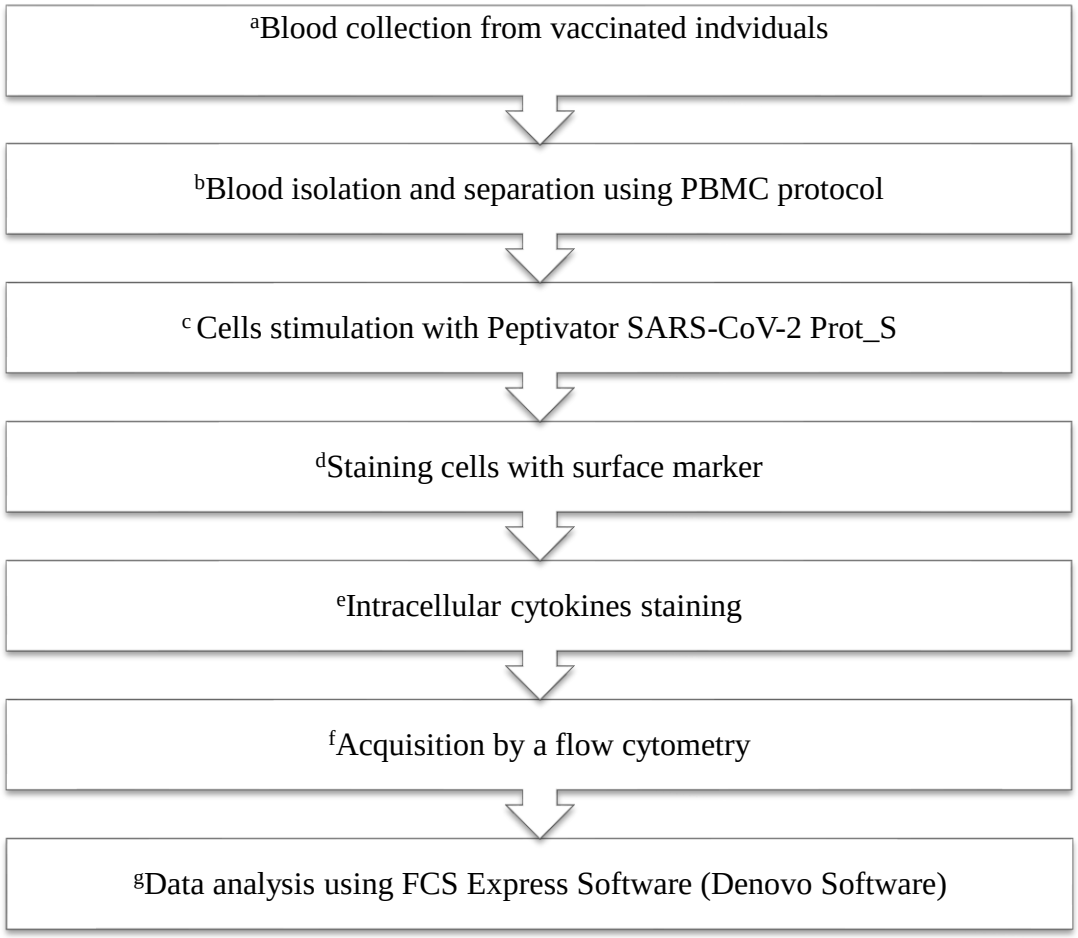

Figure 1.1 Flow chart of research methodology in this study. (a) Blood collection from individuals receiving second dose and booster (BNT162b2 and CoronaVac vaccines). (b) PBMC isolation by lymphocyte separation medium (LSM). (c) Stimulation of PBMC is to stimulate secretion of cytokines. (d) Cell surface staining (CD3/CD4/CD8). (e) Intracellular cytokines staining (IL-2, IL-4, IFN-γ and TNF-α). (f) The stained PBMC acquired on flow cytometry. (g) Data generated by flow cytometry will be analysed using Denovo software.

CHAPTER 2

LITERATURE REVIEW

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

The emergence of a new coronavirus disease has been predicted in early March 2019 (Fan et al., 2019), and the first confirmed case was reported in November 2019 in Hubei, Central China (Pekar et al., 2020). However, the novel virus was only notified during an outbreak in Wuhan, China in December 2019 (Zhu et al., 2020). COVID-19 is a contagious disease that has quickly spread throughout China and the world. The World Health Organization (WHO) declared COVID-19 a global pandemic on March 11, 2020, (Cucinotta and Vanelli, 2020). The WHO referred to the causative virus as novel coronavirus 2019 (2019-nCoV), but the International Committee of Taxonomy of Viruses (ICTV) renamed it SARS-CoV-2. The disease referred as coronavirus disease 2019 (COVID-19) (Rahman et al., 2021).

2.1.1 Structure of SARS-CoV-2

SARS-CoV-2 is a single-stranded RNA virus, like other coronaviruses (Kumar et al., 2020). It is an enveloped, positive-sense and beta-coronavirus (Lotfi et al., 2020). The coronavirus genome contains nearly 30,000 nucleotides that encodes four structural proteins: Nucleocapsid (N) protein, Membrane (M) protein, Spike (S) protein, and Envelop (E) protein, as well as several non-structural proteins (nsp) (Naqvi et al., 2020). The N protein is the most abundant in infected-host cells and is responsible for virus genome binding (Figure 2.1). The N protein binds to a ribonucleic acid (RNA) string, giving the virus shape and allowing it to replicate. RNA serves as a molecular message that allows for the production of protein required by other virus components. The M-protein is the most distributed on the viral surface, and it is presumed to be the central organizer of the coronavirus assembly (Boopathi et al.,

2020). M protein shapes the envelope and interacts with the N protein to strengthen and stabilize the nucleocapsid. M protein also protects the virus outside the cells. The E protein embedded in this layer assists in the formation of new viral particles after a SARS-CoV-2 has infected a cell (Astuti and Ysrafil, 2020).

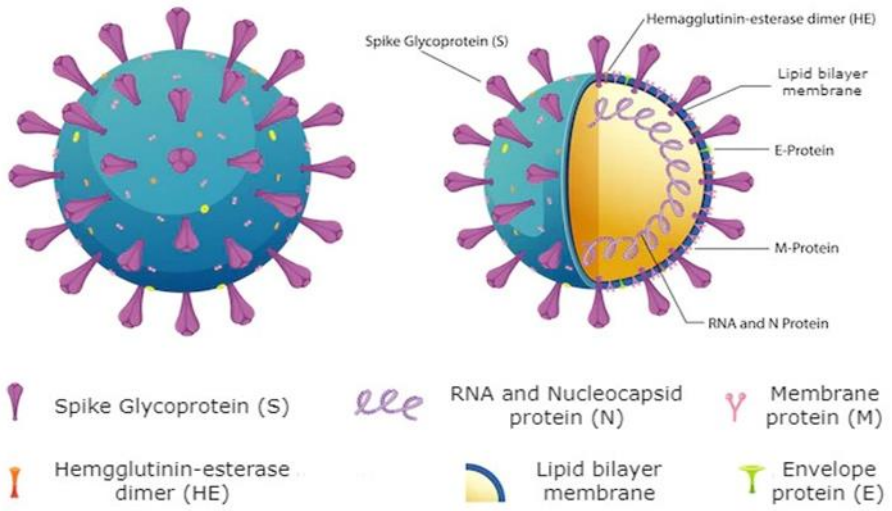

Figure 2.1 Diagram of SARS-CoV-2 particle structure. The SARS-CoV-2 structure is primarily formed by the structural protein such as spike (S), membrane (M), envelope (E), and nucleocapsid (N) protein. The S, M, and E protein are all embedded in the lipid bilayer membrane. In the virion core, the N protein interacts with the viral RNA. (Santos et al., 2020)

The spike (S) protein of SARS-CoV-2 is crucial for receptor identification and cell membrane fusion process. S-protein is crown-like appearance under the microscope acts as a gripping hook, allowing the virus to latch onto host cells and break them open for infection. The S protein is comprised of two subunits: the S1 subunit, which is responsible for receptor binding and the S2 subunit, which is responsible for membrane fusion (Zhu et al., 2021). Hemagglutinin esterase is an enzyme comprised of hemagglutinin and acetyl esterase that serves as a receptor

destroyed enzyme and is thought to be involved in coronavirus pathogenicity (Lang et al., 2020).

2.1.2 Mode of transmission

Early in the pandemic, the transmission of SARS-CoV-2 in humans is unclear (Tang et al., 2020), even though it was announced that the infection is zoonotic, meaning that it spreads from animal to human (Benvenuto et al., 2020). The initial incidences of COVID-19 have been linked to the Hunan South China Seafood Market, where snakes, birds, and other creatures such as bats were sold. Unlike the exported cases, many of the early patients worked or visited the market (Li et al., 2020). However, many patients without a history of contact with the Hunan South China Seafood Market or animals elsewhere have lately been infected by the disease, suggesting that the infection is not limited to animal-to-human transmission, but may potentially occur between humans (Gralinski and Menachery, 2020).

Current data suggest that the primary mechanism of COVID-19 disease transmission in the current epidemic is transmitted from human to human (Rahman et al., 2020). Generally, those who present symptoms (symptomatic) of COVID-19 will spread the disease to someone who was less than 6 feet away from an infected person (close contact) (Boldog et al., 2020; She et al., 2020). However, it has been reported that SARS-CoV-2 can be transmitted from an asymptomatic person within the incubation period, which is 5 to 6 days after contact (Bai et al., 2021; Rothe et al., 2020). As a result, SARS-CoV-2 potentially transmit from symptomatic to asymptomatic individuals. Asymptomatic transmission can occur from two different infection states: pre-symptomatic persons who became infected before developing symptoms and those who never developed symptoms (Johansson et al., 2021). These are types of human-to-human transmission routes; droplet transmission, aerosol

transmission, direct transmission. Transmission from animals to humans should also be taken into consideration.

A person infected with SARS-CoV-2 spreads many small respiratory particles containing the virus via coughing, sneezing, or even talking. The virus remains intact and contagious in droplets smaller than 5μm and it is suspended in the air for up to three hours. SARS-CoV-2, on the other hand, can only transmit for six feet or two meters (Doremalen et al., 2020). When these droplets containing viruses are inhaled or ingested or land on the mucous membrane, it will cause infection in people (Wang et al., 2020). The risk of infection can be reduced by employing effective droplet barriers such as masks and maintaining personal and environmental hygiene (Chang et al., 2020).

Aerosol transmission occurs not just from people with symptoms of the disease, but also from asymptomatic COVID-19-positive people (Yeo et al., 2020). The aerosols from coughs and sneezes that contaminate the nearby area are among the media for viral transmission (Koenig et al., 2020). It has been reported that the SARS-CoV-2 may survive in aerosols for at least three hours and on stainless steel and plastic surfaces for 48 to 72 hours (Putten et al., 1993). The rate of transmission may be further accelerated in close environment where the aerosol containing viruses may remain in the air for long periods and at high concentrations (Wu et al., 2020).

In hospitals, healthcare providers were seriously endangered by nosocomial infection because they performed procedures on the respiratory route, dental treatment, hemodialysis, and managed the intensive care unit (ICU) (Wang et al., 2020). WHO (2020) stated that this transmission is most easily to occur in “Three C”; crowded places, close contact settings and confined and enclosed spaces (Sok, 2021). To minimize the risk of infection, WHO recommends the use of masks, personal

protective equipment (PPE), and practicing social distance (Gordon and Thompson, 2020).

Direct contact transmission can occur when people come into direct touch with virus-contaminated items or surfaces, infecting them through the nose, mouth, or eyes (Koenig et al., 2020). It has been reported that fomites are presumed to be the main source of infectious particles in direct contact transmission (Jiang et al., 2020). Healthcare personnel are more vulnerable to SARS-CoV-2 infection through this mode of disease transmission, as there are numerous nosocomial infections (Li et al., 2020).

Frequent hand cleaning using an alcohol-based hand rub or soap and water, as well as avoiding contacting the eyes, nose, and mouth with infected hands, can help to reduce viral transmission (Lotfi et al., 2020). The modes of transmission route of SARS-CoV-2 are summarized in Figure 2.2.

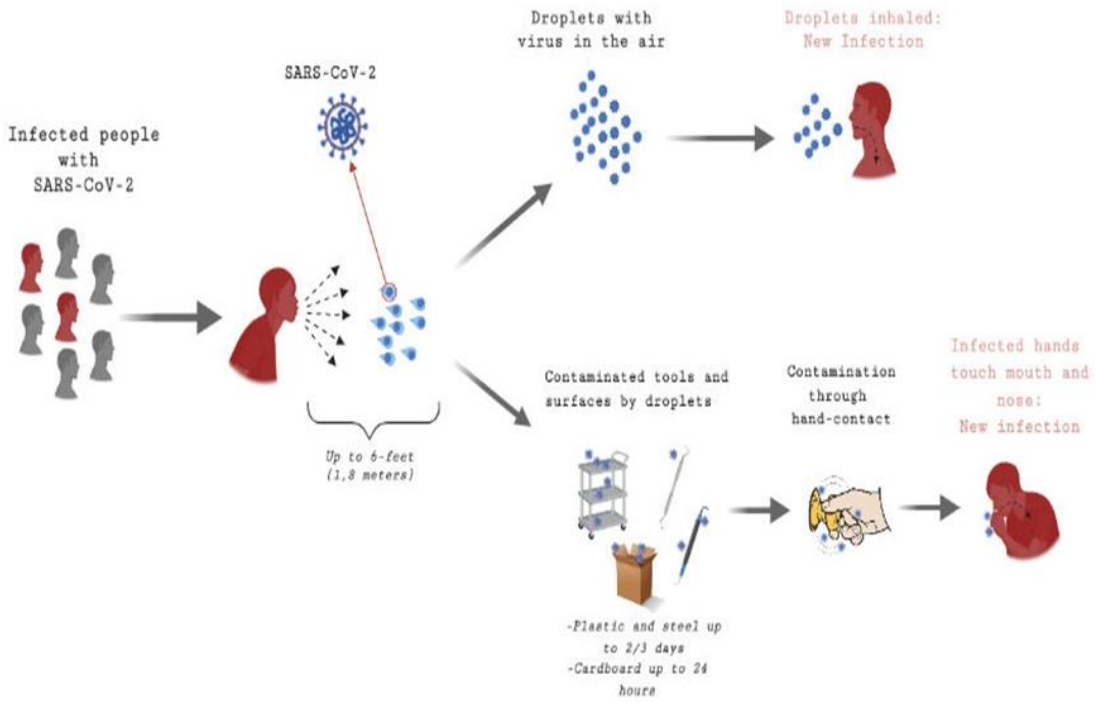

Figure 2.2 SARS-CoV-2 transmission routes include droplet, aerosol, and direct contact transmission. These transmissions contribute to the spread of COVID-19 disease in the population (Mahdi et al., 2020).

Animal can transmit SARS-CoV-2 to human. Bats may act as a reservoir for acute infection in humans since the human SARS-CoV-2 virus and the bat coronavirus, Beta-CoV/bat/Yunnan/RaTG13/2013 (bat/ RaTG13), share 96% of the same genes at the whole genome level (Zhou et al., 2020). According to Chan et al., (2020), COVID-19 transmission originated from bats but may have passed through intermediate animals sourced from the local seafood market in Wuhan City, Hubei province, China. A study conducted by Xiao et al., (2020) mentioned that an intermediate host is required for SARS-CoV-2 to transmit to humans. These findings suggest that SARS-CoV-2 may have evolved from a viral recombination between Pangolin-CoV and Bat-nCov before transmitting to humans. However, different species may have different infection risks. A study done by Shi et al., (2020) found that ferrets, cats, dogs, and

other domesticated animals are vulnerable to SARS-CoV-2. Cats can be infected with SARS-CoV-2 and spread it to other cats. But it is unknown if cats can spread the virus to their owners. Furthermore, chickens, ducks, pigs, and dogs are unlikely to get infected. It should be noted, however, that a German shepherd pet dog died two days after the owner was placed in COVID-19 quarantine. The cause of death was unknown since the owner declined to have an autopsy performed (Lotfi et al., 2020). As a result, the possibility of the virus spreading from animal to human must be considered.

2.2 Clinical manifestations

COVID-19 infection affects people of all ages. However, current data suggests that susceptibility to infection and clinical manifestations often rises with age (Zhang et al., 2020). Elderly peoples with COVID-19 infection have the worst outcomes, including the highest mortality rates (Cevik et al., 2020). A study has found that children aged under 10 years have significantly lower susceptibility to COVID-19 infection than adults (Goldstein et al., 2021). A study has shown that most of the infected children are asymptomatic or have minimal symptoms of infection as compared to adults (Team et al., 2020). The mortality rate is associated with age, underlying comorbidities, and the severity of the disease (Wu and McGoogan, 2020). The clinical manifestations in the infected people range from asymptomatic, mild to moderate, severe, and critical illness.

It is possible to be infected with SARS-CoV-2 without experiencing any symptoms (asymptomatic). However, the percentages of asymptomatic people vary and unknown. There has been very limited research into how many people who present with an asymptomatic infection develop clinical illness. It has been reported that 40% to 45% individuals with COVID-19 were asymptomatic until admission (Oran and

Topol, 2020). A study also reported that asymptomatic people shed a similar amount of virus to symptomatic people (Lee et al., 2020). Therefore, asymptomatic illnesses have the potential to spread viruses and are contagious. It has been advised that asymptomatic individuals should isolate themselves at home and follow safety measures to prevent viral transmission.

Patients with mild illness may present a variety of common symptoms, but they do not have shortness of breath, dyspnea on exertion, or abnormal imaging. In a study of nearly 370,000 confirmed COVID-19 infections from January to May 2020 in the United States, the most common presenting symptoms were cough (50%) fever (43%), myalgias (36%), and headache (34%) (Burke et al., 2020). Similar ranges of clinical results have been found in other cohort studies of individuals with confirmed COVID-19 (Huang et al., 2020). Another study discovered that nasal congestion (77–82%), sneezing (63–71%), and sore throat (61–71%) were also common symptoms (Menni et al., 2022).

In Malaysia, the most common clinical manifestations of COVID-19 reported were cough (32.2%), fever (29.5%), sore throat (14.3%), rhinorrhea (10.3%), and shortness of breath (5.3%), with anosmia and ageusia constituting a small minority of cases (2.8% and 0.7%, respectively) (Sim et al., 2020). A study found that Malaysian COVID-19 patients had significant loss of smell, taste, and chemesthesis, which showed up as early signs of infection (Lee et al., 2020). These symptoms of smell and taste loss have been found to range from 60% to 80% in many self-reported symptom studies, mostly from the United States, United Kingdom, and Europe (Giacomelli et al., 2020). These patients are in category 2, meaning that they can treat their symptoms at home and patient is called to update their status via digital electronic health record or known as Mysejahtera. Patients with mild symptoms are usually no need for

imaging or laboratory tests. Elderly patients and those with underlying comorbidities are monitored closely until clinical recovery is achieved (See et al., 2022).

Moderate illness is defined as the level of oxygen saturation (SpO_2) on room air at sea level is less than 94% during clinical assessment. Patients with moderate symptoms of COVID-19 should be closely monitored since pulmonary disease can worsen quickly (Gandhi et al., 2020). Empiric antibiotics are given if bacterial pneumonia or sepsis is suspected, and drugs are stopped if no bacterial infection is confirmed. In Malaysia, these patients are classified as category 3 and are advised to remain at home under quarantine. They are evaluated through Mysejahtera (See et al., 2022). Patients can call an emergency hotline or go to the nearest hospital if their symptoms worsen.

Patients with COVID-19 are considered to have severe disease if their SpO_2 on room air at sea level is 94%, their arterial oxygen partial pressure to fractional inspired oxygen (PaO_2/FiO_2) is 300 mm Hg, their respiratory rate is more than 30 breaths per minute, or their lung infiltrates are more than 50% ("Clinical Spectrum | COVID-19 Treatment Guidelines," 2022). Patient will be classified under category 4 since it is symptomatic with lung infection. These patients may undergo rapid clinical deterioration. Oxygen treatment should be given as soon as possible using a nasal cannula or a high-flow oxygen device (See et al., 2022). If sepsis or secondary bacterial pneumonia are suspected, the patient is start on empiric antibiotics, the patient re-evaluates every day, and stop the medications if there is no sign of bacterial infection (Sieswerda et al., 2021).

Patient with category 5 is critically ill patients who have acute respiratory distress syndrome, septic shock that may represent virus-induced distributive shock,

cardiac dysfunction, an exaggerated inflammatory response, or exacerbation of underlying comorbidities (See et al., 2022). Pneumonia is the most frequent serious manifestation of infection, characterized primarily by fever, cough, dyspnea, and bilateral infiltrates on chest imaging (Gennaro et al., 2020). In addition to pulmonary disease, patients with critical illness may also experience cardiac, hepatic, renal, central nervous system, or thrombotic disease. As with any patient in the intensive care unit (ICU), successful clinical management of a patient with COVID-19 includes treating both the medical condition that initially resulted in ICU admission and other comorbidities and nosocomial complications.

2.3 Pathogenesis

The pathogenic stages of COVID-19 are poorly understood. Infection may progress through these phases: SARS-CoV-2 invasion and replication, as well as dysregulated immune response (Li et al., 2021). Firstly, SARS-CoV-2 enters the host cells and binds to the host target cell receptor, angiotensin-converting enzyme 2 (ACE2). Non-specific signs and symptoms like fever, myalgia, headaches, and respiratory symptoms are caused by the virus active replication and release of the virus in the lung cells (Cevik et al., 2020). Sia et al., (2020) has demonstrated in hamster model research that the virus can harm the olfactory epithelium, resulting in olfactory impairment. This may explain the usual temporary loss of taste and smell observed in COVID-19 patient. The site of infection and patient symptoms can be explained by the distribution of ACE2 receptors in different tissues. For example, gastrointestinal symptoms and cardiovascular complications were caused by the ACE2 receptor on the epithelium of other organs, such as the intestine and endothelial cells in the kidney and blood vessels Varga et al., (2020) discovered lymphocytic endotheliosis in COVID-19

patients after doing postmortem examinations on the lung, heart, kidney, and liver, as well as liver cell necrosis and myocardial infarction. This study showed that the virus has a direct effect on many organs.

Most patients recover following the initial inflammatory response to SARS-CoV-2 infection, which attracts virus-specific T lymphocytes to the infection site and eliminated the infected cells there before the virus spreads. However, SARS-CoV-2 triggers an abnormal host immune response in those who develop severe illness. A study by (Xu et al., 2020a) on postmortem histology of the lung tissues of patients who died of COVID-19 has discovered the inflammatory nature of the injury, with characteristics of bilateral diffuse alveolar damage, hyaline-membrane formation, interstitial mononuclear inflammatory infiltrates, and desquamation consistent with acute respiratory distress syndrome (ARDS). These findings are similar to the lung pathology seen in severe Middle East respiratory syndrome (MERS) and severe acute respiratory syndrome (SARS) (Carsana et al., 2020). Wang et al., (2020) also showed in their study that the presence of mucus plugs with fibrinous exudate in the respiratory tract causes the severity of COVID-19 in young adults. Mangalmurti and Hunter, (2020) postulated that the damaging of lung parenchyma is caused by overproduction of pro-inflammatory cytokines that accumulate in the lungs. Additionally, some patients have multi-organ failure and septic shock. The release of highly sensitive troponin and natriuretic peptides reflects the early involvement of the cardiovascular system in COVID-19 disease (P. P. Liu et al., 2020). Focal intra-alveolar hemorrhage and the presence of platelet-fibrin thrombi in tiny artery arteries are also seen, which is consistent with the clinical setting of coagulopathy (Carsana et al., 2020).

2.4 Virulence factors in COVID-19

The virulence factors of SARS-CoV-2 specific primarily intended to avoid detection by immune cells. Host immune responses also manipulated including slowing interferon (IFN)-mediated defense and effective formation of neutralizing antibodies or activating host cell machinery that facilitates viral replication (Kumar et al., 2022). SARS-COV-2 differs from other SAR-related viruses because it has a furin cleavage site (FCS) containing multi-basic amino acids (PRRAR) at the S1/S2 intersection of the viral S protein (Coutard et al., 2020). Its high infectivity and transmissibility are cause by evolutionary gain of FCS (Johnson et al., 2020). Preliminary evidence also suggests that FCS may have contributed to the virulence of SARS-CoV-2 since it attenuated the disease in a hamster pathogenesis model. As the disease progressed, (Lucas et al., 2020) discovered a significant difference in the expression of inflammatory markers between those with mild (non-ICU) and severe (ICU) disease.

Most recent studies have reported that the virulence factors of SARS-CoV-2-specific are mainly aimed against host immune responses. However, new data also suggests that the cytokine storm in severe cases of COVID-19 was caused by SARS-CoV-2 manipulating the host into a delayed hyperactive innate immune response (Coperchini et al., 2020). According to this findings, virus-mediated tricking may lead in a dichotomous host immune response that might suppress T-cell-mediated antiviral activities (Lucas et al., 2020). SARS-CoV-2 proteins have a direct role in mediating host cell invasion. The spike protein allows the host and viral cell membranes to fuse, resulting in virus infection (Kumar et al., 2022). The N-linked glycans of the S2 subunit of the spike protein have reportedly been adapted to help the virus evade the host immune system. The N-linked glycans strategically decorated the post fusion

structure of the S2 subunit of spike protein (Cai et al., 2020). This suggests that the proteins may induce non-neutralizing antibody responses in order to evade the host immune system and offer defense against potentially harmful external factors.

Bouhaddou et al., (2020) investigated the driven molecular pathways in host cells. These findings showed strong evidence for broad phosphorylation of SARS-CoV-2 viral proteins by the host proteome, which activated host cell kinases and growth factor receptor (GFR) signaling, ultimately leading to the hijacking of host protein machinery. SARS-CoV-2 infection causes cell cycle arrest by activating casein kinase II (CK2) and p38 mitogen-activated protein kinase (MAPK), producing a variety of cytokines, and shutting down CDK1/2/5. The activation of the molecular pathway by SARS-CoV-2 explained acute inflammation and epithelial cell destruction, as well as vascular endothelial dysfunction, which are hallmarks of pulmonary tissue injury and other organs in severe COVID-19 patients.

2.5 Cellular immune response during SARS-CoV-2 infection

Once the SARS-CoV-2 enter the target cell, the host immune system detects the virus or its surface epitopes, inducing the innate or adaptive immune response (Azkur et al., 2020). The function of host innate immune cells is impaired by non-structural proteins of SARS-CoV-2, which affects the overall cytokine production (Shah et al., 2020). After host cells are infected, SARS-CoV-2 replication causes cell lysis and tissue damage. The SARS-CoV-2 is detected by pathogen recognition receptors (PRRs) on immune cells mainly Toll-like receptors 3, 7 and 8. The virus enhanced Interferon (IFN) production that may lead to uncontrolled replication. Apoptosis is induced by perforin and granzyme when infected cells present virus antigen to $CD8^+$ cells in the presence of natural killer (NK) cells that have become

cytotoxic. Dendritic cells have the ability to identify antigens and deliver them to $CD4^+$ T cells, inducing the $CD4^+$ T cells to differentiate into memory Th1 and Th17 cells, as well as memory T follicular helper (Tfh) effector cells (Santos, 2021).

The immune response is built upon the expression of a wide variety of transcription factors by distinct cell subtypes, which in turn affect cell activity and the pattern of cytokine production. Tfh cells can stimulate B-cells to differentiate into plasma cells and produce anti-SARS-CoV-2 specific IgM, IgA, and IgG antibodies. B cells generate an early response against the N protein at the onset of SARS-CoV-2 infection, but antibodies against the S protein can be found 4-8 days from the appearance of initial symptoms (Mallano et al., 2021). The ability of the adaptive immune response to overcome the challenges by the virus is demonstrated by the diversity of antibodies production during the virus infection. Macrophages found in tissue can also play a role in the presentation of antigens to $CD4^+$ T cells (Santos, 2021) (Figure 2.3).

In addition to neutralizing antibodies, there also numerous non-neutralizing antibodies in the system that aid the infection of immune cells and antigen-presenting cells (APCs) (X. Zhu and Zhu, 2020). T cells also detect viral antigens presented by class I major histocompatibility complex (MHC class I), which enhances cytokine release and cytotoxic activity in $CD8^+$ T cells. In addition, SARS-CoV-2 peptides are found to be presented to $CD4^+$ T cells via class II major histocompatibility complex (MHC class II) (Bertoletti et al., 2021; Raskov et al., 2020). Hematopoietic cells, monocyte-macrophages, and other immune cells infected with SARS-CoV-2 produce more of pro-inflammatory cytokines including TNF-α, IL-6, and IFN-α/-γ, whereas anti-inflammatory cytokines are decreased (Costela-Ruiz et al., 2020).

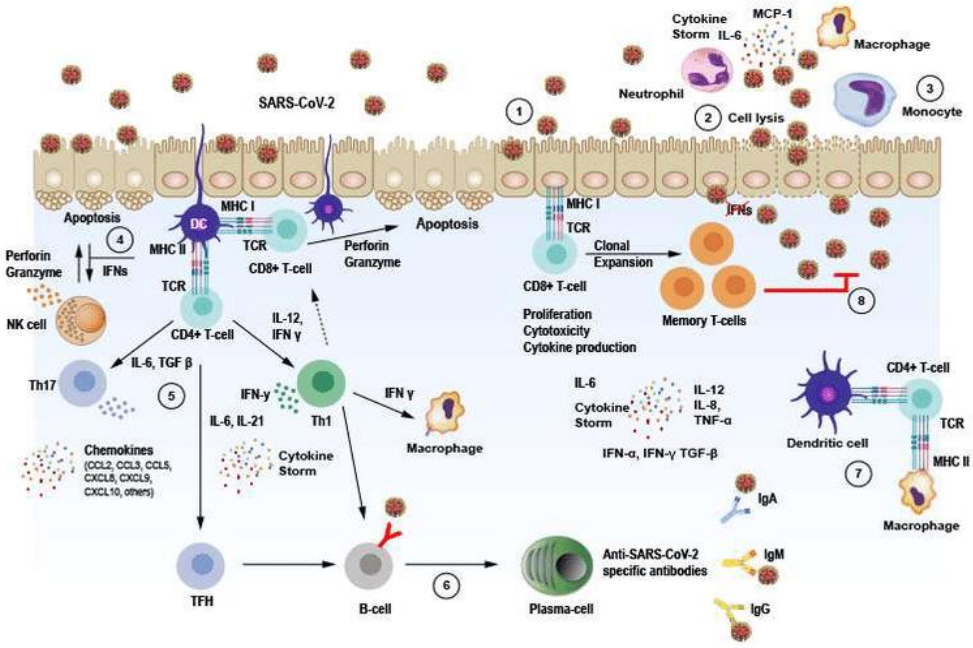

Figure 2.3 Immune response against SAR-CoV-2 infection. 1. SARS-CoV-2 infects target cells that express ACE2. 2. The virus may be able to overcome antiviral Interferon (IFN) responses, resulting in uncontrolled replication. 3. An excessive production of inflammatory cytokines may occur if neutrophils and monocytes/macrophages are recruited to the site of infection. Both the humoral and cellular immune responses cause immunological reactions. 4. The virus-infected epithelial cells may undergo apoptosis as a result of $CD8^+$ T lymphocytes presenting viral antigens and natural killer (NK) cells become cytotoxic. 5. Virus antigen is presented to $CD4^+$ T cells by Dendrictic cells and stimulate their differentiation into memory Th1 and Th17 as well as memory T follicular helper (TFH) effector $CD4^+$ T cells. 6. Plasma cells and activated B-cells generate anti-SARS-CoV-2 IgM, IgA, and IgG antibodies. 7. Antigen presented to $CD4^+$ T cells by macrophages and dendritic cells via MHC-TCR interaction. 8. Memory T cells are a subset of T cells that are produced and have the ability to provide immunity against reinfection with the same virus strain for an extended period of time (Santos, 2021).

2.6 Cytokine storm in SARS-CoV-2 infection

Cytokines are small proteins that are secreted by cells. These cytokines have a specific effect on the interactions and communications that occur between cells (J. M. Zhang and An, 2007). Different types of cytokines include chemokines, lymphokines, ILs, INFs and TNF (Ferreira et al., 2018). Cytokines are crucial part of the host immune system, as they protect the body from bacterial and viral invasion. Additionally, cytokines play a role in the development of symptoms in infections and pathogenesis (Ye et al., 2020). Cytokines will be released when the virus invade the lung endothelial cell via attachment to the ACE2 receptors on cells surface to initiates the pathogenesis of SARS-CoV-2 (Rabaan et al., 2021). Cytokines are classified into two types based on their activity during an infection: pro-inflammatory cytokines such as IL-2, IFN-γ and TNF-α, and anti-inflammatory cytokines such as IL-4 (Jamilloux et al., 2020; Song et al., 2020).

SARS-COV-2 entering lung epithelial cells trigger apoptosis and necroptotic, causing lung injury and different chemokine production. Various immunologically important cells, including dendritic cells, neutrophils, plasmacytoid dendritic cells and alveolar macrophages, are recruited within the lungs as a result of the initial release of chemokines. Various of molecular patterns, including viral particles, RNA, proteins, and other signals were recognize by Toll-like receptors (TLRs) on alveolar macrophages. This, in turn, stimulates the transcription and release of pro-inflammatory cytokines. However, these pro-inflammatory cytokines further activate other immune cells such as B-cells, T cells, NK cells, and other macrophages, which secrete a large number of cytokines that can lead to cytokine storm. As a result of this dysregulated and hyperinflammatory response, cytokine storm may cause harm to many organs. (Fara et al., 2020). Many studies have suggested that the pro-

inflammatory is the major factors such as IL, TNF and IFN, play important role in the prognosis of the COVID-19 disease. The onset of several clinical symptoms, including headaches, dizziness, chills, fatigue, and fever, is strongly correlated with the increased of IFN- secretion. Like IFN-γ, TNF-α induces flu-like symptoms along with fever, malaise, and fatigue. It can also cause lung damage, heart failure, vascular leaking, and synthesis of acute-phase protein (Shimabukuro-Vornhagen et al., 2018) (Figure 2.4)

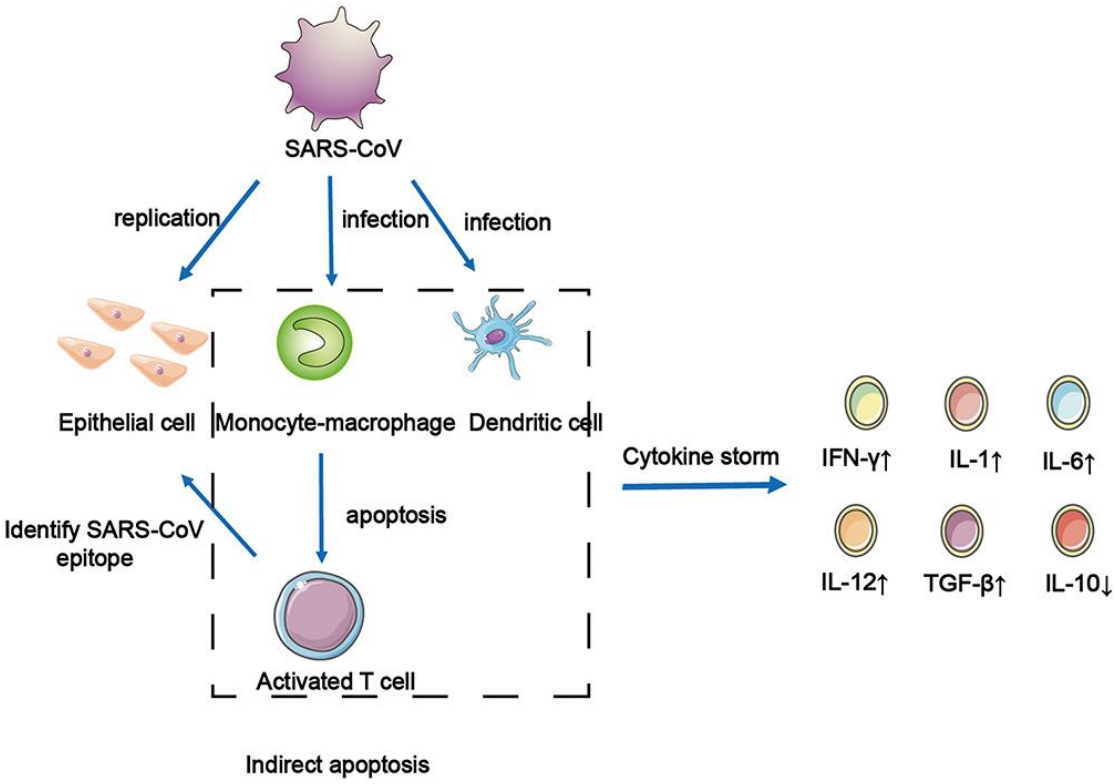

Figure 2.4 The infection and replication of SARS-CoV in epithelial cells and immune cells forming cytokine storm. Infection and replication of SARS-CoV occurs in epithelial cells. The SARS-CoV virus infects monocytes, macrophages, and dendritic cells, which causes activated T cells to induce apoptosis. During an immunological response, the cells represented by the dotted frame can secrete a variety of cytokines, which contributes further to the formation of a cytokine storm (Liu et al., 2021).

Cytokine storms can also result in a serious clinical complication known as acute respiratory distress syndrome (ARDS). ARDS is caused by an overactive

immune response rather than a viral load (Grasselli et al., 2021). It can lead to respiratory epithelium damage, which is most serious complication of COVID-19, with a high mortality rate (Xu et al., 2020b). The study showed that aberrant pathogenic T lymphocytes produce larger quantities of cytokines such as granulocyte–macrophage colony-stimulating factor (GM-CSF), which leads to an inflammatory storm and severe lung injury (Zhou et al., 2020). In addition to those mentioned above, cytokines play a significant role in other complications of COVID-19. Collectively, excessive production of cytokines can result in cytokine storms, which can cause uncontrolled tissue damage (Ragab et al., 2020). Restraining the uncontrolled cytokine infiltration by immunization helps control COVID-19-induced organ damage (Wan et al., 2020).

2.7 Immune response induced by COVID-19 vaccine

There are many different types of vaccines, and each one provides protection in a different way. Nevertheless, the body is left with a supply of "memory" T-lymphocytes as well as B-lymphocytes after receiving any type of vaccine (Turner et al., 2021). These lymphocytes will remember how to fight off that virus in the future. The production of T- and B-lymphocytes following vaccination normally takes a few weeks. As a result, it is possible for a person to become infected with the virus that causes COVID-19 either before or just after vaccination, and then become ill due to the vaccine not having enough time to provide protection against the disease (“Understanding How Vaccines Work | CDC,” 2022). After receiving a vaccination, the natural process of the body developing immunity might occasionally induce symptoms such as fever (Alrowdhan et al., 2022). These symptoms are natural indicators that the body is working to build up its immunity.

Generally, all the vaccines can trigger an immune response and are effective against SARS-CoV-2, even at different levels. Inactivated virus vaccine work classically stimulating $CD4^+$ T-cells by activating APCs, in the presence of IFN-γ. This, in turn, leads to the production of antibodies by B lymphocytes as well as the activation of CD8 T-cells, which promote the death of infected cells (García-Montero et al., 2021). Vaccines based on viral vectors destructed the infected cells by stimulating a strong antibody production and cytotoxic response, SARS-CoV-2 transgene expression and mimicking the characteristics of natural infection (Lundstrom, 2020). In turn, products of mRNA-based vaccines are picked up by APCs, where they are identified by TLR, resulting in the activation of type I interferon, which has the potential to stimulate a Th1 response. Additionally, the antigenic protein is produced by the host cells, and it favours presentation through MHC I, which triggers a cytotoxic response. This is very similar to what happens when a virus infects the host (Cagigi and Loré, 2021). Thus, the type of vaccine may affect the immune system in a different way, resulting in immunological responses.

The mechanism of action of the mRNA vaccine is shown in figure 4.6. When a single dose of the COVID-19 vaccine is injected into the arm, the contents of the vaccine penetrate body cells at the injection site, along with the genetic components for the spike protein. Human cells are responsible for producing the spike protein, which is then presented on the surface of those cells. This activates Th cells, Killer T cells, and B cells, all of which are essential to the immunological response. The spike protein is detected by the Th cells, which then alert the immune cells. Killer T cells that are specifically looking for the spike protein. B cells are responsible for the production of antibodies, and these antibodies particularly identify the spike protein. T and B cells develop immune memory of the spike protein, which allows them to

respond quickly the next time they come into contact with it. Antibodies stay in the body for a while, ready to bind to the spike protein and stop the virus from infecting new cells. If there is a need in the future, B cells can produce more antibodies (Santos, 2021).

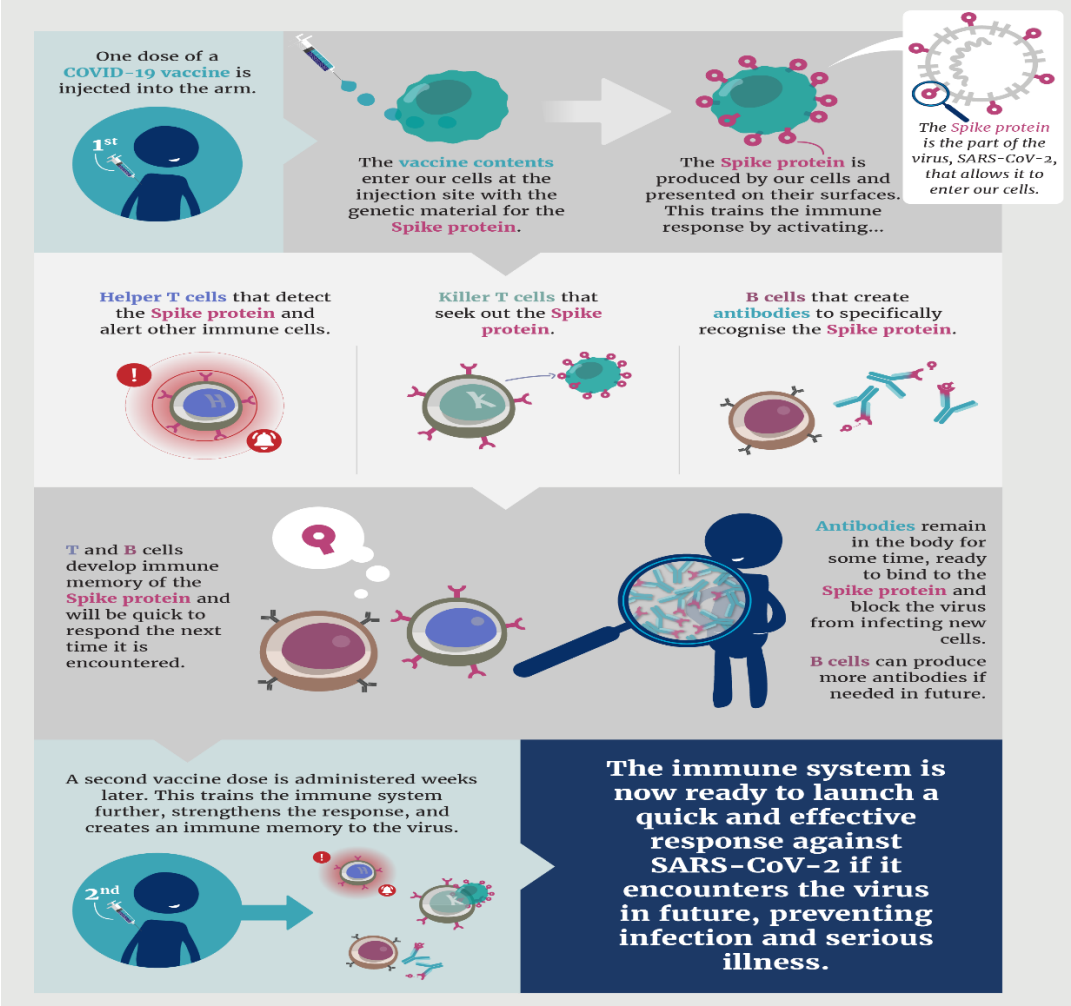

Figure 2.6 Mechanism of mRNA vaccine. It is use mRNA that has been synthesized in a laboratory in order to instruct the body cells on how to produce a protein, or even just a portion of a protein, that will cause an immune response to take place inside the bodies (Santos, 2021).

2.8 $CD4^+$ and $CD8^+$ cell immunity

T lymphocytes are classified into two subsets based on the presence of the CD4 and CD8 antigens. The $CD4^+$ T cells, together with B lymphocytes, and $CD8^+$ T lymphocytes, help to coordinate the immune response by stimulating other immune cells to fight infection (Luckheeram et al., 2012). T lymphocytes that express CD4 are known as T helper (Th) cells. Th cells regulate the adaptive immune response based on the cytokines they secrete. $CD4^+$ T helper cells are classified into five types: Th1, Th2, Th17, Treg (T-regulatory), and Tfh (Alberts et al., 2002). However, the most common Th cells are classified as Th1 and Th2. Th1-type cytokines are produced by Th1 cells, while Th2-type cytokines are produced by Th2 cells. Th1 tends to produce a proinflammatory response with TNF-α being the most abundant cytokine (Aleebrahim-Dehkordi et al., 2022).

Antibodies that solely target intact extracellular viral particles may have a limited role in decreasing viral spread inside the host, since SARS-CoV-2 can spread from cell to cell without exposure to the extracellular environment (Fenrich et al., 2020). T cells, as would be anticipated for a viral infection, play a crucial role in mediating the host response to SARS-CoV-2 infection by defeating infected cells, supporting B cell activity and antibody responses, and decreasing the risk of vaccine-induced enhanced disease (Sadarangani et al., 2021) . The study observed both decreased and increased levels of $CD8^+$ and $CD4^+$ T cells in COVID-19 patients (Chen and John Wherry, 2020). A stronger clonal activation of $CD8^+$ T lymphocytes in both the lungs and the blood has been linked with milder disease and recovery (Liao et al., 2020; Wen et al., 2020).

Patients who have recovered from COVID-19 have both virus-specific $CD8^+$, and $CD4^+$ T cells, as well as virus-specific $CD8^+$ memory T cells (Grifoni et al., 2020a;

Sekine et al., 2020). However, it is unclear how significant they are regarding providing protection against a following infection or serious illness. During an acute infection, the Th1 cells secreted - IFN, and it has been hypothesized that a Th1 cell-biased phenotype linked with a less severe disease. This, in turn, considers the current COVID-19 vaccine has been developed to elicit responses that are biased towards Th1 cells phenotype (Weiskopf et al., 2020).

There is some evidence that suggests that individual with better protection from COVID-19 shows higher level of IFN-γ secreted by T cells against the S protein, nuclear proteins and membrane proteins of SARS-CoV-2. This is measured by enzyme-linked immunosorbent spots (Wyllie et al., 2021). Additionally, individuals with mild disease conditions have more effective Thf cell responses in the germinal center, which promotes an increase in plasma blast numbers and boosts antibody production (Kuri-Cervantes et al., 2020). Most studies showed that the adoptive transfer of antigen-specific T lymphocytes could prevent immunodeficient mice against infection after a challenge with the SARS-CoV-2-related coronaviruses SARS-CoV and MERS-CoV (Zhao et al., 2016). It was discovered that the passive transfer of NAbs was protective in non-human primate models, whereas the removal of $CD8^+$ T cells in these same species was shown to be impaired protection, indicating that both components play a role (McMahan et al., 2021). In addition to high titers of NAbs, or even in place of them, evidence from human and animal studies has suggested that a robust cytotoxic $CD8^+$ T cell response and a Th1 cell-biased $CD4^+$ T cell effector response would result in protective immunity against COVID-19 (Jeyanathan et al., 2020). A better understanding of immune responses to SARS-CoV-2, especially robust CD4+ and $CD8^+$ T cells is crucial to developing effective vaccines and therapeutics and ending the current pandemic.