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Alexzander A. A. Asea
Punit Kaur *Editors*

Coronavirus Therapeutics – Volume I

Basic Science and Therapy Development

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Preface

Currently, the entire world is in the grips of an unprecedented Coronavirus epidemic that has affected all facets of normal life. There is a huge need for clear and concise information that can be used to educate and combat this disease. *Coronavirus Therapeutics Volume I* is the concise collection of articles focused on the basic science behind this viral outbreak and the efforts aimed to understand the molecular mechanisms of the infection. In addition, it discusses the research aimed at developing efficient therapies aimed at combatting this global disease.

The book *Coronavirus Therapeutics Volume I* provides the most comprehensive review on contemporary knowledge on the origin and structural biology and molecular aspects of Coronaviruses. This also describes the etiology and pathogeny and therapeutic targets including vaccine drug candidates against Coronaviruses in the light of current scientific knowledge. Using an integrative approach to the understanding of Coronaviruses structure, function, and immunobiology, the contributors provide a synopsis of novel mechanisms by which Coronaviruses can be treated.

Key basic and clinical research laboratories from major universities, academic medical hospitals, and biotechnology and pharmaceutical laboratories around the world have contributed chapters that review present research activity and importantly project the field into the future. The book is a must read for graduate students, medical students, basic science researchers, and postdoctoral scholars in the fields of Translational Medicine, Clinical Research, Human Physiology, Biotechnology, Natural Products, and Cell and Molecular Medicine, and pharmaceutical scientists and researchers involved in Drug Discovery.

Toledo, OH, USA

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Editors Biography

Alexzander A. A. Asea is a highly innovative and accomplished world-renowned clinical and basic research scientist and visionary executive leader who has exceptional experience spearheading clinical and basic science research, training, education, and commercialization initiatives within top ranked academic biomedical institutes. Prof. Dr. Asea's initial findings studying the effects of Hsp72 on human monocytes led to the proposal of a novel paradigm that Hsp72, previously known to be an intracellular molecular chaperone, can be found in the extracellular milieu where it has regulatory effects on immuno-competent cells – a term now called chaperokine. Prof. Dr. Asea is also president and CEO of NampEVA BioTherapeutics, a company dedicated to providing affordable medicines to patients in emerging nations. Prof. Dr. Asea has authored more than 320 scientific publications including peer-reviewed articles, reviews, books, book chapters, editorials, and news headliners in a wide range of biomedical-related disciplines. Prof. Dr. Asea is the editor-in-chief of the widely successful book series Heat Shock Proteins (Springer Nature Publishing) and is an editorial board member of numerous scientific peer-reviewed journals. Prof. Dr. Asea is at the University of Toledo College of Medicine and Life Sciences in Toledo, USA.

Punit Kaur is an expert in onco-proteogenomics, with extensive training and experience in quantitative mass spectrometry imaging, protein chemistry, and biomarker discovery. Dr. Kaur's main research focus is on the use of heat-induced nanotechnology in combination with radiotherapy and chemotherapy in cancer stem cell therapy. Dr. Kaur has published more than 80 scientific articles, book chapters, and reviews, and currently serves as editorial board member of the *European Journal of Cancer Prevention* and the *Journal of Proteomics and Bioinformatics*. Dr. Kaur is the associate editor of the highly successful Heat Shock Proteins book series by Springer Nature. Currently, Dr. Kaur is at the University of Toledo College of Medicine and Life Sciences in Toledo, USA.

Origin and Structural Biology of Novel Coronavirus (SARS-CoV-2)

1

Rahul Mallick and Asim K. Duttaroy

Abstract

Introduction: A recent rapid outbreak of infection around the globe has been caused by a novel coronavirus, now known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which was first identified in December 2019 in Wuhan city of Hubei province, People's Republic of China.

Methods: We reviewed the currently available literature on coronaviruses.

Results: Coronaviruses are a group of enveloped viruses with non-segmented, single-stranded, and positive-sense RNA genomes. Although 13 variation sites in open reading frames have been identified among SARS-CoV-2 strains, no mutation has been observed so far in envelop protein. The origin and structural biology of SARS-CoV-2 in details are discussed.

Conclusions: Origin and structural biology will help the researchers identify the virus's mechanism in the host and drug design. Currently, no clinical treatments or prevention strategies are available for any human coronavirus.

Keywords

ACE2 · COVID-19 · Furin-like cleavage site · Heparan sulfate proteoglycans · non-structural proteins · Open reading frame · Polyproteins · Receptor binding domain · SAR-CoV-2 · Spike protein · S1/S2 cleavage site · S2′ cleavage site

Abbreviations

AA	amino acid
ACE2	Angiotensin-converting enzyme 2
COVID-19	Coronavirus disease 2019
E	Envelope protein
FAO	Food and Agriculture Organization of the United Nations
FP	Fusion peptide
HR1/2	Heptad repeat 1/2
IFP	Internal fusion peptide
IFN	Interferon
LFA-1	Lymphocyte function-associated antigen 1
M	Membrane protein

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MERS-CoV	Middle East respiratory syndrome-related coronavirus
NSP	Non-structural protein
NF-κB	Nuclear factor-kappa B
NLRP	NOD-like receptor
NTD	N-terminal domain
N	Nucleocapsid protein
ORF	Open reading frame
RBD	Receptor binding domain
SP	Signal peptide
S	Spike protein
SARS-CoV	Severe acute respiratory syndrome-related coronavirus
SARS-CoV-2	Severe acute respiratory syndrome-related coronavirus 2
TD	Transmembrane domain
WHO	World Health Organization
2019-nCoV	2019-novel coronavirus

1.1 Introduction

Emerging and re-emerging pathogenic microorganisms have always been the major threats to human civilization. In the twenty-first century alone, five respiratory virus (three coronavirus strains and two influenza virus strains) outbreaks have occurred worldwide. Human respiratory tract infection by coronaviruses was first characterized in 1960 (Kahn and McIntosh 2005). A recent rapid outbreak of infection around the globe was caused by a novel coronavirus, which was first identified in December 2019 in Wuhan city of Hubei province, People's Republic of China. Later it was declared a pandemic on 11 March 2020 by the World Health Organization (WHO) (Corman et al. 2020; Wang et al. 2020b; WHO (Press release) 2020). According to the guidelines of the World Organisation for Animal Health (OIE) and the Food and Agriculture Organization of the United Nations (FAO), the infectious disease was officially announced as coronavirus disease 2019 (COVID-19) on 11 February 2020 by WHO (WHO 2020). The disease was previously known as a 2019-novel coronavirus (2019-nCoV), Wuhan flu, and Wuhan pneumonia (Zhou et al. 2020; Stobbe 2020; Campbell 2020). COVID-19 is caused by severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) (WHO 2020). Within two decades, COVID-19 is the third outbreak of highly contagious coronavirus in humans, followed by a severe acute respiratory syndrome-related coronavirus (SARS-CoV) in 2002–2003 and the Middle East respiratory syndrome-related coronavirus (MERS-CoV) in 2012 (De Wit et al. 2016). More than 50 million people from 217 countries and territories, along with 2 international conveyance, are affected by SARS-CoV-2 as of 30 October 2020 (https://www.worldometers.info/coronavirus/). The case fatality rate for COVID-19 has been estimated at around 3% so far (https://www.worldometers.info/coronavirus/). This chapter focuses on the origin and structural biology of SARS-CoV-2 infection, based on the available data on SARS-CoV-2 and other coronaviruses.

1.2 Phylogenetics and Taxonomy of SARS-CoV-2 Virus

Enveloped positive-sense single-stranded RNA virus, SARS-CoV-2, belongs to a group of viruses known as coronaviruses that cause respiratory, enteric, hepatic, and neurological diseases in mammals and birds (Zhu et al. 2020). Six coronavirus species have been identified so far and can infect humans (Su et al. 2016). Four of them (HKU1, HCoV-OC43, HCoV-NL63, and HCoV-229E) are known to infect immunocompromised individuals (Zhu et al. 2020). The other two zoonotic origin species (SARS-CoV and MERS-CoV) from the *Betacoronavirus* genus have been lethal for humans (Cui et al. 2019; Perlman and Netland 2009). SARS-CoV-2 is the new strain of species SARS-CoV (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses 2020). Figure 1.1 describes the taxonomy of coronavirus. In December 2019, SARS-CoV-2 caused an outbreak of coronavirus disease 2019 (COVID-19). Phylogenic analysis has shown that, like other SARS-related coronavirus strains (including the strain of the 2003 SARS outbreak),

Virus classification

Realm: *Riboviria*
Order: *Nidovirales*
Suborder: *Cornidovirineae*
Family: *Coronaviridae*
Subfamily: *Orthocoronavirinae*
Genus: *Betacoronavirus*
Subgenus: *Sarbecovirus*
Species: *Severe acute respiratory syndrome-related coronavirus*
Individuum: *SARS-CoV-2*

Fig. 1.1 Taxonomy of SARS-CoV-2

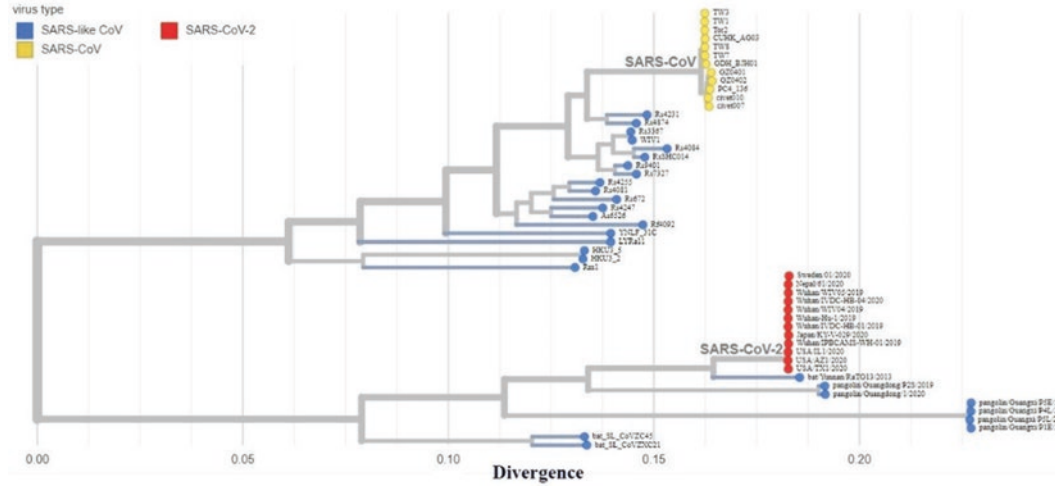

Fig. 1.2 Phylogeny of SARS-like coronaviruses. The complete genome sequences of coronaviruses was obtained and analyzed with Nextstrain. (Last updated on 26 February 2020) (https://github.com/blab/sars-like-cov)

SARS-CoV-2 is also a member of subgenus *Sarbecovirus and* species SARS-CoV (Nextstrain 2020; GISAID EpifluDB 2020). SARS-CoV-2 genome has the following similarities: 96% identity to the sequence of Yunnan-RaTG13 (nearest bat precursor), 90% identity to the sequences of Guangdong-1/PS2 (nearest pangolin precursors), 88% identity to the sequences of SL-CoVZC45/ZXC21 (bat precursor), 80% SARS-CoV, and only 50% identity to the sequence of MERS-CoV (Lu et al. 2020; GISAID EpifluDB 2020). Despite mutations at the nucleotide or amino acid (AA) level, SARS-CoV-2 strains are highly homologous (~99.99%) (Wang et al. 2020a). Figure 1.2 describes the phylogenic analysis of the SARS viruses.

1.3 Structural Biology of SARS-CoV-2 Virus

SARS-CoV-2 virions are around 50–200 nm in diameter, which contains four main structural proteins: spike protein (S protein), an envelope protein (E protein), nucleocapsid protein (N protein), and membrane protein (M protein) (Xu et al. 2020; Wu et al. 2020; Chen et al. 2020). S, E, and M proteins form a viral envelope/surface together, while N protein holds an RNA genome of the SARS-CoV-2 virus (Wu et al. 2020). SARS-CoV-2 virus nucleotide is around 30k long (GISAID EpifluDB 2020). Like SARS-CoV, the SAR-CoV-2 genome contains about 10 open-reading frames (ORFs), and 2/3 of the viral genome is first ORFs (ORF1a/b), which are

translated into polyproteins required for viral replication and transcription (Li et al. 2020). One-third of the SARS-CoV-2 genome contains other ORFs, which encodes structural and accessory proteins that interrupt the host's innate immune response (Li et al. 2020). Only 13 variation sites in ORFs (1a, 1b, S, 3a, M, 8, and N regions) have been identified in SARS-CoV-2 stains (Wang et al. 2020a). The functions of accessory proteins encoded by ORFs of SARS-CoV-2 are still to be known.

1.4 Nonstructural Proteins of SARS-CoV-2 Virus

Usually, ORFs of the SARS-CoV-2 virus are translated into polyproteins required for viral replication and transcription. ORF1 encoded polyproteins (pp1a and pp1ab) responsible for the formation of replicase-transcriptase complex (Fehr and Perlman 2015; Li et al. 2020). Following the viral entry, 16 nonstructural proteins (NSPs) are produced from these polyproteins by essential viral proteases, for example, main protease and papain-like protease (Table 1.1) (Báez-Santos et al. 2015; Li et al. 2020). These two proteins act like scissors, snipping the different NSPs' links and freeing them to do their jobs. NSP1 reduces the synthesis of infected host cell's proteins. The sabotage forces the infected cell to make more viral proteins and prevents it from assembling antiviral proteins that could stop the virus. Stabilizing mutation of mysterious NSP2 has made SARS-CoV-2 more contagious than SARS-CoV (Angeletti et al. 2020). Large NSP3 modifies infected cellular proteins. Healthy cells tag old cellular proteins for degrain order to avoid cell toxicity. But the coronavirus can change the balance of proteins, possibly by reducing the cell's ability to fight the virus by removing the tags. New copies of the virus are formed inside (NSP3, NSP4, and NSP6 induced) double-membrane vesicles. Like NSP3, NSP5 cuts free other NSP proteins to carry out their jobs. NSP7 and NSP8 help NSP12 make new copies of the RNA genome, ultimately end-

Table 1.1 Functions of prospective SARS-CoV-2 virus NSPs

Proteins	Functions
NSP1 (cellular saboteur)	Degrades host mRNA, blocks host translation and innate immune response (Fehr and Perlman 2015)
NSP2 (mystery protein)	Binds with prohibitin proteins (Fehr and Perlman 2015)
NSP3 (large multidomain transmembrane protein) (untagging and cutting)	Ubiquitin-like 1 and glutamic acid-rich acidic domains (interact with N protein) (Báez-Santos et al. 2015; Fehr and Perlman 2015)
	ADP-ribose-1′-phosphatase domain (promotes cytokine expression) (Fehr and Perlman 2015)
	Papain-like protease/ deubiquitinase domain (cleaves viral polyprotein and blocks host innate immune response) (Fehr and Perlman 2015)
	SARS-unique domain (binds oligonucleotides known to form G-quadruplexes) (Báez-Santos et al. 2015)
	Nucleic acid-binding domain (nucleic acid chaperon function) (Báez-Santos et al. 2015)
	Transmembrane domains (putative metal-binding region) (Báez-Santos et al. 2015)
	Ubiquitin-like 2, G2M, Y domains (unknown functions) (Fehr and Perlman 2015)
NSP4 (transmembrane scaffold protein) (bubble marker)	Responsible for double-membrane vesicles assembly (Fehr and Perlman 2015)
NSP5 (main protease) (protein scissor)	Cleaves viral polyprotein (Fehr and Perlman 2015)
NSP6 (transmembrane scaffold protein) (bubble factory)	Able to proliferate membrane, induces perinuclear vesicles localization (Angelini et al. 2013).
	Blocks translocation of the STAT1/2/IRF9 complex (Kopecky-Bromberg et al. 2007).

(continued)

Table 1.1 (continued)

Proteins	Functions
NSP7 (copy assistant)	Acts as clamp for RNA polymerase by forming complex with NSP8 (Fehr and Perlman 2015)
NSP8 (copy assistant)	Acts as clamp for RNA polymerase by forming complex with NSP7 (Fehr and Perlman 2015)
NSP9 (interest on nucleus)	RNA-binding protein (Fehr and Perlman 2015)
NSP10 (genetic camouflage)	Forms heterodimer with NSP14 and NSP16 to stimulate viral exoribonuclease and 2′-*O*-methyltransferase respectively (Fehr and Perlman 2015)
NSP11	Short peptide at the end of ORF1a (Chan et al. 2020)
NSP12 (copy machine)	RNA-dependent RNA polymerase (Fehr and Perlman 2015)
NSP13 (unwinding)	RNA helicase domain and RNA 5′ triphosphatase activity (Fehr and Perlman 2015)
NSP14 (proof-reader)	N7 Methyltransferase activity to add 5′ cap to viral RNAs (Fehr and Perlman 2015)
	Viral exoribonuclease activity as proofreading of viral genome (Fehr and Perlman 2015)
NSP15 (cleaning up)	Viral endoribonuclease (Fehr and Perlman 2015)
NSP16 (camouflage assistant)	2′-*O*-methyltransferase activity to shield viral RNA from melanoma differentiation associated protein 5 (Fehr and Perlman 2015)

The NSPs are encoded into two large ORFs (ORF1a and ORF1b). ORF1a and ORF1b encode the replicase genes rep1a and rep1b, respectively. rep1a and rep1b translate into polyproteins, pp1a and pp1ab, respectively. pp1a and pp1ab cleave into 16 NSPs by papain-like protease and main protease

ing up inside new viruses. NSP9 may infiltrate tiny channels in the infected cell's nucleus to facilitate molecules' movement in and out of the nucleus. Host cells have antiviral proteins that find viral RNA and shred it.

NSP10 functions with NSP16 to camouflage the virus's genes so that they are not attacked. NSP11 of ORF1a overlaps the part of the same sequence of ORF1b in the RNA genome. NSP12 assembles genetic letters into new virus genomes. Virus RNA is wound into intricate twists and turns. NSP13 is responsible for unwinding virus RNA. Therefore, other proteins can read their sequence and make new copies. As NSP12 duplicates the coronavirus genome, it sometimes adds a wrong letter to the original copy. NSP14 cuts out these errors so that the correct genetic letter can be added instead. NSP15 chops up leftover virus RNA to hide from the infected cell's antiviral defenses.

1.5 Unique Class I Spike Glycoprotein of SARS-CoV-2

Primary coronavirus tropism is the crown (Latin "corona")-shaped S protein (~1200 AA) long) on its surface, which allows the virus to attach and fuse with the host cell membrane (Woo et al. 2010; Coutard et al. 2020; Wu et al. 2020). Structural proteins also help assemble and release new copies of the virus. Like other human-infecting coronaviruses, the following cleavage class I S protein of SARS-CoV-2 generates two subunits: S1 (attachment domain) subunit and S2 (fusion and transmembrane domain) subunit (Hoffmann et al. 2020; Andersen et al. 2020; Coutard et al. 2020). Figure 1.3 shows the S protein of SARS-CoV-2 with a focus on favorable putative sites. But the virus is highly pathogenic and transmissible comparing with other beta coronaviruses of linage b due to its polybasic furin-like cleavage site in S protein (Walls et al. 2020; Andersen et al. 2020; Coutard et al. 2020). Also, the receptor-binding domain (RBD) of SARS-CoV-2 S protein has a high affinity for the human angiotensin-converting enzyme 2 (ACE2) receptor, which increases virulence (Andersen et al. 2020). Also, the S protein of SARS-CoV-2 can interact with CD147 for cellular invasion (Wang et al. 2020c).

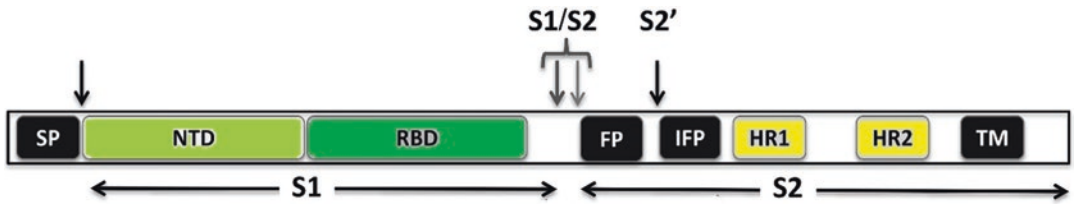

Fig. 1.3 S protein of SARS-CoV-2 with a focus on favorable putative site. S protein consists of S1 subunit [N-terminal domain (NTD) and receptor binding domain (RBD)] and S2 subunit [fusion peptide (FP), internal fusion peptide (IFP), heptad repeat 1/2 (HR1/2), and transmembrane domain (TM)] along with signal peptide (SP). Cleavage sites of SP, S1/S2, and S2′ are indicated by arrows. (The figure is modified and adapted from Coutard et al. 2020)

1.6 Attachment Capability of S1 Subunit of SARS-CoV-2

S1 subunit of S protein is known for the virus's host-specific attachment capability. N terminal S1 subunit comprises two distinct domains: N-terminal domain (NTD) and RBD (Li 2012; Coutard et al. 2020). Both domains are critical as receptor recognition (Li 2012). Although the RBD is known to bind the cell surface receptor ACE2, the function of NTD of SARS-CoV-2 is not well investigated. NTD has been suggested to facilitate the initial attachment by recognizing sialic acids (as receptor determinants) in the host cell membrane (Schwegmann-Weßels and Herrler 2006; Ou et al. 2020; Hoffmann et al. 2020).

1.7 Fusion Capability and Viral Infectivity of S2 Subunit of SARS-CoV-2

Several viral proteins such as fusion peptide (FP), second proteolytic site (S2′), internal fusion peptide (IFP), heptad repeat 1 and 2 (HR1 and 2), and transmembrane domain (TM) form S2 subunit complex (Coutard et al. 2020). FP and IFP are thought to be responsible for virus entry (Lu et al. 2015). Like another type, I fusion proteins such as Ebola GP and HIV gp41, SARS-CoV-2 HR1, and HR2 may form canonical six-helix bundle (Lu et al. 2015). Even it remains unknown if FP, IFP, HR1 and 2, and TM function individually or synergistically. Further investigation is required for a better understanding of functionality of the S2 subunit.

1.8 Multiple Cleavage Sites of SARS-CoV-2 Glycoprotein

Proteolytic cleavage regulates numerous processes in health and disease. One key player is the ubiquitously expressed serine protease furin, which cleaves many proteins at polybasic recognition motifs. The enzymatic activity of furin is exploited by numerous viral pathogens to, enhance their virulence and spread. Seven mammalian secretory preprotein convertases (PC1, PC2, furin, PC4, PC5, PACE4, and PC7) cleave precursor proteins at specific single or paired basic amino acids (AA) within the canonical motif (**R/K**)-(**2X**)**n**-(**R/K**)↓, where **n** = 0, 1, 2, or 3 and **X** = AA (Seidah and Chretien 1999). The preprotein convertases, especially furin (highly expressed in the lung), are known to cleave viral envelope glycoproteins to enhance cellular tropism and pathogenesis (Coutard et al. 2020). And polybasic motif in viral envelope glycoproteins is furin-like cleavage-specific and associated with the hypervirulence of the SARS-CoV-2 (Coutard et al. 2020). Different pathogenic RNA viruses, for example, HIV, Ebola virus with polybasic envelop protein cleavage site(s) are on focus due to their higher pathogenicity (Table 1.2). Similar to MERS-CoV, two polybasic furin-like cleavage sites (S1/S2 and S2′) were discovered in SARS-

Table 1.2 Comparison of canonical envelop protein polybasic cleavage sites of different RNA viruses

Virus	Envelop protein	Cleavage site
SARS-CoV-2	S protein	SP**R**RA**R**↓S**V**AS (S1/S2)
SARS-CoV-2	S protein	SKPS**KR**↓S**F** (S2')
SARS-CoV	S protein	LKPT**KR**↓S**F** (S2')
MERS-CoV	S protein	TP**R**SC**R**↓S**V**PG (S1/S2)
HKU1	S protein	SR**R**KR**R**↓S**I**SA (S1/S2)
HCoV-OC43	S protein	KN**R**RS**R**↓GAITT
HIV	Gp160	VQ**R**E**KR**↓A**V**
Human CMV	gB	HK**R**T**KR**↓S**T**
Influenza virus H5	HA	**R**K**R**K**KR**↓G**L**
Avian H5N1 A/HK/98	HA	**R**E**R**K**R**K**KR**↓G**L**
Yellow Fever Virus	PrM	SR**R**S**RR**↓A**I**
Zika Virus	PrM	AR**R**S**RR**↓A**V**
Ebola Virus	GP	GR**R**T**RR**↓EA

SARS-CoV-2 and other RNA viruses display the canonical (R/K)-(2X)n-(R/K)↓ motif (red bold alphabets) at cleavage site(s). The hydrophobic residues (except alanine) (black bold alphabets) at P2' position of cleavage site favor the cleavage by furin/furin-like proteases

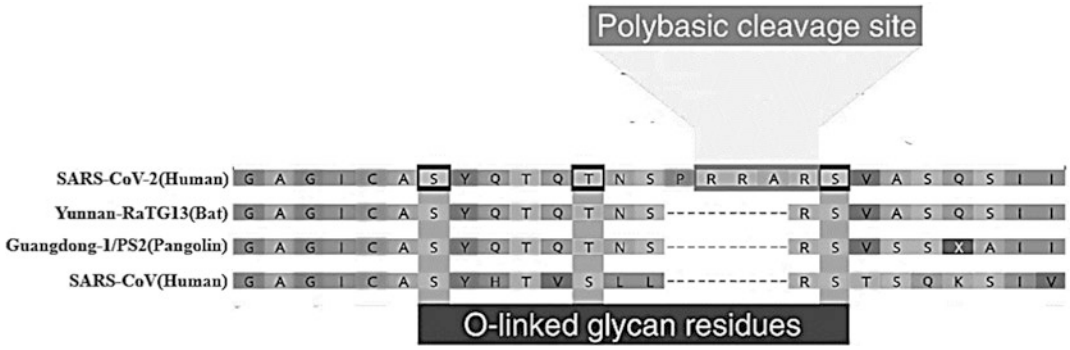

Fig. 1.4 Polybasic cleavage site of closely related Betacoronaviruses. Alignment of SARS-CoV-2 against the most closely related viruses. Polybasic furin cleavage site (light green box) and predicted related O-linked glycans (dark green box) at S1/S2 junction of SARS-CoV-2 genome sequence. (The figure is modified and adapted from Andersen et al. 2020)

CoV-2 so far (Coutard et al. 2020). But the role of furin-like cleavage sites in SARS-CoV-2 S protein on viral pathogenesis or replication is less studied. Like SARS-CoV, SARS-CoV-2 contains the conserved site sequence **AYT↓M** between RBD and FP, which can be cleaved by other proteases (e.g., cathepsin L, TMPRSS2) rather than proprotein convertases (Coutard et al. 2020; Hoffmann et al. 2020). The conserved sequence **AYT↓M** could be cleaved following furin cleavage at S1/S2 site. This possibly demonstrates if S protein of SARS-CoV-2 wouldn't cleave at furin-like cleavage sites during viral endocytosis; S protein would indeed cleave at conserved sequence **AYT↓M**.

1.9 Glycobiology of SARS-CoV-2

Insertion of proline before furin cleavage site (-RRAR-) results in the addition of O-linked v that flank at the cleavage site (S1/S2) (Hoffmann et al. 2020; Andersen et al. 2020). Figure 1.4 depicts the polybasic cleavage sites. The function of the O-linked glycan of SARS-CoV-2 has not been studied much. It is predicted that O-linked glycan

could create a mucin-like domain to protect epitopes or key residues on S protein. Different viruses use the mucin-like domain to conserve immunoevasion (Bagdonaite and Wandall 2018). Therefore, O-linked glycan could be a crucial determinant for vaccine discovery of SARS-CoV-2.

1.10 Mutations in the Receptor-Binding Domain

Surface proteins of SARS-CoV-2 have around 98% and 76% identical AA sequence of Yunnan-RaTG13 (bat precursor) and SARS-CoV, respectively (GISAID EpifluDB 2020). The most variable part of the coronavirus genome seems to be receptor-binding sites (RBDs) in the S protein (Andersen et al. 2020). Figure 1.5 represents the schematic diagram of the ŔBD site. With 30 mutations (13 interface mutations) in RBD it indicates how changes could allow switching the

Fig. 1.5 RBD (ACE2 contact residue) of closely related Betacoronaviruses. Alignment of SARS-CoV-2 against the most closely related viruses. Key residues in the RBD of S1 subunit that make contact to the ACE2 receptor are marked with blue boxes in both SARS-CoV-2 and related viruses. Six key AA residues involved in interaction with ACE2 receptor are 99% similar between Guangdong-1/PS2 (Pangolin) and SARS-CoV-2. But only one key AA residue is similar between Yunnan-RaTG13 (Bat) or SARS-CoV and SARS-CoV-2. (The figure is modified and adapted from Andersen et al. 2020)

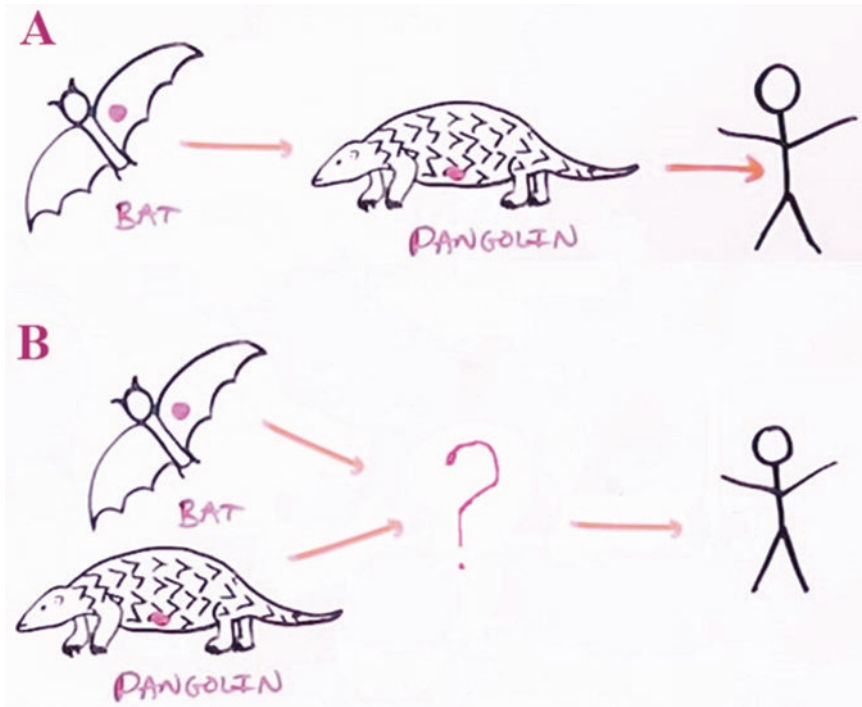

Fig. 1.6 Possible origins of SARS-CoV-2. The coronavirus can transmit from natural reservoir to human through intermediate host. The possible routes are (**a**) bat to human through pangolin or (**b**) reassortment of coronaviruses from bat and pangolin into another intermediate host

host from bat to human (GISAID EpifluDB 2020). As a positive-sense RNA virus, the COVID-19 virus can mutate in humans for its adaptation. Although second closest relative, Guangdong-1/PS2 (pangolin precursors) has approximately 91% similarity with S protein of SARS-CoV-2, RBD of Guangdong-1/PS2 that is much closer to SARS-CoV-2 (99% identical) than Yunnan-RaTG13 Fig. 1.6 (Zhang et al. 2020; Cyranoski 2020; GISAID EpifluDB 2020). So, the probable pangolin origin of COVID-19 outbreak cannot be denied. Seemingly natural selection in the animal host before the zoonotic transfer or natural selection in humans following zoonotic transfer can be the reason of the origin of human SARS-CoV-2 infection (Andersen et al. 2020). Figure 1.6 shows the possible origin of the SARS-CoV-2. Additionally, these genomic comparisons suggest that the SARS-CoV-2 could result from recombination between Yunnan-RaTG13 and Guangdong-1/PS2 (pangolin precursors) viruses. Recombination two divergent positive-sense RNA viruses must have infected the same organism simultaneously (Barr and Fearns 2010; Cheng et al. 2007). The recombination mechanism had already been described to explain the origin of SARS-CoV (Graham and Baric 2010). Therefore, it is questionable in which organism did this recombination occur: bat, pangolin, or another species? Further research is needed for definitive conclusions. But two features of the SARS-CoV-2 virus – its distinct backbone and the mutations in the RBD of the S protein – rule out laboratory manipulation as a potential origin for SARS-CoV-2.

1.11 Promising Envelop Protein of SARS-CoV-2

Like in SARS-CoV, E protein could be involved in SARS-CoV-2 assembly, budding, and envelope formation (Alam et al. 2020). Interestingly no mutation has been observed so far among E protein of SARS-CoV-2 strains (Wang et al. 2020a). Similar to SARS-CoV, E protein of SARS-CoV-2 may form channels in ERGIC/Golgi membranes to permeabilize calcium ions to trigger NOD-like receptor 3 (NLRP3) inflammasome, leading to IL-1β overproduction (Nieto-Torres et al. 2015). E protein latches onto proteins that help turn host genes on and off. Ultimately, E protein has a significant role in the viral life cycle, which could be a promising target for vaccine development (Schoeman and Fielding 2019).

1.12 Nucleocapsid Protein of SARS-CoV-2

Nucleocapsid protein (N protein) protects the viral RNA by stabilizing it inside the virus (Chang et al. 2014). N protein induces apoptosis and inhibits type I interferon (IFN) production, cell cytokinesis, and proliferation (Zebin et al. 2020). N protein also regulates several pathways, for example, AP-1 signal transduction pathway, nuclear factor kappa B (NFκB) pathway, and transforming growth factor-beta signaling (Zebin et al. 2020).

1.13 Accessory Proteins of SARS-CoV-2 Virus

Accessory proteins modulate the environment in infected cells to make easier for viral replication. However, the roles of the accessory proteins (ORF3a, ORF6, ORF7a, ORF8, ORF9b, and ORF10) of SARS-CoV-2 in viral replication are yet to be known fully. KAUST metagenomic 154 analysis platform (KMAP, www.cbrc.kaust.edu.sa/kmap) has insight that accessory proteins of SARS-CoV-2 encoded by five gene clusters (ORF3, ORF6, ORF7, ORF8, ORF9, which are similar to those of other *beta coronaviruses*; Alam et al. 2020). Endoplasmic reticulum-induced ORF3a protein is thought to be responsible for apoptosis and suppression of type I (IFN) signaling through PERK pathway (Minakshi et al. 2009). Whereas ORF3a upregulates fibrinogen expression and activate NFκB and NLRP3 inflammosome to trigger inflammation (Siu et al. 2019; Minakshi et al. 2009).

ORF3a makes holes in the infected cell membrane for new viruses to escape out. Vesicle-bound ORF6 could play a vital role in the replication cycle of the virus (McBride and Fielding 2012). It also blocks some of the own virus-fighting proteins of the host cells. However, short truncated ORF6 of SARS-CoV-2 may not block signals that the infected cell would send out to the immune system (Lokugamage et al. 2020). Accessory protein ORF7a binds with lymphocyte function-associated antigen 1 (LFA-1), induces apoptosis, and activates NFκB and IL-8 promoter (McBride and Fielding 2012). When newly formed viruses escape a cell, the cell can plexus them with proteins called tetherin. Virus liberator ORF7a cuts down an infected cell's supply of tetherin to allow more of the viruses to escape. Studies showed that ORF8 of SARS-CoV-2 was distinct from other betacoronaviruses (Chan et al. 2020). Even intracellular stress pathways and NLRP3 inflammosome triggering the aggregation motif **VLVVL** of SARS-CoV ORF8b are absent in the novel coronavirus ORF8 (Chan et al. 2020). Alam et al. predict that secretory ORF8 could function as ligand-binding molecule (Alam et al. 2020). More research is needed to define the function of ORF8. Cellular protein Crm1 interacts with ORF9b encoded protein to induce apoptosis (McBride and Fielding 2012). ORF9b can block IFN. Unlike SARS-CoV, host immune response modulating ORF3b seems to be absent in SARS-CoV-2 due to overlap the same sequence of ORF3a. Multilocalized accessory protein encoded by ORF3b upregulates cytokines (except type I IFNs) and chemokines by modulating transcription factor RUNX1b, induces AP1 transcriptional activity through activation of JNK and ERK pathway, arrests cell growth in G0/G1 phase, and induces apoptosis and necrosis (McBride and Fielding 2012). Surprisingly, lacking of ORF3b doesn't affect much in the infective ability of the virus (McBride and Fielding 2012). ORF7b overlaps this same sequence of ORF7a in RNA genome, but it is not clear what, if anything, the gene does. ORF7b encoded protein has not been localized in the Golgi compartment of SARS-CoV-2 (Schaecher et al. 2007). Although ORF7b encoded nonstructural (type III integral transmembrane) protein is responsible for apoptosis, IFN-dependent reporter gene expression, and type I IFN response, but deletion of ORF7b enhances viral growth might explain the reason of more tissue damage by SARS-CoV-2 (Pfefferle et al. 2009). The nonconserved ORF7b in SARS-CoV-2 could affect infection property. But unique ORF10 could be an artifact annotation for SARS-CoV-2 (Alam et al. 2020).

1.14 Polarity of the RNA of SARS-CoV-2

SARS-CoV-2 belongs to group IV in Baltimore classification (Baltimore 1971). Positive-sense (5′–3′) viral RNA genome directly serves as messenger RNA, which is capable of translating into viral proteins (skipping transcription step) in the host cell during viral replication. Vulnerable SARS-CoV-2 RNA genome protects its genome integrity from environmental factors and host factors (attack by nucleases and other RNA-modifying enzymes that comprise the cellular intrinsic or innate immune response) through different mechanisms (Barr and Fearns 2010).

1.15 Conclusion

An outbreak of a novel, zoonotic coronavirus occurred in December 2019 in the city of Wuhan, China, and has now affected almost the entire world, with the maximum confirmed cases being 52,312,843 and 1,287,350 deaths as of 11 November 2020 (https://www.worldometers.info/coronavirus/). There have been rapid advances in what we know about the pathogen, how it infects cells and causes disease, and clinical characteristics of disease. Currently, no clini-

cal treatments or prevention strategies are available for any human coronavirus. Origin and structural biology will help the researchers to identify the mechanism of action of the virus in host and to drug design.

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Conflict of Interest The authors report no conflict of interest.

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2 The Molecular Virology of Coronaviruses with Special Reference to SARS-CoV-2

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Abstract

Introduction: Coronaviruses (CoVs) are large, enveloped and positive-sense RNA viruses which are responsible for a range of upper respiratory and digestive tract infections. Interest in coronaviruses has recently escalated due to the identification of a newly emerged coronavirus named severe acute respiratory syndrome 2 (SARS-CoV-2), which is the causative agent of the COVID-19 pandemic. In this chapter, we summarise molecular virological features of coronaviruses and understand their molecular mechanisms of replication in guiding the control of the global COVID-19 pandemic.

Methods: We applied a holistic and comparative approach to assess the current understanding of coronavirus molecular virology and identify research gaps among different human coronaviruses.

Results: Coronaviruses can utilise unique strategies that aid in their pathogenicity, replication and survival in multiple hosts. Replication of coronaviruses involves novel mechanisms such as ribosomal frameshifting and the synthesis of both genomic and sub-genomic RNAs. We summarised the key components in coronavirus molecular biology and molecular determinants of pathogenesis. Focusing largely on SARS-CoV-2 due to its current importance, this review explores the virology of recently emerged coronaviruses to gain an in-depth understanding of these infectious diseases.

Conclusions: The presented information provides fundamental bottlenecks to devise future disease control and management strategies to curtail the impact of coronaviruses in human populations.

2.1 Introduction

Coronaviruses are so named based on their crown-like structures from the appearance of envelope glycoproteins covering their surfaces, visible when observed under electron microscopy (Neuman et al. 2006). They are the largest group of viruses belonging to the *Nidovirales* order, which includes *Coronaviridae*, *Arteriviridae*, *Mesonivirdae* and *Roniviridae* families (Groneberg et al. 2005). The *Coronaviridae* family is further sub-divided into four different genera: *Alphacoronavirus, Betacoronavirus, Gammacoronavirus* and *Deltacoronavirus.* Originally, the viruses were divided into these genera based on serology but

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are now grouped based on their phylogenetic clustering (Ashour et al. 2020).

There are seven known types of human coronavirus. Common human coronaviruses that circulate in populations include the alpha coronaviruses such as human coronaviruses 229E and NL63, along with the beta coronaviruses, including human coronaviruses OC43 and HKU1 (Singhal 2020). Collectively, they are responsible for around 15% of all common cold infections worldwide. Other known coronaviruses have recently emerged via zoonotic transfer. This is when a coronavirus in animals evolves and mutates in a way that can then make people sick by becoming a new human coronavirus. Examples of this include the beta coronaviruses, severe acute respiratory syndrome 1 (SARS-CoV-1), the Middle East respiratory syndrome (MERS-CoV) and the recent novel coronavirus severe acute respiratory syndrome 2 (SARS-CoV-2) that causes COVID-19 disease. These three coronaviruses all share a common ancestor in bats.

SARS-CoV-1, MERS-CoV and SARS-CoV-2 all appear to have emerged from bat coronaviruses which entered the human population via intermediate hosts where the virus acquired advantageous mutations, allowing easy transmission to humans. Phylogenetic analysis showed that SARS-CoV-2 shares around 88–89% similarity to two bat-derived coronaviruses named bat-SL-CoVZC45 and bat-SLCoVZXC21 but remains more distantly related to SARS-CoV-1 (sharing 79% similarity) and MERS-CoV (less than 50% similarity) (Lu et al. 2020b). Further analysis has identified SARS-CoV-2 to share 98.7% nucleotide similarity to the partial RNA-dependent RNA polymerase (RdRp) gene of the bat coronavirus strain (bat-SL-CoVZC45) which circulates in the horseshoe bat *Rhinolophus sinicus* (Chen et al. 2020a). Based on phylogenetic analysis and genomic insights, it is likely that SARS-CoV-2 has originated from bats and has passed into humans via bat droppings associated with contaminated materials or via intermediate animal hosts in the wet market in Wuhan or its surrounding areas (Zhou et al. 2020b). Unlike SARS-CoV-2, the intermediate hosts for SARS-CoV-1 and MERS-CoV have been successfully identified as civet cats and camels, respectively. The SARS epidemic ended with around 8000 cases and 774 deaths, whereas the MERS outbreak had around 2500 cases and 858 deaths (Mahase 2020). The difference is, whilst the SARS epidemic ended, MERS infections still occur to this day from spillover events from camels straight into human populations. The COVID-19 pandemic has resulted in far more cases and deaths, and continues to cause much more damage than SARS and MERS combined.

Bats have been recognised as the natural reservoirs of a large variety of viruses, including over 500 coronaviruses found solely in horseshoe bats from China (Hu et al. 2017). Many of these bats in China have been found to harbour SARS-like coronaviruses that share similar viral proteins, such as S and N proteins with SARS-CoV-2 (Benvenuto et al. 2020). Phylogenetically, other coronaviruses related to MERS-CoV have also been found in many bat species (Cui et al. 2019). The current SARS-CoV-2 outbreak is therefore unlikely the last coronavirus outbreak that we will see, and bat coronaviruses will continue to jump into human populations in the future unless action is taken to prevent this. Investigating and understanding the bat origin of human coronaviruses is therefore essential in preventing and predicting future coronavirus outbreaks (Cui et al. 2019).

2.2 Why Is SARS-CoV-2 Proving More Difficult to Control than SARS-CoV-1?

There are many reasons as to why SARS-CoV-2 is proving much more difficult to control than the previous coronavirus outbreaks. First, the SARS-CoV-1 spillover animal reservoirs, mainly civet cats, were quickly identified in the outbreak (Shi and Hu 2008). Therefore, this animal was effectively culled, removing over 10,000 animals and was successful in breaking the chain of transmission into humans (Watts 2004). For SARS-CoV-2, despite bats being the likely reservoir hosts for the virus, their ecological separation from humans makes it likely that another species

has acted as an intermediate or amplifying host. The intermediate animal in question that allowed SARS-CoV-2 to acquire the necessary mutations for efficient human transmission remains largely unknown (Zhang and Holmes 2020). Therefore, culling cannot be used as a preventative measure to stop zoonotic transmission. Studies suggest that the pangolin coronavirus (pangolin-CoV-2020) is closely related to the SARS-CoV-2 receptor-binding domain (RBD); however, results do not support that SARS-CoV-2 emerged directly from the pangolin-CoV-2020 (Lam et al. 2020). Studies are ongoing to identify the intermediate animal source of the COVID-19 outbreak.

In addition to the intermediate host of SARS-CoV-2 transmission remaining a question, it has been identified that this coronavirus can not only be transmitted from human to human, but also from humans into animals. SARS-CoV-2 viral RNA has been detected in domesticated animals such as dogs, cats and minks (Shi et al. 2020). A large outbreak of coronavirus cases in minks occurred in Denmark, with some human cases confirmed to had contracted the virus directly from a mink. The country took action to cull these animals to prevent any further transmission into humans (Oreshkova et al. 2020). In a case from the Bronx Zoo in New York, a Malaysian Tiger was also found to contract SARS-CoV-2 as confirmed by RT-PCR by the National Veterinary Services Laboratories (NVSL) of the US Animal and Plant Health Inspection Service (APHIS) in Iowa (Leroy et al. 2020). Infection of three more tigers and three lions at the same zoo then quickly followed in succession to this case, also exhibiting signs of disease. It has been recognised that SARS-CoV-2 identified in these cats has not then spread to other animals or back into humans. Despite this, and the number of animal cases remaining minimal in comparison to human-to-human transmission, these observations provoked many violent reactions towards cats and dogs as potential reservoirs in the household (Leroy et al. 2020; Shi et al. 2020).

During the SARS-CoV-1 outbreak, a high incidence of cases was observed within a hospital setting among health-care workers, especially during the early stages of the outbreak. This was due to most cases first originating in hospitals where SARS patients were being treated (Webb et al. 2004). Throughout the whole period of the SARS epidemic, there were a series of infection control and protective measures implemented in the affected hospitals to prevent nosocomial infections. Measures such as avoiding face-to-face contact with SARS patients whilst caring for them and appropriate sterilisation of apparatus and equipment significantly reduced the risk to health-care workers contracting the virus. By reducing contact in the hospital setting, a barrier was formed between nurses and patients and therefore reduced transmission rates observed in health-care workers. In contrast, SARS-CoV-2 is transmitted by widespread community transmission. Therefore, it is proving extremely difficult to control and easily form a barrier to transmission between individuals as the virus is not largely in one given setting and has such high prevalence in populations.

The transmission time of the two seemingly similar coronaviruses has been found to vary. SARS-CoV-1 patients were found not to transmit the virus until 24–36 h after the initial onset of symptoms along with a lack of asymptomatic cases in the outbreak (Anderson et al. 2004). This permitted an efficient contact tracing of the virus, aiding in the prevention of its spread and mapping of the disease within populations (Klinkenberg et al. 2006). However, for the current SARS-CoV-2 outbreak, an abundance of asymptomatic and mild cases have been identified which poses a major issue in contact tracing as the asymptomatic carriers can transmit the virus to others whilst remaining unaware that they are infected with the virus themselves (Zhou et al. 2020a). Therefore, the tracing and mapping of affected individuals with SARS-CoV-2 is proving much more difficult to measure effectively and other methods to identify infected individuals need to be implemented such as effective diagnostics and mass testing.

SARS-CoV-2 has spread much faster than SARS-CoV-1, despite them sharing very similar genomes and viral structures. This information poses the question as to why SARS-CoV-2 has

spread much quicker. Research has shown that the SARS-CoV-2 spike (S) protein binds to its receptor angiotensin converting enzyme 2 (ACE2) much more tightly than SARS-CoV-1, exhibiting an affinity that is 10–20 times higher than SARS-CoV-1 (Wrapp et al. 2020; Shang et al. 2020). Therefore, the easier SARS-CoV-2 can bind to the cell, the easier it is for the virus to infect the cell. Further research into the structural comparison of the S proteins of both viruses and their individual interactions with the ACE2 receptor is required to gain an understanding as to how this may occur.

2.3 Genomic Organisation

Coronaviruses possess a helically symmetrical nucleocapsid which is notably uncommon for positive-sense RNA viruses; this contains their extremely large RNA genome that can be as great as 33.5 kilobases (kb) which comprises several open reading frames (ORF) (Kumar et al. 2020a) (Fig. 2.1a). Due to their high sequence similarity, the structure of SARS-CoV-2 appears the same as SARS-CoV-1 with no significant difference in their ORFs and non-structural proteins (nsps) (Kumar et al. 2020a). Coronavirus genomes contain a 5′ cap structure and 3′poly (A) tail which allows it to act as mRNA and hence be translated directly by replicase polyproteins (Fehr and Perlman 2015) (Fig. 2.1b). The first ORF that encodes the replicase proteins comprises two-thirds of the viral genome, with the accessory and structural proteins making up the remaining 13 ORFs. This ORF can be classified as ORF1a/b as it encodes the two large polyproteins that are proteolytically cleaved to generate nsps (nsp1–nsp16) found at the 5′ end of the viral RNA.

The 5′ end of the genome contains a leader sequence and an untranslated region (UTR) that contains structures necessary for replication and transcription of viral RNA (Fehr and Perlman 2015). At the beginning of each structural or accessory gene located at the 3′ end of the genome is a transcriptional regulatory sequence (TRS) that is needed for expression of these genes as they exist as sub-genomic RNAs (sgRNAs). The structures required for replication and synthesis of viral RNA are also located within the 3′ UTR. Coronavirus genomic structure is therefore organised as 5′-leader-UTR-replicase-S (Spike)-E

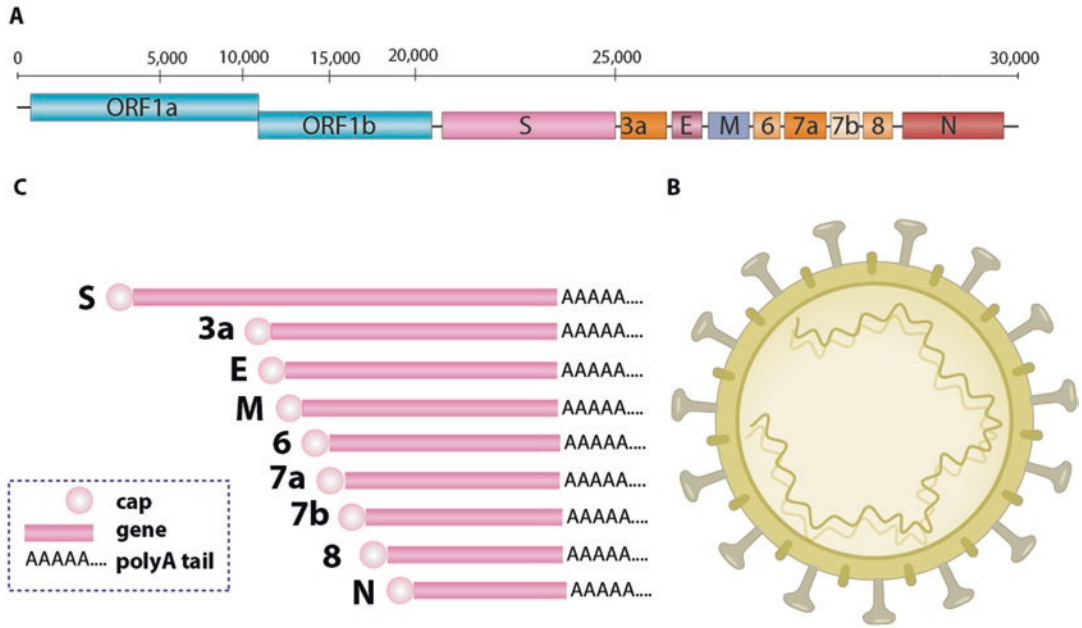

Fig. 2.1 Genome organisation (**a**), virus morphology and (**b**) transcripts for most prominent structural genes (**c**)

(Envelope)-M (Membrane)-N (Nucleocapsid)-3′UTR-poly(A) tail (Fig. 2.1c). Within the 3′ region are the structural and accessory genes existing as a nested set of sgRNAs (Fehr and Perlman 2015). Studies have shown that accessory proteins are non-essential for replication in tissue culture but have been proven vital in viral pathogenesis (Zhao et al. 2012; Liu et al. 2014).

2.4 Virion Morphology

The single-stranded positive-sense RNA genome of coronaviruses is contained within the virion core and is coated by nucleocapsid proteins (Fig. 2.1b). The nucleocapsid-associated RNA is encased within a lipid envelope, formed solely from lipids taken from host cells during the budding process. This envelope is studded with a range of viral glycoproteins which are responsible for attachment to the host cell and carry the main antigenic epitopes, particularly those recognised by neutralising antibodies. Coronavirus particles contain four main structural proteins – S, E, M and N – which are all encoded in the 3′ end of the viral genome and are essential in virion assembly and infection (Chen et al. 2020b). The S protein is the most prominent envelope glycoprotein that forms the club-shaped projections on the virion membrane that give the corona 'halo' appearance and are responsible for the attachment of the virus to host cell receptors (Beniac et al. 2006).

The S protein of SARS-CoV-2 demonstrates sequence conservation to related S proteins identified in other coronaviruses. The S protein is a trimeric class I fusion protein that facilitates attachment to host cell receptors (Bosch et al. 2003). When cleaved by a host cell furin-like protease, it can be divided into two polypeptides called S1 and S2, which make up a receptor binding domain (RBD) and a fusion domain, respectively (Abraham et al. 1990). The RBD is highly variable, which is common for viruses. This has arisen from increased evolutionary pressure on the protein due to contact with the immune system. The fusion domain is much more conserved, containing a fusion peptide which fuses the viral membrane to the host membrane upon infection and forms the stalk of the spike molecule (de Groot et al. 1987).

The E proteins of coronaviruses are highly divergent but share a common structure (Godet et al. 1992). Data suggest that the E protein is a transmembrane protein with an N-terminal ectodomain and a C-terminal endodomain. The role of the E protein is to mediate assembly and release of the virus but has been suggested to have other roles such as ion channel activity required for pathogenesis of coronaviruses such as SARS-CoV-2 (Nieto-Torres et al. 2014).

The M protein is a small protein that has three transmembrane domains and is the most common structural protein found in the virion. M proteins do not have a signal sequence despite being inserted into the endoplasmic reticulum (ER) membrane (Fehr and Perlman 2015). Studies suggested that the M protein exists as a dimer and therefore can assume two different conformations, hence allowing it to not only bind the nucleocapsid, but also cause membrane curvature (Neuman et al. 2011).

The N protein is the only protein located in the nucleocapsid of the virion. The N protein is composed of two domains: the N terminal domain (NTD) and the C terminal domain (CTD). Both domains bind RNA via different mechanisms (Hurst et al. 2009), but both are required for optimal RNA binding. Phosphorylation causes a structural change of the N protein which increases its affinity for viral RNA over non-viral RNA (Fehr and Perlman 2015). Two RNA substrates are associated with the N protein: the transcriptional regulatory sequence (TRS) and genomic packaging signal. The genomic packaging signal binds the CTD of the N protein (Kuo and Masters 2013). The N protein can also bind the M protein and nsp3 which is part of the replicase complex (Sturman et al. 1980). Interactions between these proteins likely aids in connecting the viral genome to the replicase-transcriptase complex (RTC) and hence packaging the genome into virions to be released.

The coronaviruses follow a sequential sequence of events to complete their replication cycle. These steps are discussed below and are outlined in Fig. 2.2.

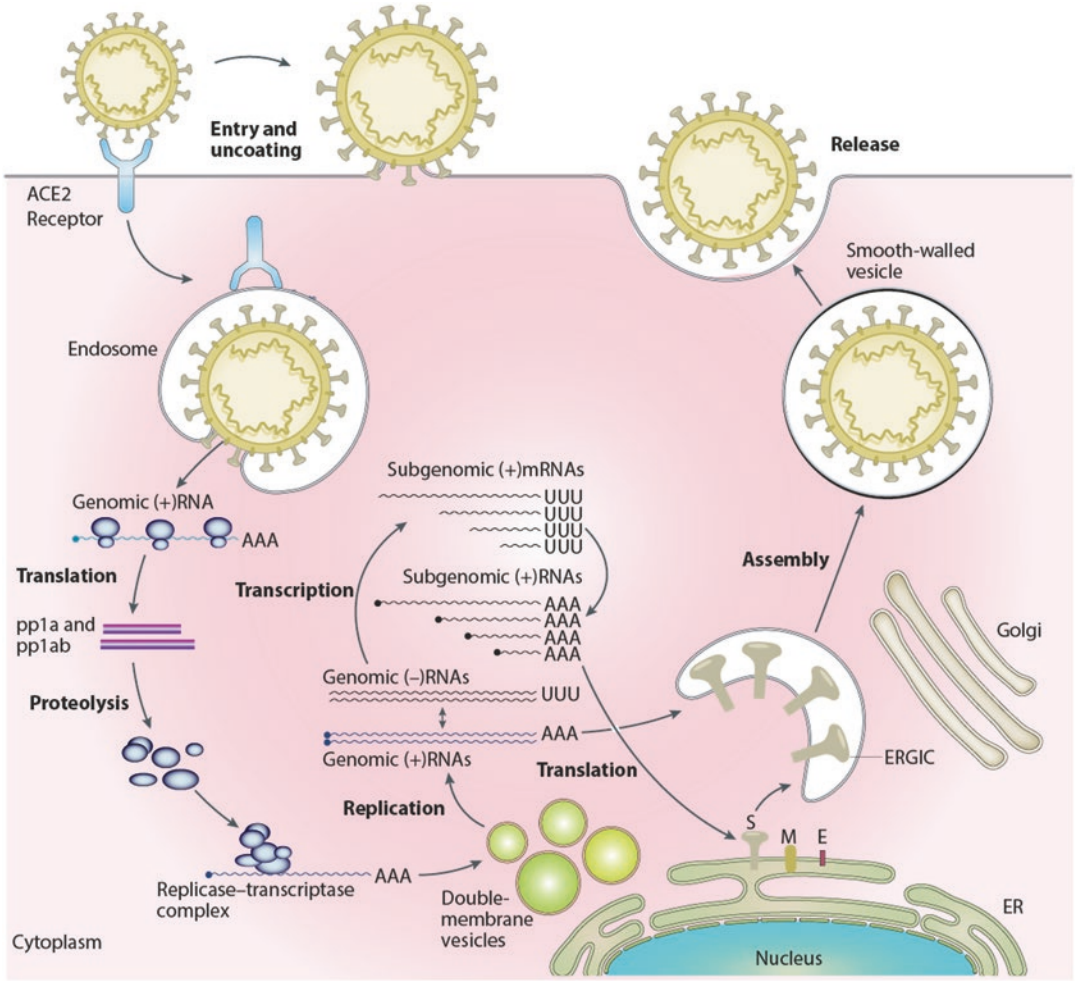

Fig. 2.2 Replication cycle of coronaviruses

2.5 Viral Attachment and Entry

Coronavirus entry into host cells is driven by interactions between the viral S glycoprotein and its cellular receptor which requires priming by host cell proteases (Kumar et al. 2020b). This method of entry is generally highly species specific; however, the adaptation of SARS-CoV-1 and SARS-CoV-2 to enter the human population has contradicted this belief (Masters 2006; Hoffmann et al. 2020). ACE2 is the receptor for both SARS-CoV-1 and SARS-CoV-2 entry, whereas MERS-CoV uses the cellular receptor dipeptidyl peptidase 4 (DPP4). Focusing here on SARS-CoV-1 and SARS-CoV-2, cell entry is dependent upon the binding of the surface unit of the S protein known as S1 which contains the RBD. Binding of this to the host cellular receptor ACE2 facilitates the attachment of the virus to the surface of target cells. After receptor binding, the virus needs to gain access to the cytosol of the host cell (Fig. 2.2). This is achieved via proteolytic cleavage of the S protein by cellular proteases such as the serine protease TMPRSS2 (Hoffmann et al. 2020).

Priming involves cleavage of the S protein at two sites in the S2 portion of the protein. The first cleavage is important to separate the RBD and fusion domains of the S protein (Belouzard et al. 2009). The second cleavage at the S2′ site exposes

the fusion peptide. Fusion of viral and cellular membranes can then occur, as conformation of the virus changes and the S2 subunit pushes up the fusion domain to come into contact with the host cell membrane, allowing the virus to enter the host cell cytosol (Fehr and Perlman 2015). Studies suggest that the S protein of SARS-CoV-2 differs from that of SARS-CoV-1. SARS-CoV-1 has six amino acids critical in the receptor binding domain with the ACE2 receptor, but studies have shown that five of these amino acids differ in the S protein of SARS-CoV-2 (Yi et al. 2020, Coutard et al. 2020). In addition, SARS-CoV-2 has acquired a polybasic cleavage site that is predicted to enable cleavage via other cellular proteases, not solely TMPRSS2, which increases the transmissibility of the virus (Qiao and Olvera de la Cruz 2020).

Following binding and conformational changes to the S protein, fusion of the viral envelope protein with host cell membranes allows entry into the cell (Coutard et al. 2020). Fusion generally occurs at acidified endosome membranes, but some coronaviruses can also fuse at the plasma membrane. After membrane fusion, the viral RNA genome is released into the cytoplasm of the host cell and the RNA becomes uncoated to allow translation to generate two polyproteins (pp1ab) and the transcription of both genomic and sgRNAs by discontinuous transcription (Kumar et al. 2020b).

2.6 Viral Replication and Transcription

As mentioned earlier, coronaviruses within the order *Nidovirales* share similar genomes in which two-thirds of their viral RNA is composed of the ORF1a/b; this is translated into two large polyproteins to yield 16 nsps which constitute the RTC. The remainder of their genome is transcribed as a nested set of sgRNAs to produce structural and accessory proteins (Fehr and Perlman 2015).

Upon entry into the host cell, ribosomes recognise the positive-sense coronavirus RNA and translate one ORF into a protein before recycling it. Within the ORF1-encoded large polyprotein are two viral proteases whose role is to rearrange membranes taken from the rough endoplasmic reticulum (RER) of host cells to form double-membrane vesicles and the RTCs required for viral replication and transcription to occur (Fig. 2.2). Around 60% of the time, the host ribosome will read through the RNA and reach a stop codon at the end of ORF1a and will stop translating the RNA. This poses the question, how can all 27 proteins be expressed from just a single strand of RNA? It appears that the coronavirus has evolved to solve this problem, whilst still obeying the eukaryotic rules of gene expression.

Coronaviruses have evolved a unique mechanism to ensure translation of the whole replicase gene within ORF1 can occur as it encodes two large ORFs (rep1a and rep1b) which express the two polyproteins (pp1a and pp1b). In order to express both polyproteins, when the ribosome reaches a stop codon at the end of ORF1a, a frameshift event can occur, and instead of halting, the ribosome will continue to read the RNA to generate an ORF1a/b fusion. This can take place because the virus utilises a slippery sequence (5′-UUAAAC-3′) near the stop codon (Baranov et al. 2005). When the ribosome reaches this, it tends to slip out of the (rep1a) reading frame and switches to the (rep1b) ORF. This occurs at a higher frequency in coronaviruses in comparison to other viruses, because downstream of this slippery sequence near the ORF1a/b junction is an RNA pseudoknot structure that causes the ribosome to pause over the slippery sequence and changes the reading frame by moving back by one nucleotide. This occurs before the ribosome ultimately melts the pseudoknot structure and extends translation into rep1b, resulting in the translation of pp1ab (Brierley et al. 1989). It is unknown exactly why coronaviruses utilise frameshifting to control their protein expression, but it has been hypothesised that this is to control the ratio of rep1a and rep1b proteins (Fehr and Perlman 2015). Another possible theory is to delay the production of rep1b until the products of rep1a have created the environment necessary for replication of the viral RNA (Araki et al. 2010).

The two replicase polyproteins pp1ab yielded are subsequently cleaved by proteolysis to produce the individual nsps 1-11 and 1-16, respectively, which are important in the unusually complex replication/transcription machinery of coronaviruses (Ziebuhr et al. 2000). Included within this are a 3C-like proteinase, an RdRp and an ATPase/helicase in addition to nsps with various other functions (Ziebuhr et al. 2000). These proteins assemble into a multi-subunit polymerase complex, known as the RTC, which creates an environment that is suitable for RNA synthesis, to mediate the transcription and replication of the viral genome (Fehr and Perlman 2015).

2.6.1 The Role of Replicase-Transcription Complexes (RTCs)

Coronavirus RNA synthesis follows the translation and assembly of the viral RTCs. It has been recognised that for positive-sense RNA viruses RTCs are invariably associated with double-membrane structures that are induced by the virus (van Hemert et al. 2008). The double membranes formed by the virus are derived from the RER and are interconnected by their outer membrane which is continuous with the RER itself. The role of these membranes remains poorly characterised but is thought to protect the viral RNA from the immune system of the host cell and from attack from exonucleases present in the host cell cytoplasm. They also serve as a basis for the synthesis of RNA and facilitate the concentration and compartmentalisation of viral macromolecules and factors needed for replication and transcription in a specified membrane space (van Hemert et al. 2008). Membrane-bound RTCs are hence central to driving RNA synthesis. Therefore, gaining a better understanding of their structure and function is vital in identifying the biochemistry of coronavirus replication and could also prove valuable in developing antiviral approaches.

RTCs consist of a central RdRp enzyme in addition to other virus-encoded enzymes and host factors essential in driving the synthesis of the viral RNA. The replicase holoenzyme complex requires the integration of an RNA polymerase enzyme in addition to exonuclease and capping activity. Nsp12 acts as the catalytic subunit that possesses RdRp activity and is the central enzyme of the RTC (Ahn et al. 2012). Research by Subissi et al. (2014) has identified that the RdRp (nsp12) needs to associate with nsp7 and nsp8 to activate its ability to replicate long stretches of RNA. This is because nsp7 and nsp8 are understood to act as processivity clamps for the RNA polymerase (Zhai et al. 2005). The nsp12-nsp8-nsp7 subcomplex is well defined as the minimal core component compulsory for facilitating coronavirus RNA synthesis (Peng et al. 2020). For complete transcription and replication of the RNA genome, many other nsps are required to assemble into a holoenzyme complex including nsp13, which encodes the RNA helicase domain, and nsp14, which encodes the exoribonuclease (ExoN), thought to confer a proofreading function on the RdRp and possesses capping activity (Fehr and Perlman 2015; Ivanov et al. 2004). In addition, nsp10 and nsp16 also comprise the holoenzyme, but their exact functions for RNA synthesis currently remain poorly defined (Ziebuhr, 2005). The components of this holoenzyme complex are associated via protein–protein interactions.

Integral membrane replicase proteins function in vesicle biogenesis and the recruitment of the factors necessary for viral transcription and amplification. Three proteins predicted to possess transmembrane components are nsp3, nsp4 and nsp6, which are involved in vesicle formation and modifying cellular endomembranes whilst directing the RTC to this scaffold (Kanjanahaluethai et al. 2007; Oostra et al. 2007). Nsp3 and nsp4 when expressed alone drive the formation of double membranes which involves the interaction of luminal loops of these proteins that is essential in causing the curvature of the RER membrane (Hagemeijer et al. 2014).

Research by V'Kovski et al. (2019) aimed to identify the full breadth of proteome components associated with these viral RTCs by using murine coronavirus (MHV) proximity labelling. Results

showed that interestingly, in addition to the recruitment of transcription machinery, translation machinery was also detected in close proximity to the viral RTC. This highlighted the critical participation of translation initiation factors during coronavirus replication (V'Kovski et al. 2019). The viral proteins present in RTCs are largely non-structural and instead have roles in replication and transcription (nsp2–10 and nsp12–16). Finding of studies conducted by V'Kovski et al. (2019) have identified that nsp1, which has been previously identified as a key pathogenicity factor found in coronaviruses, was not present in the MHV RTC proteome. This is because nsp1 is rapidly cleaved from the polyproteins pp1ab as it is not an integral component of coronavirus RTCs (Huang et al. 2011). Nsp1 has instead been found to interfere with host cell translation by inducing the degradation of cellular mRNAs (Lokugamage et al. 2015). Nsp1 is specific for host mRNA as it recognises the 5′ sequence on viral RNA and hence does not cleave it and instead targets host mRNA that lacks this distinct sequence. Therefore, nsp1 restricts host cell gene expression by encouraging host mRNA degradation which aids in the virus's ability to shunt gene expression away from the host and direct it towards the virus as an immune evasion tactic. By degrading host mRNA and hence suppressing host gene expression, the generation of proteins involved in the innate immune response, including those composing the interferon (IFN) response, is also suppressed (Kamitani et al. 2006). This is advantageous to the coronavirus in delaying the IFN response generated towards the virus as a successful immune-evasion strategy.

In addition to nsp1, several assembly virion proteins and structural proteins such as S, M and E proteins were also identified to be absent in RTCs of the MHV (V'Kovski et al. 2019). The reason behind their absence from the RTC is that viral assembly does not occur in RTCs and hence these proteins play roles elsewhere in the replication cycle of the virus. Accessory proteins and structural proteins that are not found in the RTC are usually specific to different viral species but are frequently dispensable in viral replication in tissue culture cells, yet they play key roles in viral–host interaction *in vivo*. SARS-CoV-1 and SARS-CoV-2 share similar accessory proteins but have notable variations, particularly those involved in the interaction with the innate immune response, perhaps in counteracting the IFN response (Liu et al. 2014).

2.6.2 Discontinuous Transcription

Viral RNA synthesis produces both genomic and sgRNAs, the latter of which serve as mRNAs for the structural genes, S, E, M and N, along with several accessory genes which are all positioned at the 3′ end of the RNA. These genes exist as a nested set of sgRNAs so that all of the positive-sense sgRNAs are 3′ co-terminal with the full-length viral genome (Fehr and Perlman 2015). The use of sgRNAs is a property unique to the order *Nidovirales,* and this method allows both genomic and sgRNAs to be transcribed from a mirror set of sub-genomic negative-strand intermediates sharing a common, short 5' leader sequence that has been derived from the 5′ end of genomic RNA (van Vliet et al. 2002). The joining of 'leader' and 'body' sequences occurs during negative RNA strand synthesis. Joining the leader and mRNA sequences is the TRS which precedes each transcription unit and is located just downstream of the genomic leader sequence (Lai and Cavanagh 1997; van Vliet et al. 2002). The mechanism by which the 'leader' and 'body' TRS segments fuse during production of sgRNAs appears exclusive to coronavirus replication.

As previously mentioned, the viral enzyme complex of coronaviruses contains RdRp as the core catalytic subunit that is used to produce negative-strand RNA. During negative-strand synthesis beginning at the 3′ end of the genome, the RdRp moves along copying the genome and pauses at any one of the TRS sequences in the body of the genome (TRS-B). After this, the RdRp either continues elongation onto the next TRS or alternatively switches the template to amplify the leader sequence at the 5′ end of the genome, which is directed by complementarity of the TRS-B to the leader TRS-L (Fehr and Perlman, 2015). This occurs by a process known

as discontinuous transcription and results in the generation of negative sense sgRNAs which are then used as a template by the same RdRp to produce positive-sense sgRNAs (Fig. 2.2). Despite the sgRNAs consisting of numerous ORFs, it is only the first ORF from their 5′ end that gets translated into the individual viral protein (Jain et al. 2020; Sola et al. 2015). During the synthesis of negative-strand RNA, leader–body fusion occurs (van Vliet et al. 2002). Evidence supporting this method includes the presence of anti-leader sequences located at the 3′ end of negative-strand sgRNAs (Sethna et al. 1991).

Coronaviruses are able to utilise both homologous and nonhomologous recombination due to the ability of RdRp to switch strands (Lai et al. 1985). It is believed the negative-strand intermediates produced from discontinuous transcription participate in RNA recombination via a copy-choice mechanism (Keck et al. 1987). Discontinuous transcription results in lots of promiscuous polymerase jumping and facilitates an extraordinarily high recombination rate in coronaviruses of around 25% (Irigoyen et al. 2016). Most positive-sense RNA viruses have low recombination rates, so this appears to be a unique feature found only in coronaviruses which may prove significant in discovering how coronaviruses have evolved to maintain such a large genome. Following viral replication and sgRNA synthesis, the structural proteins S, E and M are translated and inserted into the endoplasmic reticulum (ER) (Fehr and Perlman 2015) (Fig. 2.2).

2.7 Viral Assembly and Release

The coronavirus structural proteins including S, E and M are inserted into the ER and move along a secretory pathway into the endoplasmic reticulum–Golgi intermediate compartment (ERGIC) (Krijnse-Locker et al. 1994) (Fig. 2.2). Within the ERGIC, structural proteins combine with viral genomes encapsulated by the N protein and bud into membranes of the ERGIC (de Haan and Rottier 2005). Studies have shown that fusion of encapsulated genomes within the ERGIC space enhances viral envelopment (Siu et al. 2008). The M protein is crucial in directing the protein interactions within the ERGIC necessary for coronavirus assembly. However, for this to be effective, the M protein must be expressed alongside the E protein so that virus-like particles (VLPs) are formed. Evidence suggests that this is why these two proteins fuse together to form the coronavirus envelopes, yet the role of the E protein in virion assembly remains unknown (Bos et al. 1996). The S protein is then incorporated into the virions, and its ability to traffic to the ERGIC to interact with M protein is essential for its inclusion within the virion (Fehr and Perlman 2015). The M protein also binds the nucleocapsid, and it is this interaction that promotes the completion of virion assembly (Hurst et al. 2005). Once assembled, the mature virions are transported to the plasma membrane via smooth-walled vesicles thought to be derived from Golgi sacs, but this remains poorly defined. The vesicles fuse with the plasma membrane at the cell surface and the virions are released by exocytosis (Fig. 2.2).

2.8 Pathogenesis

SARS-CoV-2, like other coronaviruses, largely targets and effects cells located in the upper respiratory tract. Infection can be mild, with some individuals unaware that they are infected with the virus at all, whereas other cases can be much more severe. In severe cases, infected individuals may exhibit symptoms such as coughing and chest pain, fever, weakness and muscle pain, which are all signs of an upper respiratory tract infection (Panahi et al. 2020). Notably, some affected individuals also lose their sense of taste and smell after contracting the virus, which for some does not return for a long time (Gautier and Ravussin, 2020). As some serious cases progress, patients may develop pneumonia, suffer hypoxaemia and possibly even respiratory failure which can lead to death. In around 80% of cases, the affected individual is either asymptomatic or only exhibits a mild to moderate form of the disease, whereas 20% will develop more serious

symptoms that require medical attention and hospital admission with 5% of these requiring ventilation support via an intensive care unit (ICU) (Wu and McGoogan 2020). Domingo et al. (2020) effectively summarise the pathogenic processes of SARS-CoV-2 as existing as four feedback loops that are chained or occurring simultaneously, suitably labelled the 'four horsemen' of viral invasion. They describe these feedback loops as being the viral loop, the hyperinflammatory loop, the hypercoagulation loop and the non-canonical renin–angiotensin system (RAS) axis loop, respectively. The viral feedback loop includes the evasion of host immune responses allowing uncontrolled viral replication which in turn activates a hyperactive form of the adaptive immune response. The inflammatory loop involves the inflammatory response of the host causing a cytokine storm and also leaves the lungs without a critical defence mechanism as the inflammatory side of RAS is induced. The coagulation loop is described by Domingo et al. (2020) as being a hypercoagulable state caused by inflammation and coagulation in a continuous feedback loop. The result of these four feedback loops on the infected individual is a hypercoagulable and hyperinflammatory state that results in immune-mediated lung damage and ultimately respiratory distress syndrome.

2.9 Immune Interactions

The IFN response is a key arm of the innate immune system. Interestingly, it has been shown that SARS-CoV-1 and MERS-CoV induce very little, if any, IFN response in most cells (Spiegel et al. 2005); however, it is currently unknown if SARS-CoV-2 follows this trend. Many putative IFN antagonists have been identified in the SARS-CoV-1 genome, such as nsp1, 3, 7, 15 and 16, ORF3a, -3b and -6 that act as accessory proteins and the structural proteins M and N (Konno et al. 2020, Lu et al. 2011). This virus and possibly SARS-CoV-2 possess a multi-pronged approach that aims to dampen the early IFN response to the virus, which is key in viral pathogenesis.

SARS-CoV-1 has been linked to delayed type I IFN signalling and therefore immune toxicity. An experiment was undertaken using knockout mice lacking an IFN-α receptor that were then infected with the virus. Results showed that all mice lacking the receptor and infected with SARS-CoV-1 survived, whilst 80% of the wild-type mice died. These results indicate that the IFN response is linked directly to disease pathogenesis (Channappanavar et al. 2016). SARS-CoV-1 was found to replicate to higher initial titres due to a delayed IFN response which would otherwise attack the virus at an earlier stage. As the IFN response is delayed, it has been proven that it cannot prevent the initial bouts of viral replication. However, since it drives aberrant recruitment of pathogenic inflammatory monocyte macrophages (IMMs), it results in the activation of the innate immune response which can subsequently lead to cell cytotoxicity (Jain et al. 2020).

A dysregulated innate immune response is therefore a leading contributor to coronavirus-mediated pathology as it results in rapid viral replication, inflammatory cell infiltration and the production of proinflammatory cytokine and chemokines from infected cells as well as from the infiltrating cells. Generated immune responses pose a threat to the host's own cells, for example, those located in the alveoli. Inflammatory cell infiltration can cause damage to lung tissue, the deterioration of lung function and could result in lung failure and respiratory distress syndrome in some cases (Gralinski and Baric 2015). Studies on patients who have recovered from SARS-CoV-1 have shown that both neutralising antibody titres and B cell responses against the coronavirus were low and short-lived (Tang et al. 2011). Therefore, it has been predicted that after a few years post infection most individuals would not mount a sufficient protective response against the virus. It is currently unknown how the immune response against SARS-CoV-2 is generated, how effective it is and how long it remains. Therefore, this poses the question that if this is the case, what implications does this have for the circulation of the virus and possible vaccine strategies? Due to the damaging inflammatory responses generated towards the infiltrating virus

causing harm to the host, immunotherapy directed towards these responses may prove an effective treatment strategy.

2.10 Treatment and Drug Targets

There is currently no specific antiviral drug or vaccine for the treatment of coronavirus pathologies, including the current SARS-CoV-2 (Cascella et al. 2020). However, numerous vaccines using varied approaches are being generated and are in different stages of testing as of November 2020. Most treatments for the virus are focused on the management of symptoms of infection and supportive therapy. Non-invasive (NIV) and invasive mechanical ventilation (IMV) has proved useful in cases of respiratory failure in coronavirus patients. Intensive care is required for the most severe and complicated cases of the disease (Cascella et al. 2020). Despite there being no antiviral treatments currently approved for SARS-CoV-2, many approaches have been explored and proposed. For example, the use and effectiveness of lopinavir–ritonavir has been tested on SARS-CoV-2 patients but to little success as no benefit was observed with this treatment in comparison to the standard care currently being administered (Cao et al. 2020). Chloroquine immunomodulatory therapy has also been proposed as an antiviral strategy to combat coronavirus infection. A trial by Gautret et al. (2020) presented that use of hydroxychloroquine was positively associated with viral load reduction and disappearance. However, the use of this drug is linked to many dangerous potential side effects including a higher risk of cardiac arrhythmias (Mercuro et al. 2020). A therapeutic approach using the combined use of IFN-α2b and ribavirin has also been tested for its effectiveness against SARS-CoV-2 and when administered was identified as a potential treatment option if used early in the course of infection, but still warrants further testing (Falzarano et al. 2013; Zhou et al. 2020c).

A review by Prajapat et al. (2020) has recently discussed many possible protein structures that could act as potential targets for the development of an effective therapeutic approach against coronaviruses. Among these possible approaches are protein targets belonging to the N protein, proteases and S protein of the coronavirus. The S and N proteins of SARS-CoV-2 are widely considered as the most promising candidates for vaccines (Prajapat et al. 2020).

2.10.1 Targeting the S Protein of SARS-CoV-2 and Other Potential Vaccine Strategies

The destruction of the S protein of SARS-CoV-2 would prevent its attachment to host cell membrane and therefore poses a promising vaccine strategy. Alternatively, a vaccine that can safely introduce the S protein of SARS-CoV-2 to prime an individual's immune system to respond to the S protein of the actual virus if they later become infected. A study by Zhang et al. (2020) mapped the immunodominant sites of S and N proteins by using samples collected from recently discharged COVID-19 patients. SARS-CoV-2 N protein–specific antibody levels in the sera of patients correlated with the neutralising antibody titres; however, not all sera contained antibodies specific for the RBD fragment of the S protein. The high immunogenicity of SARS-CoV-2 N protein during infection proved successful in revealing its potential as an antigen for the development of therapeutic strategies, but in this instance the effectiveness of targeting the S protein needed further investigations (Zhang et al. 2020).

Many monoclonal human antibodies have been previously identified to bind to the RBD of SARS-CoV-1 and hence prevent its attachment to ACE2 and avert *in vitro* replication. Learning from past experiences, monoclonal antibodies are now recognised as potential antivirals (Lu et al. 2020a). However, despite their similarity, these ACE2-blocking antibodies do not bind the RBD of SARS-CoV-2 (Wrapp et al. 2020). Therefore, the investigation of another potential monoclonal antibody (CR3022) has been conducted that is directed explicitly against SARS-CoV-1, allosterically blocking viral binding to

ACE2 and inhibiting cell entry (Huo et al. 2020). The CR3022 also possesses the ability to cross-react with the RBD of SARS-CoV-2. This study identified that CR3022 does not block the RBD of SARS-CoV-2, but it is successful in neutralising the virus because it is non-competitive with the ACE2 receptor site. The binding of CR3022 to the pre-fusion state of the S protein makes it unstable and results in cleaving and formation of post-fusion state. Therefore, the CR3022 destabilises the S protein before fusion, cleaving the S1 subunit and resulting in virus inactivation (Huo et al. 2020). For success of this method, the antibody-bound virus must be incubated before the cytopathic effect develops for effective neutralisation. A synergistic approach using an epitope similar to CR3022 with a higher affinity for the S protein combined with antibodies that efficiently bind the RBD may prove successful in preventing viral entry. Moreover, a vaccine targeted against the RBD of the S protein of SARS-CoV2 may reduce the range of immune responses directed towards viral entry and thereby diminish the immune pathology observed in patients (Dandekar and Perlman 2005; Tseng et al. 2012).

2.11 Conclusions

The observation of SARS-CoV-2 exhibiting a high level of homology to SARS-CoV-1 has allowed researchers to utilise this virus as a basis to gain an understanding of many components of the molecular virology of the new coronavirus. Despite this, many key aspects and components of the novel coronavirus SARS-CoV-2 remain unknown. Further research into the molecular virology of SARS-CoV-2 is essential to gain an understanding of key aspects of the novel coronavirus, such as the role of the polybasic site on the S protein in transmission of the virus and the pathways that are induced by membrane remodelling for RTCs. The biochemical activities and roles of various proteins within the RTC also remain unknown. Therefore, studies must be completed to determine how the virus efficiently coordinates genome replication and transcription. Although there have been suggestions as to why coronaviruses have such a large genome, it remains unproven as to why they possess this feature and how they are able to retain sufficient mutation rates that allow for adaptation and trans-species spillover. Other aspects such as determination of the roles of accessory proteins in the coronavirus genome need to be investigated and their impact on the *in vivo* growth and virulence of the virus needs to be assessed. Defining the exact mechanisms of coronavirus pathology and how it interacts with the host's immunopathological responses is required to improve vaccine strategies and aid in efficiently controlling the current pandemic.

On a larger scale, it is essential that studies are carried out to determine the effect of possible vaccine strategies that could be implemented against the virus. It is unknown whether infected individuals themselves or vaccines containing parts of the virus result in mounting long-term protective immune responses. It is also speculated what type of immunity would be gained from vaccines and whether people can become reinfected with the same strain of the SARS-CoV-2 virus. There is a desperate requirement for more information from elderly patients on their immunology and inflammatory responses to aid in identifying what parallels should be examined in animal models for vaccine testing.Compliance with Ethical Standards

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3 Epidemiology, Transmission, and Molecular Immunopathology of SARS-CoV-2

Rahul Mallick and Asim K. Duttaroy

Abstract

Introduction: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-associated coronavirus pandemic has posed a global health emergency.

Methods: This chapter focuses on the epidemiology and transmission immunopathology of SARS-CoV-2 infection based on the available data on SARS-CoV-2 and other coronaviruses.

Results: The virus is transmitted by inhalation or contact with infected droplets, and the incubation period ranges from 2 to 14 days. The case fatality rate is estimated at 6%. Following binding with cell surface angiotensin-converting enzyme 2 (ACE2) receptor, the SARS-CoV-2 enters the host cell and replicates by using host machinery to cause disease.

Conclusions: Cytokine storm due to COVID-19 has challenged the treatment outcome.

Keywords

ACE2 · COVID-19 · Cytokines · Epidemiology · SARS-CoV-2 · Spike protein transmission

Abbreviations

2019-nCoV	2019-novel coronavirus
ACE2	Angiotensin-converting enzyme 2
ARDS	Acute respiratory distress syndrome
COVID-19	Coronavirus disease 2019
E	Envelope protein
FAO	Food and Agriculture Organization of the United Nations
HLA	Human leukocyte antigen
IFN	Interferon
IRF	Interferon regulatory transcription factor
M	Membrane protein
MERS-CoV	Middle East respiratory syndrome-related coronavirus
MHC	Major histocompatibility complex
N	Nucleocapsid protein
NK	Natural killer
NLRP	NOD-like receptor
NSP	Nonstructural protein

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OIE	World Organisation for Animal Health
ORF	Open reading frame
$PI(3,5)P_2$	Phosphatidylinositol-3,5-bisphosphate
RBD	Receptor binding domain
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I-like receptor
S	Spike protein
SARS-CoV	Severe acute respiratory syndrome-related coronavirus
SARS-CoV-2	Severe acute respiratory syndrome-related coronavirus 2
TLR	Toll-like receptor
TPC2	Two pore channel subtype 2
WHO	World Health Organization

3.1 Introduction

A recent rapid outbreak of infection around the globe was caused by a novel coronavirus, which was first identified in December 2019 in Wuhan City of Hubei Province, People's Republic of China. Later it was declared a pandemic on March 11, 2020, by the World Health Organization (WHO) (Corman et al. 2020; D. Wang et al. 2020a, b; (WHO) (Press release) 2020). According to the guidelines of the World Organisation for Animal Health (OIE) and the Food and Agriculture Organization of the United Nations (FAO), the infectious disease was officially announced as coronavirus disease 2019 (COVID-19) on February 11, 2020, by WHO (2020). The disease was previously known as a 2019-novel coronavirus (2019-nCoV), Wuhan flu, and Wuhan pneumonia (Zhou et al. 2020; Stobbe 2020; Campbell 2020). COVID-19 is caused by severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) (WHO 2020). Within two decades, COVID-19 is the third outbreak of highly contagious coronavirus in humans, followed by a severe acute respiratory syndrome-related coronavirus (SARS-CoV) in 2002–2003 and the Middle East respiratory syndrome-related coronavirus (MERS-CoV) in 2012 (De Wit et al. 2016). More than 5 million people from 210 countries and territories along with 2 international conveyance were affected by SARS-CoV-2 as of May 30, 2020 (https://www.worldometers.info/coronavirus/). The case fatality rate for COVID-19 has been estimated at around 6% so far (https://www.worldometers.info/coronavirus/). This chapter focuses on the epidemiology and transmission immunopathology of SARS-CoV-2 infection, based on the available data on SARS-CoV-2 and other coronaviruses.

3.2 Epidemiology of COVID-19

Following the detection of the first case in mid-November 2019, SARS-CoV-2 had spread through human-to-human transmission in almost 210 countries and territories within 5 months (https://www.worldometers.info/coronavirus/; Chan et al. 2020a, b; Ma 2020). As the epidemic spreads due to the gathering of people and/or travel to other areas, travel restrictions will help to manage the epidemic (Kucharski et al. 2020). Also, 60–70% population would need to be infected for getting herd immunity against COVID-19 (BBC 2020a; Lintern 2020). The estimated time from developing symptoms and death is 6–41 days with a median of 14 days (Rothan and Byrareddy 2020). Also, 80% of COVID-19-infected people who died were aged ≥60 years, and 75% of the patients had comorbid health conditions (BBC 2020b). People under 18 years of age are at lower risk of COVID-19 (Aylward, Bruce (WHO); Liang 2020). The studies found no sexual transmission through vaginal route and no vertical transmission of COVID-19 (P. Cui et al. 2020; H. Chen et al. 2020). The extent of genome mutation and herd immunity will control the virulence (Saplakoglu 2020).

3.3 Reservoir of SARS-CoV-2

The bat is thought to be the primary reservoir for all coronavirus-related human outbreaks (Cui et al. 2019). Although WHO considers the bat to be the natural reservoir, the original source of

COVID-19 is still a mystery (Cohen 2020; Aylward, Bruce (WHO); Liang 2020). SARS-CoV-2 shares 96% of its genome with RaTG13 from *Rhinolophus affinis* from Yunnan Province, China (Zhou et al. 2020). But Texas-based scientists claim reassortment in coronaviruses and engagement of Malayan pangolins in SARS-CoV-2 virus origin (Wong et al. 2020). Although it is hard to assure that pangolins would be the intermediate host (Cyranoski 2020).

3.4 Routes of Transmission of SARS-CoV-2

Transmission of COVID-19 occurs via respiratory droplets (within the range of approximately 27 feet) and fomites during unprotected contact between the infector and the infectee (Centers for Disease Control and Prevention 2020; Bourouiba 2020; Aylward, Bruce (WHO); Liang 2020). Although the possibility of airborne transmission cannot be ruled out (Morawska and Cao 2020; Santarpia et al. 2020). Still, it is not known how much concentration of viable SARS-CoV-2 virus is needed to infect a human being. Van Doremalen et al. showed the viability of SARS-CoV-2 virus in the air (up to 3 hours), copper (4 hours), cupboard (24 hours), and plastic and stainless steel (up to 3 days) (van Doremalen et al. 2020). In another study, Chin et al. could not detect viable infectious SARS-CoV-2 virus from printing and tissue paper after 3 hours, wood or cloths on day 2, and glass or banknote on day 4, but infectious SARS-CoV-2 was detectable on the outer surface of the surgical mask on day 7 (Chin et al. 2020). A recent study suggested that COVID-19 transmission would be reduced due to high temperature and high humidity (Wang et al. 2020a, b). SARS-CoV-2 is extremely stable at 4 °C, but at 70 °C SARS-CoV-2 virus inactivates within 5 min (Chin et al. 2020). As viable SARS-CoV-2 virus is present on stool sample and ultimately sewage water, so the fecal–oral route could be considered as another determinant for COVID-19 transmission (DutchWaterSector 2020; Aylward, Bruce (WHO); Liang 2020). Evidence suggests that human-to-human transmission occurs mainly in families, but nosocomial and/or other close settings could amplify the transmission rate (Aylward, Bruce (WHO); Liang 2020). If the median incubation period of COVID-19 is around 5 days, then the subclinical infection might be the potential source of disease transmission (Kupferschmidt 2020; R. Li et al. 2020a, b; Lauer et al. 2020). Thus far, a reproductive number of this virus is estimated around 6, which means that the infected person can infect approximately six other people (Sanche et al. 2020).

3.5 Molecular Infection Biology of SARS-CoV-2

In order to reproduce and establish infection, the virus must enter the host organism. Tissue tropism of the virus depends on the surface proteins of virus and host factors (physical barrier, local temperature, pH, and host defenses). S protein of SARS-CoV-2 virus is the vital determinant of viral entry, which binds to angiotensin-converting enzyme 2 (ACE2) expressing host epithelial cell through receptor binding domain (RBD) (X. Li et al. 2020a, b). S protein of SARS-CoV-2 has 10–20 times stronger affinity for human ACE2 receptor than that of SARS-CoV (Xia et al. 2020). ACE2 regulates renin–angiotensin system homeostasis (Cheng, Wang, and Wang 2020). ACE2 received attention due to its protective mechanism in lung. Along with angiotensin II receptor type 2, ACE2 protects from severe acute lung injury (Cheng, Wang, and Wang 2020). The potential target ACE2 is expressed in upper and lower respiratory tract (polarized epithelial cells and type II pneumocytes), oral cavity (epithelial cells), esophagus (upper and stratified epithelial cells), intestine (enterocytes), gall bladder (cholangiocytes), heart (myocardial cells), breast, adrenal gland, kidney (proximal tubular cells), urinary bladder (urothelial cells), eye (conjunctival fibroblast and epithelial cells and corneal epithelial cells), brain (neuronal cells), and male organ (testis and seminal vesicle) (Xu et al. 2020a, b; Uhlén et al. 2015; Ren et al. 2006; Zhao et al. 2020; Xin 2007; Doobay et al. 2007; Zou et al. 2020). Direct or indirect contact with mucus

membrane in the nose, mouth, and eyes can transmit COVID-19 virus (Zheng 2020). Droplets and fomites containing SARS-CoV-2 find their prospective target ACE2 receptors at respiratory tract primarily as a portal of entry. But due to the highest expression of ACE2 receptor into enterocytes (and cholangiocytes), the intestinal wall cannot be ignored as the portal of entry of COVID-19 virus (Xu et al. 2020a, b). Studies found that stools were positive for SARS-CoV-2 for 1–12 days in patients following negative results in respiratory samples (Gu et al. 2020). Gastric pH may reduce the infective capacity of SARS-CoV-2 as previous studies showed how pH influenced the viability of SARS-CoV in the stool (WHO 2003). Although Chin et al. claimed an extreme stability of SARS-CoV-2 in a wide range of pH values (3–10) at room temperature (22 °C) (Chin et al. 2020).

Following binding to ACE2 receptor, two-step sequential protease cleavage occurs in S protein of SARS-CoV-2 by host proteases: priming cleavage on S1/S2 and activating cleavage on S2' sites (Ou et al. 2020; X. Li et al. 2020a, b) Priming cleavage on S1/S2 site is regulated by host proteases, for example, cathepsin L and TMPRSS2 (Shen et al. 2017; Hoffmann et al. 2020; X. Li et al. 2020a, b). Also, ACE2 receptor is proteolytically cleaved by TMPRSS2 and/or ADAM17 to propagate SARS-CoV-2 coronavirus entry (Simmons et al. 2013). SARS-CoV-2 primarily enters the cell through endocytosis as shown in Fig. 3.1 (Ou et al. 2020). Phosphatidylinositol-3,5-bisphosphate ($PI(3,5)P_2$) is important for early to late lysosomal maturation. As the critical SARS-CoV-2 viral entry regulators, phosphatidylinositol 3-phosphate 5-kinase primarily synthesizes $PI(3,5)P_2$ in the early endosome, while two-pore channel subtype 2 (TPC2) in endosome is one of the fundamental downstream effectors of $PI(3,5)P_2$ (Ou et al. 2020). Cathepsin L (highly expressed in lung and bronchus) is essential for endosomal cell entry and might be responsible for activation of membrane fusion through proteolysis on S1/S2 site (Najjar et al. 2015; Ou et al. 2020; Uhlén et al. 2015). The fusion peptide can expose and insert into the host cell membrane due to the acidic environment of the endosome (Shen et al. 2017). Due to the conformational change, HR1 and HR2 domains of S2 subunit form canonical six-helical (coiled-coil complex) fusion core that can bring viral and host cell (endosomal) membrane close together for syncytium formation (Xia et al. 2020). Ou et al. also showed that without exogenous protease, for example, trypsin priming or activation, S protein of SARS-CoV-2 could trigger directly by endocytosis for rapid disease progression (Ou et al. 2020). Although cathepsin L is dispensable, membrane-bound TMPRSS2 could play the role of viral entry in the tissues where TMPRSS2 is highly expressed, for example, gastrointestinal tract (Hoffmann et al. 2020; Uhlén et al. 2015). At neutral pH, transmembrane TMPRSS2 activates S protein that results in syncytium formation, which lead to the release of the viral RNA genome into the host cell cytoplasm (Shen et al. 2017; Hoffmann et al. 2020; Xia et al. 2020). But the role of furin/furin-like protease in SARS-CoV-2 virus entry is obvious. Similar to MERS-CoV, the SARS-CoV-2 has polybasic furin-like cleavage sites and may have a similar mechanism of action (Coutard et al. 2020). Endogenous furin expression in various tissues (e.g., upper respiratory tract, salivary gland) may influence viral entry through cleavage of S2' site during fusion-mediated viral entry (Mille and Whittaker 2014; Belouzard, Chu, and Whittaker 2009; Uhlén et al. 2015). However, one cannot rule out the possibility of cross-talk between heparan sulfate proteoglycans in the host cell surface and furin-like cleavage sites in SARS-CoV-2 (Pasquato et al. 2007; Belouzard et al. 2009) as high level of heparan sulfate proteoglycans may cause coagulopathy during COVID-19 (Wang et al. 2019). Recently, neuropilin-1 has been highlighted due to its binding capacity of furin-cleaved S-protein to potentiate SARS-CoV-2 infectivity (Cantuti-Castelvetri et al. 2020). More studies are needed to study the furin-like cleavage sites (Fig. 3.1).

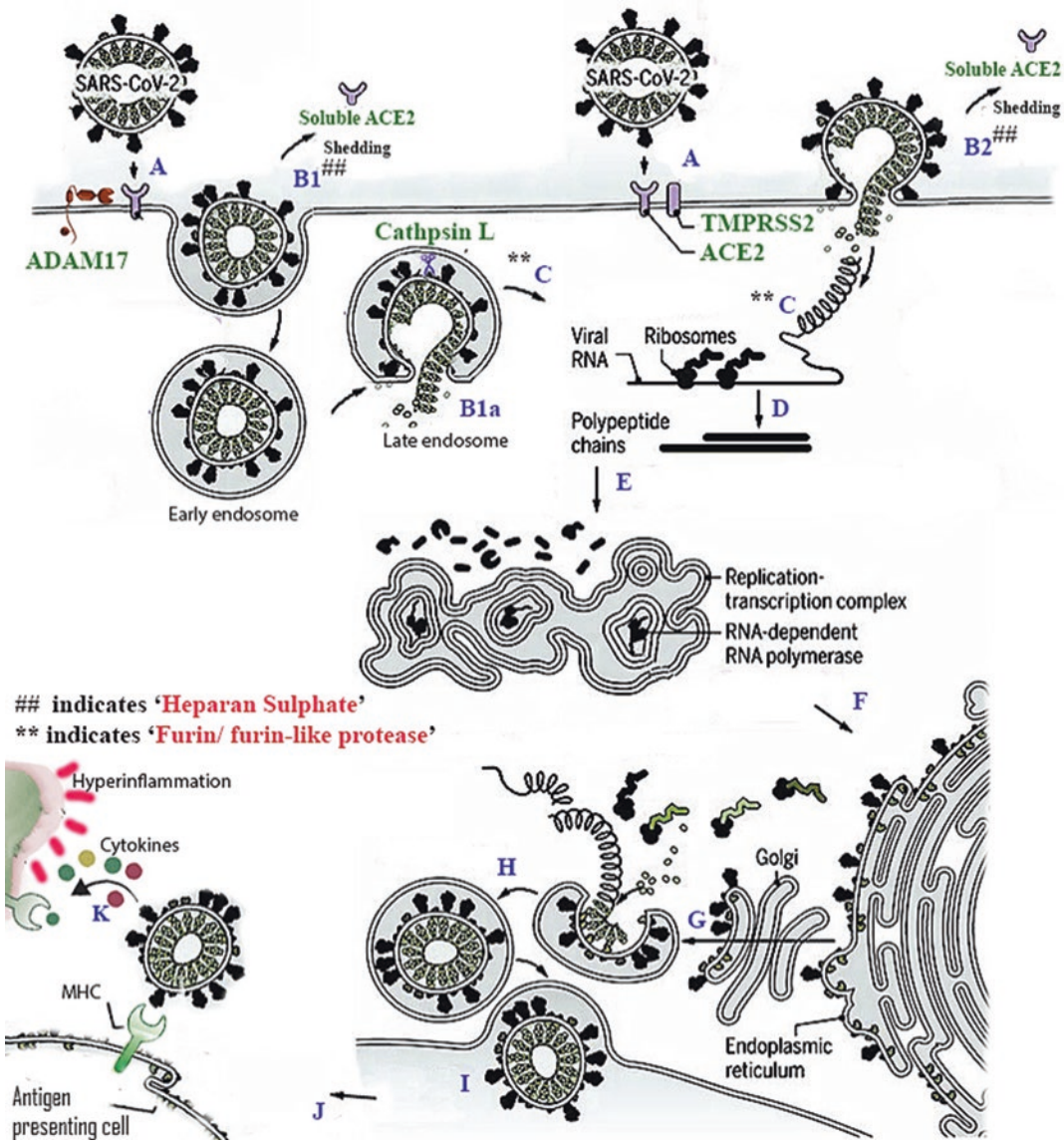

Fig. 3.1 SARS-CoV-2 virus life cycle. (**A**) SARS-CoV-2 binds with host cell ACE2 receptor, resulting in fusion, either (**B1**) endocytosis or (**B2**) priming cleavage by TMPRSS2; shedded ACE2 also augments viral entry by either activated ADAM17 or TMPRSS2. (**B1a**) Endocytosed virus S protein is also cleaved by cathepsin L; (**C**) virus RNA genome is liberated into cytoplasm and (**D**) translation into polypeptides takes place into endoplasmic reticulum derived double membrane vesicles; (**E**) polyproteins are cleaved into NSPs; (**F**) translation of structural proteins and RNA replication; (**G**) trafficking of newly synthesized proteins and RNA from endoplasmic reticulum to Golgi body; (H) packaging of virion in the budding vesicle; (**I**) mature virus releases via exocytosis; (**J**) SARS-CoV-2 antigens presentation to antigen-presenting cells via MHCs/HLAs; (**K**) stimulated antigen-presenting cells release cytokines to enhance proinflammatory response. (In both pathways, furin/heparan sulfate could cleave S2' site for successful viral entry and replication.) (Adapted and modified from https://www.genetex.com/Research/Overview/infectious_diseases/SARS-CoV-2?fbclid=IwAR0T8T8J75gdjt1z6Uuvh6KEdsJNDO6Ja8xuhso5Q0SDlkdkwATY077cMxo)

3.6 Replication Packaging and Release of SARS-CoV-2

Viral replication step starts by hijacking host translational machinery (a reticulovesicular network of double-membraned vesicles with interconnected outer membranes originated from the endoplasmic reticulum) (Báez-Santos et al. 2015). The released SARS-CoV-2 RNA genome into host cell cytoplasm is translated into two polyproteins, structural and accessory proteins (Báez-Santos et al. 2020). This translational microenvironment protects viral RNA from host nucleases and innate immune response (Báez-Santos et al. 2015). The cleavage of the polyproteins forms 16 nonstructural proteins (NSPs) (Chan et al. 2020a, b; X. Li et al. 2020a, b). The NSPs assembled replicase-transcriptase complex in double-membraned vesicles are responsible for synthesizing a full-length negative RNA strand template (Fehr and Perlman 2015; X. Li et al. 2020a, b). The RNA strand replicates to genomic RNA and individual subgenomic RNA continuously (Guo et al. 2020a, b; Fehr and Perlman 2015). Subgenomic RNA serves as mRNA, which is directly translated to viral structural proteins and trafficked into the endoplasmic reticulum–Golgi intermediate compartment (Fehr and Perlman 2015). It is noteworthy mentioning that furin is thought to cleave S1/S2 site during S protein biosynthesis (Mille and Whittaker 2014).

The Spike protein (S protein), an envelope protein (E protein), nucleocapsid protein (N protein), and relatively abundant membrane protein (M protein), along with newly generated complete RNA genome, form virion in budding Golgi vesicles (Fehr and Perlman 2015). Virion-containing vesicles fuse with the host cell membrane, and mature SARS-CoV-2 viruses are released by exocytosis (Y.-R. Guo et al. 2020a, b; X. Li et al. 2020a, b). The newly formed SARS-CoV-2 virus attacks another neighboring cell and goes on. Due to strong evidence of immune and lung damage, pulmonary epithelium and immune cells suggest to be the primary target for SARS-CoV-2 (Xu et al. 2020a, b; X. Li et al. 2020a, b). Similar to SARS-CoV and MERS-CoV, the studies showed widespread immunopathology and/or extrapulmonary dissemination and replication of SARS-CoV-2 among infected patients (Gu et al. 2005; Xu et al. 2020a, b). Postmortem study analysis of deceased SARS-CoV-2 and SARS-CoV-infected patients found other potential target sites such as neurons, epithelium of renal tubules, intestinal mucosa, and macrophages in various organs (Liu et al. 2020; Poyiadji et al. 2020; Gu et al. 2005; Xu et al. 2020a, b). As part of the upper respiratory tract, the olfactory epithelium has also come into focus due to COVID-19-associated anosmia (Brann et al. 2020).

3.7 Host Immune Response of SARS-CoV-2 Infection

Following virus entry into the host cell, the antigen presentation is the key determinant of T-cell immune response triggering. T cells identify major histocompatibility complex (MHC)/human leukocyte antigen (HLA)-bound processed cell surface antigens only. Mainly with the help of MHC class I (e.g., HLA-A, HLA-B, HLA-C) molecules virus-infected host cells are presented to CD8+/cytotoxic T cells, but the MHC class II (HLA-DM, HLA-DR, HLA-DP, HLA-DQ, HLA-DOA and HLA-DOB) also contributes in antigen presentation to CD4+ /helper T cells (Wosen et al. 2018; Hansen and Bouvier 2009). Not only macrophages, dendritic cells, or B cells, but also aerodigestive epithelial cells (enterocytes, columnar ciliated epithelial cells, and type II pneumocytes) are present in MHC class II (Wosen et al. 2018). Variability of HLA genes make the differences in immune response and disease severity among individuals (Nguyen et al. 2020). Similar to SARS-CoV, studies across 145 HLA alleles showed that individuals with HLA-B*46:01 allele are vulnerable to COVID-19 disease (X. Li et al. 2020a, b; Austin Nguyen et al. 2020). HLA-A*25:01 and HLA-C*01:02 alleles are also shown to be related to the susceptibility of SARS-CoV-2, while HLA-A*02:02, HLA-B*15:03, and HLA-C*12:03 might be related to the protection from SARS-CoV-2 infection (Austin Nguyen et al. 2020). Due to

limited comprehensive COVID-19 pathogenesis study, we focused on previous reports of related viruses. HLA-DR B1*1202 and HLA-DR0301 are least and top, binding MHC class II molecules for SAR-CoV (X. Li et al. 2020a, b). Even HLA-DRB1*11:01 and HLA-DQB1*02:0 are associated with MERS-CoV infection (Li et al. 2020a, b).

Th1-type immune response plays a vital role in adaptive immunity. Following antigen presentation, B- and T-cell-mediated humoral and cellular immune response occurs respectively to eliminate the virus and preclude disease progression. The humoral immune response plays a vital role to limit infection at later phase and prevent reinfection in the future. Like other acute viral infections, a typical pattern of IgM and IgG antibodies against SARS-CoV-2 is seen. SARS-CoV-2-induced seroconversion starts gradually from approximately day 5 and switches to IgG by around day 14 (Guo et al. 2020a, b). At the end of week 12, the SARS-specific IgM antibodies disappeared, while the IgG antibody against SARS-specific S, M, E, and N proteins can last long (Prompetchara et al. 2020; X. Li et al. 2020a, b). In vitro cross-reactivity of SARS-CoV-2 sera with SARS-CoV suggests possible mounting of the humoral immune response (Prompetchara et al. 2020). As COVID-19 disease is associated with lymphocytopenia, the number of blood CD4+ and CD8+ T cells of COVID-19 patients is significantly reduced (Shi et al. 2020; X. Li et al. 2020a, b). But CD4+ and CD8+ T cells are overactivated (Xu et al. 2020a, b). Similar results had been observed in acute phase response in SARS-CoV virus infection and CD4+ and CD8+ memory T cells sustained for 4 years in recovered patients to perform T cell proliferation, DTH response, and production of interferon-γ (IFN-γ) (X. Li et al. 2020a, b). CD8+ T cell responses were higher in SARS-CoV infection (which is crucial for lung pathology) than CD4+ T cell responses (Prompetchara et al. 2020). In severe cases, higher multifunctional CD4+ T cells (IFNγ, TNF-α, and IL-2), CD8+ T cells (IFNγ, TNF-α and degranulated state), and Th2 cytokines (IL-4, IL-5, IL-10) were detected compared with mild or moderate SARS-CoV infection (Prompetchara et al. 2020).

Only a few studies are available on host innate immune response to SARS-CoV-2 infection. Gradual increment of total neutrophil counts, C-reactive protein, and many innate cytokines suggest highly proinflammatory condition in COVID-19 disease progression and severity (Prompetchara et al. 2020). The innate immune response against viral infection mainly depends on the type I IFN responses. First-line defense against RNA viruses comprises Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and NOD-like receptors (NLRPs) to detect viral genome and its replication intermediates (Jensen and Thomsen 2012). Innate immune cells [pneumocytes/epithelial cells, granulocytes, monocytes, macrophages, dendritic cells, and natural killer (NK) cells] recognize viral invasion by either the endosomal TLRs (TLR3, TLR7, and TLR8) and the cytosolic double-stranded RNA sensor RIG-I/MDA-5 (Artis and Spits 2015; Lim et al. 2016; Prompetchara et al. 2020). Adaptor proteins (MyD88 and MAVS)-induced downstream signaling are recruited upon recognition by TLRs and RLRs (Lim et al. 2016). As the first line of defense, the recognition events lead to interferon regulatory transcription factor 3 or 7 (IRF3/7) and NFκB-induced type I IFN (IFN-α and IFN-β) and other proinflammatory cytokines (e.g., TNF-α, IL-6) expression (Lim et al. 2016; Prompetchara et al. 2020). Type I induction is essential in early phase of disease to halt virus propagation within the host and modulate innate and adaptive immune responses. STAT1/2-IRF9 complex-mediated IFN-stimulated genes (e.g., antiviral enzyme RNAse L, proinflammatory chemokine CXCL10) transcription follows activation of JAK-STAT pathway via type I IFN (Prompetchara et al. 2020). During the early phase of infection, SARS-CoV-2 viral (structural and nonstructural) proteins could use multiple strategies to dampen type I IFN-mediated immune response (Prompetchara et al. 2020). But the influx of hyperinflammatory neutrophils, monocytes, and macrophages due to delayed type I IFN immune

response compensates early viral control and could result in deteriorating consequences to the infected host. Additionally, SARS-CoV-2 E and open reading frame 3a (ORF3a) proteins may trigger inflammasome sensor NLRP3 to secrete IL-1β and induce pyroptosis (an inflammatory form of cell death) (Chen et al. 2019; Nieto-Torres et al. 2015). Simply saying, the innate immune response could be protective or destructive for SARS-CoV-2-infected patients and may open the window for immune intervention. Due to highly effective innate immunity, very few severe cases were reported in young patients. So, the innate immune response is the key determinant for disease outcome.

3.8 Immune Evasion by SARS-CoV-2

Clinical and experimental data showed that similar to SARS-CoV or MERS-CoV, SARS-CoV-2 deals with host immune response (Prompetchara et al. 2020). The SARA-CoV-2 virus can evade immune detection and dampen human immune responses, which explain why the incubation period of SARS-CoV-2 virus is higher than the influenza virus. Innate immune responses are inhibited at the level of type I interferon recognition and signaling, while adaptive immune evasion is processed by MHC class I and class II downregulation, which would reduce T cell activation markedly (Prompetchara et al. 2020). Like other betacoronaviruses, intermediate products of SARS-CoV-2 may avoid host recognition within double-membrane vesicles during virus replication process (Knoops et al. 2008; Oudshoorn et al. 2017). M protein has been shown to suppress RIG-I-induced activation of IRF3 (Lui et al. 2016), while deubiquitinase (DUB) activity in the infected cell and inhibitory activity against IRF3 activation by NSP3 may play a vital role in immune evasion (Frieman et al. 2009; Yang et al. 2014; Li et al. 2016). NSP1 and NSP6 can block the phosphorylation of STAT1 and the translocation of the STAT1/2/IRF9 complex, respectively, to prevent activation of an antiviral function within the infected cell and the enhancement of the IFN response (Wathelet et al. 2007; Kopecky-Bromberg et al. 2007). Therefore, downregulating IFN response directly or indirectly can cause an unbalance production of proinflammatory cytokines and infiltration of inflammatory cells lead to severe form of COVID-19.

3.9 Cytokine Release Syndrome on COVID-19

Although heat shock protein 70 is a vital anti-inflammatory chaperone to inhibit cytokine storm, it is gradually suppressed in chronic metabolic diseases/disorders such as diabetes and obesity (Heck et al. 2020). Also, it has been observed that male patients with COVID-19 are at risk of worse outcome since males don't have estrogen–heat shock response axis-based cardioprotection. Unlike females, deficiency of androgen receptors causes diminished activation of heat shock factor 1 and heat shock protein 70 in males (Heck et al. 2020). Acute respiratory distress syndrome (ARDS) and multiple organ failure are the fundamental causes of death due to COVID-19 (X. Li et al. 2020a, b). As the initiator of ARDS, deadly uncontrolled cytokine storm results from the release of large amounts of proinflammatory cytokines (IFN-α, IFN-γ, IL-1β, IL-6, IL-12, IL-18, IL-33, TNF-α, TGFβ, etc.) and chemokines (CCL2, CCL3, CCL5, CXCL8, CXCL9, CXCL10, etc.) by the immune effector cells (X. Li et al. 2020a, b).

3.10 Conclusion

This new virus outbreak has challenged the economic, clinical, and public health worldwide. More so, future outbreaks of viruses and pathogens of zoonotic origin are likely to continue. Therefore, apart from curbing this outbreak, efforts should be made to devise comprehensive measures to prevent future outbreaks of zoonotic origin. The pandemic has largely affected education systems all around the globe. Due to the COVID-19 shortage of supply of grocery essen-

tials, personal protective equipment, and disruption of operation of factories and logistics have been observed. A lot of people have lost their jobs due to the outbreak, which strongly impacted on world economy. If it continues this way, then many countries may face famines. Sports, traveling, festivals, religious services, and other social services are being interrupted due to the pandemic. Suspicion, hostility, xenophobia, racism, and even domestic violence have been noted in different parts of the world. Health workers have mixed experience of appreciation and harassment in different parts of the world. Despite a lot of negative impacts, positive impacts on the environment and climate have been observed all around the globe.

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Conflict of Interest The authors report no conflict of interest.

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Epidemiology and Etiopathogeny of COVID-19

4

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Abstract

Despite the recent announcement of the new pathogenic coronavirus to man, SARS-CoV2, a large number of publications are presented to the scientific community. An organized and systematic review of the epidemiological, etiological, and pathogenic factors of COVID-19 is presented. This is a systematic review using the databases MEDLINE, EMBASE, Web of Science, SCIELO; the descriptors coronavirus, SARS-CoV-2, etiology, epidemiology, pathophysiology, pathogenesis, COVID-19, with publications from December 2019 to January 2021, resulting in more than 800 publications and 210 selected. The data suggest that COVID-19 is associated with SAR-CoV-2 infection, with the transmission of contagion by fomites, salivary droplets, and other forms, such as ver-

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tical and fecal–oral. The bat and other vertebrates appear to be reservoirs and part of the transmission chain. The virus uses cell receptors to infect human cells, especially ACE2, like other coronaviruses. Heat shock proteins have different roles in the infection, sometimes facilitating it, sometimes participating in more severe conditions, when not serving as a therapeutic target. The available data allow us to conclude that COVID-19 is a pandemic viral disease, behaving as a challenge for public health worldwide, determining aggressive conditions with a high mortality rate in patients with risk factors, without treatment, but with the recent availability of the first vaccines.

Keywords

Epidemiology · Etiopathogenesis · Covid-19

4.1 Etiology of COVID-19

4.1.1 Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Gobalenya et al. 2020) – which has been provisionally coined "2019-nCoV" (CDC 2020; WHO 2020a, b), sometimes called "Wuhan coronavirus" (Huang 2020), or "COVID-19 virus" (WHO 2020a, b) – is a single-stranded positive RNA virus–linear genome (Changchuan 2020). It is contagious in humans and is responsible for the etiology of COVID-19 morbidity, as well as related to the ongoing pandemic in the year 2020 (Wee et al. 2020; Chan et al. 2020). This chapter reveals through systematic review the aspects involving viral biology of SARS-CoV-2, the epidemiology, and pathogeny of COVID-19.

4.1.2 History: Hypotheses About the Origin of the Virus

Although the SARS-CoV-2 viral strain was discovered in Wuhan, the capital of the central province of China (Zhou et al. 2020a, b, c), it is still unclear what the original source of viral transmission to humans is, neither when did this virus had become pathogenic (Andersen et al. 2020; Eschner 2020; Cohen 2020; Yu et al. 2020).

The COVID-19 virus has a phylogeny similar to coronaviruses observed in bats, from which it probably originated (Gobalenya et al. 2020; Perlman 2020; Benvenuto et al. 2020). A genomic study of bats of the species *Rhinolophus affinis* (Fig. 4.1), from the province of Yunnan, in southwest China, where a varied landscape is observed covering snow-capped mountains, extensive rice paddies, lakes, and deep gorges, showed a 96% similarity to SARS-CoV-2 (Gobalenya et al. 2020; Perez and Montagnier 2020). However, it is thought that before the aforementioned virus was introduced into the human species, it could be involved in an intermediate animal reservoir, such as the pangolin (*Manis pentadactyla*), mammals of the order *Pholidota* that live in tropical areas of Asia and Africa, which present the body covered with scales, habits similar to anteaters and that adopt a curled shape when threatened (WHO 2020a, b; Shield, 2020) (Fig. 4.2).

Fig. 4.1 Illustration representing *Rhinolophus affinis*. (Source: own authorship)

Fig. 4.2 Illustration representing the *Manis pentadactyla*. (Source: own authorship)

From a taxonomic point of view, SARS-CoV-2 is classified as a strain of the coronavirus species related to severe acute respiratory syndrome (SARS-CoV) (Gobalenya et al. 2020). On February 7, 2020, researchers in the city of Guangzhou, Guangdong Province, China, a large port city in the northwest of Hong Kong, on the banks of the Pearl River, found 99% identical to SARS-CoV-2 in pangolin biological material (Cyranoski 2020), differing only in only one amino acid (Xiao et al. 2020). It is worth mentioning a complicating factor that despite the legal protection by Chinese law about pangolin there is still an illegal trade in pangolins for use in traditional Chinese medicine (Ling et al. 2016). Reinforcing this idea, microbiologists and geneticists in Texas (USA) observed evidence of rearrangement in coronavirus, suggesting the involvement of pangolins in the origin of SARS-CoV-2 (Wong et al. 2020a, b). It should be noted, however, that the coronaviruses observed in pangolins, until the present moment, share only 92% of the SARS-CoV-2 genome, which makes the hypothesis that pangolins are intermediate hosts fragile. In comparison, the SARS virus responsible for the 2002–2004 outbreak shared 99.8% of its genome with the coronaviruses of the Asian Palm Civet (*Paradoxurus hermaphroditus*) (Fig. 4.3), small mammal, light, generally arboreal, omnivorous with 1.7–2.7 kg of nocturnal and solitary habits that lives in several regions of South and Southeast Asia, adapting very well in different types of habitats. This species feeds on fruits such as papaya, bananas, palm fruit, and, also coffee beans, because of the sweet taste of the outer skin of these beans (Cyranoski 2020) (Fig. 4.4).

Fig. 4.3 Illustration representing *Paradoxurus hermaphrodites*. (Source: own authorship)

4.1.3 Viral Biology

4.1.3.1 Adsorption and Penetration

Structural Proteins from SARS-CoV-2

As a member of the Coronaviridae family, SARS-CoV-2 has four structural proteins with a relevant role in its pathogenicity, especially concerning cell penetration and viral dissemination, namely spike protein (S), membrane protein (M), envelope protein (E), and nucleocapsid protein (N) (Wu et al. 2020a, b, c; Malik 2020; Shereen et al. 2020) (Fig. 4.5).

Protein S

Protein S is highly glycosylated, with a class I fusional trimeric structure and a molecular mass of approximately 150 kDa. It is responsible for mediating the connection between the virus and the receptors on the surface of the host cell, resulting in a membrane fusion or endocytosis and the subsequent viral entry into the target cell (Malik 2020; Bosch et al. 2003). This transmembrane glycoprotein is capable of generating homotrimers that project themselves from the viral surface, being the first viral component to encounter the target cell (Fig. 4.6) (Hoffman et al. 2020).

SARS-CoV-2 uses the same host receptor as SARS-CoV, the human angiotensin converting enzyme 2 (ACE2), which is a membrane-anchored carboxypeptidase, highly expressed by the respiratory epithelium, including the type I and type II alveolar epithelium (Hoffmann et al. 2020; Zhou et al. 2020a, b, c; Perrota et al. 2020). After binding to ACE2, protein S undergoes a process of cleavage by host proteases, resulting in the formation of two distinct functional subunits, S1 and S2 (Fig. 4.7). The first interacts with the host receptor (Letko et al. 2020; Ou et al. 2020) (Fig. 4.8), the second introduces the virus into the cytoplasm of the target cell and/or to fuse host cell membranes (Fig. 4.9) (Hulswit et al.

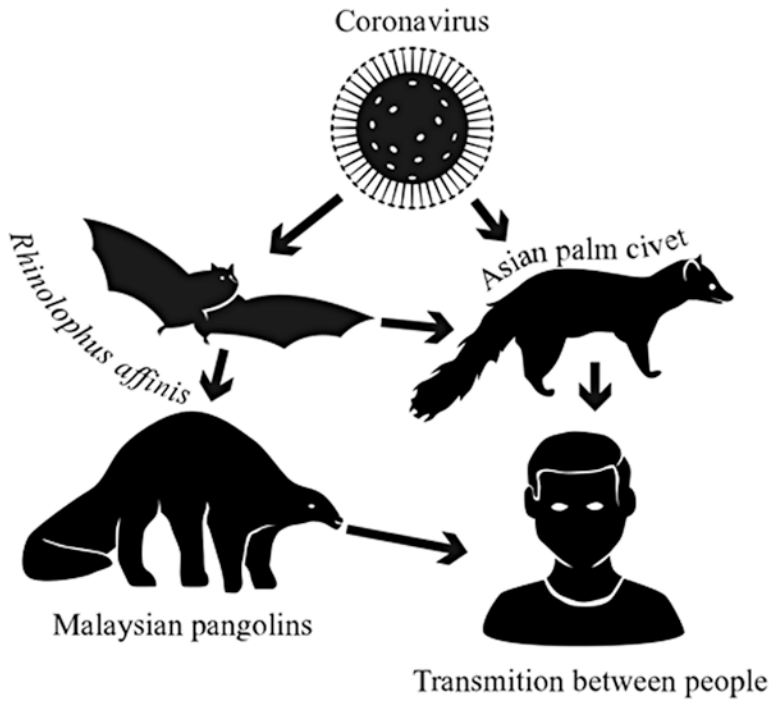

Fig. 4.4 Ecology of the emerging coronaviruses SARS-CoV and SARS-CoV-2, originating in bats, causing infections in humans after circulation in intermediate hosts (civet and pangolin). (Source: own authorship)

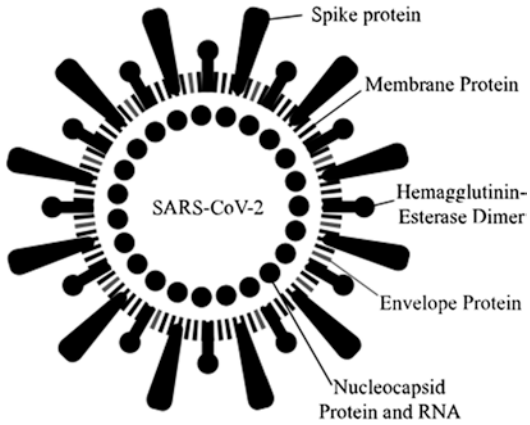

Fig. 4.5 SARS-CoV-2 structural proteins. (Source: own authorship)

2016; Li et al. 2020a, b, c, d, e, f, g; Zhai et al. 2020; Malik 2020; Coutard et al. 2020).

Protein N

It is the only glycoprotein, among the other structural proteins, directly linked to viral genomic RNA and which is not synthesized in the endoplasmic reticulum (ER) of the host cell, acting on viral assembly and budding (de Haan and Rottier 2005).

Protein E

It is a transmembrane protein with ion channel activity, abundantly expressed during the viral replication cycle within the infected cell. However, only a small part of it is incorporated into the virus envelope. With an important role in the assembly and viral maturation, it is responsible for inducing the curvature of the membrane and preventing the aggregation of protein M (de Haan and Rottier 2005).

Protein M

Structural protein is more abundant in SARS-CoV-2, being responsible for defining the shape of the viral envelope (Malik 2020). It interacts with the other three structural glycoproteins in SARS-CoV-2. With the glycoprotein N, it forms a complex (protein N–RNA) stabilizing the

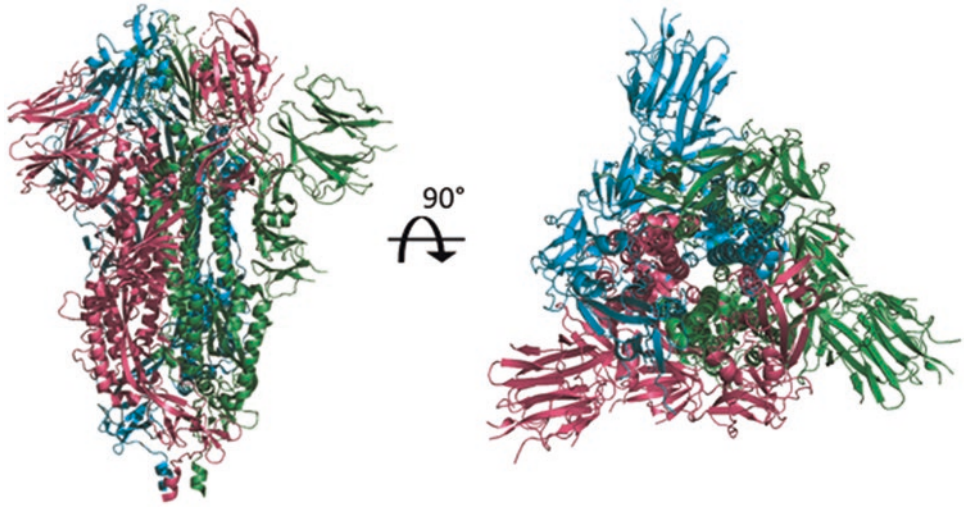

Fig. 4.6 Overview of the SARS-CoV-2 protein S. The assembly of the SARS-CoV-2 protein trimer S is colored in green; cyan and magenta are shown in the cartoon representation. This figure was elaborated using the coordinates of the structure deposited in the protein database (code PDB: 6M0J). (Source: own authorship)

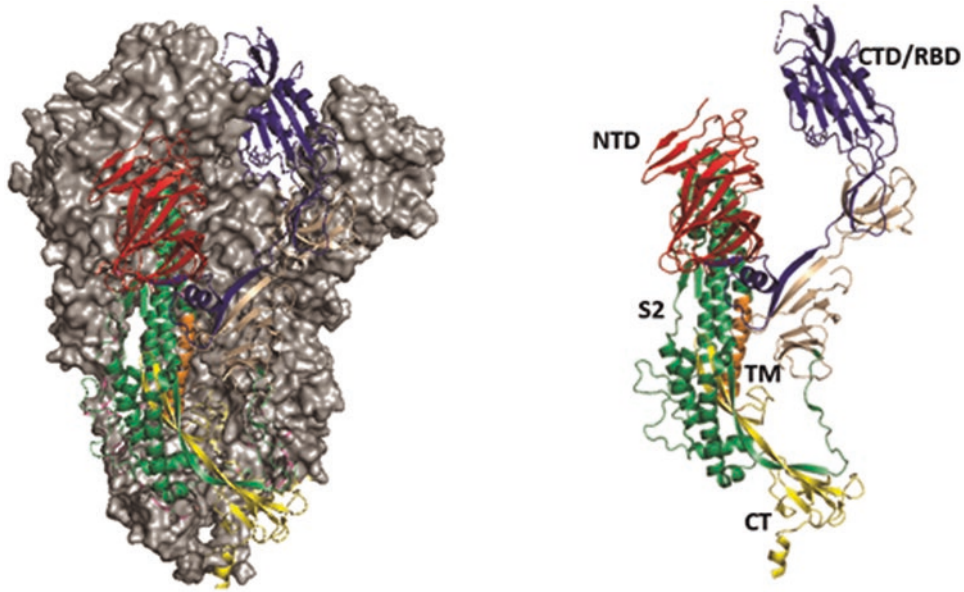

Fig. 4.7 Representation of SARS-CoV-2 protein S domains. A cartoon representation of the domains: NTD (red), CTD/RBD (blue), S2 (green), TM (orange), and CT (yellow). This figure was elaborated using the coordinates of the structure deposited in the protein database (code PDB: 6M0J). (Source: own authorship)

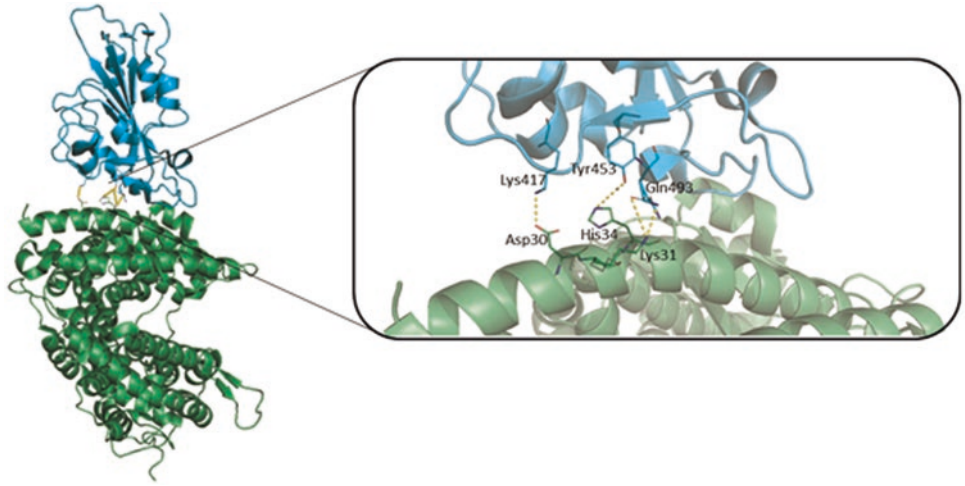

Fig. 4.8 Representation of the complex structure of SARS-CoV-2-CTD linked to the human receptor ACE2. The structures SARS-CoV-2-CTD and ACE2 are represented in cartoons, in cyan and green, respectively. The residues involved in the SARS-CoV-2-CTD-ACE2 connection are labeled and represented on sticks. Yellow strokes represent polar contacts. This figure was elaborated using the coordinates of the structure deposited in the protein database (code PDB: 6M0J). (Source: own authorship)

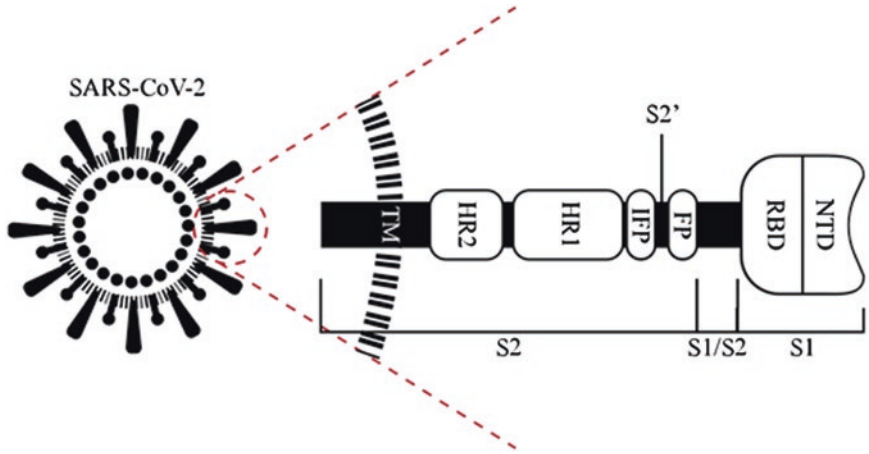

Fig. 4.9 Schematic drawing of protein S and its respective domains. (Source: own authorship)

nucleocapsid and the heart of the virion, also helping to complete the viral assembly (Escors et al. 2001). Together with glycoprotein E, they make up the viral envelope and interact to produce and release particles that are similar to virus (VLPs) (Vennema et al. 1996).

Adesão e internalização Viral

The adhesion of the SARS-CoV-2 virus to the target cell occurs through protein S, which interacts with ACE2 cell receptors (Angiotensin-Converting Enzyme 02), present in a variety of cells in the human body, especially present in pulmonary cells. This process is similar to the key-lock fitting (Uzunian 2020). This process depends on two proteases: transmembrane protease/serine subfamily member 2 (TMPRSS2), which cleaves and activates the S protein, allowing the virus to bind to the ACE2 receptor, adhering to the cell membrane allowing its

internalization in the endosomes (Hoffmann et al. 2020), and Furina, which has a similar role to TMPRSS2 (Liu and Saif 2020). The role of TMPRSS2 in the adhesion and internalization of SARS-CoV-2 has been demonstrated by Danish researchers who used nafamostat mesylate, a short-acting anticoagulant, used to treat pancreatitis and during hemodialysis to prevent fibrinogen proteolysis (Al-Horani and Desai 2014; Sadahiro et al. 2018), and also a TMPRSS2 inhibitor. Several trials are underway to demonstrate the blocking action of TMPRSS2 on viral internalization, a promising therapeutic target (Holmes and Enjuanes 2003).

In addition to ACE2, recent works show that SARS-CoV-2 protein S also binds to CD147 (Wang et al. 2020a, b). CD147 is also known as Basigin and as an extracellular matrix metalloproteinase inducer. It is present in the membrane of several cell types, including epithelial cells, endothelial cells, and leukocytes (Kasinrerk et al. 1992). It is a determinant protein for the OK blood group system and appears to have an important role in the adhesion and internalization of SARS-CoV-2.

On the other hand, virial internalization occurs with the participation of lysosomal cathepsins in host cells, which are the proteases involved in the activation process of glycoprotein S in the receptor-dependent endosomal penetration pathway, which also has prior activation by furin for interaction with ACE2, allowing the internalization of SARS-CoV-2 by endocytosis in endosomal vacuoles (Shang et al. 2020; Walls et al. 2020). Concerning the coronaviruses, the current evidence suggests that viral internalization can vary between viral types and host cell types, involving endocytosis, possibly dependent on clathrin or caveolae and independent mechanisms involving lipid rafts. The first is a protein that plays an important role in the process of forming membrane vesicles inside eukaryotic cells; the second represents small invaginations of the plasma membrane in many types of vertebrate cells, especially in endothelial cells, adipocytes, and notochord cells (Glebov 2020).

Despite important efforts of the world scientific community in recent years, to understand the viral biology of SARS-CoV-2, many unknowns remain.

4.1.3.2 Stripping

In this process, the capsid is removed by the action of cellular enzymes in the lysosomes, exposing the viral genome. Besides, the eclipse phase is observed, where there is no increase in the amount of infectious particles in the host cell. In SARS-CoV-2 infections, the viral binding process carried out by protein S to the ACE2 receptor and transmembrane protease/serine subfamily member 2(TMPRSS2) initiates viral adhesion and penetration, followed by the action of trypsin, still outside the cell and of furin and cathepsin acting on the intracytoplasmic endocytic vesicle, allowing for the decapsulation and exposure of genomic RNA, allowing for the next phase of viral replication (Millet and Whittaker 2014).

4.1.3.3 Transcription

SARS-CoV-2 is a positive, spherical, enveloped RNA genome virus. In common with typical mammalian mRNAs, the CoV genome has a Cap structure in the 5′ region and a poly (A) sequence at the 3′ end (Masters 2006). But, unlike most mammalian mRNAs, the CoV genome carries multiple open reading frames (ORFs) between the 5′ and 3′-terminal untranslated region (UTRs), both containing cis-action signals involved in RNA replication (Brian and Baric 2005; Masters 2006).

Viral synthesis comprises the formation of structural and nonstructural proteins from the processes of transcription and translation. The synthesized messenger RNA will bind to the ribosomes, encoding the synthesis of viral proteins. The first proteins to be synthesized are called structural because they will form the viral particle. The late ones are nonstructural proteins, which participate in the viral replication process. Thus, the CoV genome translates four most important structural proteins, which are spikes (S1 and S2), membrane protein (M), envelope protein (E), and nucleocapsid protein (N), all encoded at the 3′ end of the genome.

Once the virus has been linked and penetrated the host cell, the next stage in the coronavirus life cycle is the translation of the replicase gene from the virion's genomic RNA. The replication and transcription of the coronavirus genome occur in cytoplasmic membranes and involve coordinated processes of continuous and discontinuous synthesis of RNA, mediated by viral replication, a huge protein complex encoded by the 20-kb replicase gene (Sola et al. 2015). Coronaviruses encode two or three proteases that cut replicase polyproteins. Then, many of the nonstructural proteins (nsp) are grouped in the replicase-transcriptase complex (RTC) to create a suitable environment for RNA synthesis, and ultimately, they are responsible for RNA replication and transcription of subgenomic RNAs.

The synthesis of viral RNA follows the translation and assembly of viral replicase complexes and produces both genomic and subgenomic RNAs. Subgenomic RNAs serve as mRNAs for the structural and accessory component genes that reside adjacent to the replicase-transcriptase complex (RTC). All positive-sense subgenomic RNA is 3′ co-terminal with the viral genome and thus forms a set of grouped RNAs, a distinctive property of the Nidovirales order. Coronaviruses are also known for their ability to recombine using homologous and nonhomologous recombination (Keck et al. 1987). The ability of these viruses to recombine is connected to the chain-changing ability of RNA-dependent RNA polymerase (RdRp). Recombination is likely to play a significant role in viral evolution and is the basis for targeted recombination of RNA, a reverse genetic tool used to create viral recombinants at the 3′ end of the genome (Malik 2020).

4.1.3.4 Synthesis

Being mandatory intracellular parasites, viruses rely heavily on the structures and functions of host cells to complete their life cycle and also use the infected cell's translation apparatus to express their proteins. In several cases, viruses have been shown to affect the host's translation mechanism to achieve efficient viral protein synthesis and replication, while mRNA cell translation is inhibited (Hilton et al. 1986; Narayanan et al. 2008a, b; Siddell et al. 1980; Siddell et al. 1981a, b). In eukaryotic cells, translation occurs in the cytoplasm and essentially involves four stages: initiation, elongation, termination, and recycling (Kapp and Lorsch 2004).

Several studies have shown CoV-mediated control at the start of translation. On the other hand, we don't have a deep knowledge if CoVs also affect the stages of elongation, termination, or recycling in the synthesis of proteins in the host. It is also unknown whether the synthesis of proteins encoded by CoV is regulated in the stages of termination of translation in infected cells. It is commonly accepted that most CoV proteins are synthesized by a Cap-dependent translation mechanism (Nakagawa et al. 2016).

4.1.3.5 Assembly, Maturation, and Release

In this process, the M protein is important, being the protein that is structurally arranged to play an important role in determining the shape of the viral envelope. It should be noted that this protein can bind to all other structural proteins. The connection with protein M helps to stabilize nucleocapsids or N proteins and promotes the completion of viral assembly by stabilizing the complex: protein N-RNA-RNA within the virus. The last component is the envelope or protein E, which is the smallest protein in the SARS-CoV structure that plays a role in the production and maturation of these viruses (Teva et al. 2009; Schoeman and Fielding 2019). Following viral synthesis, the subgenomic proteins will be converted into structural and accessory proteins, such as M, S, and E proteins, which will later be isolated in the endoplasmic reticulum and then moved to the intermediate compartment of ER-Golgi Intermediate Compartment (ERGIC). In time, the genomic program replicated in the previous step binds directly to the N protein in the nucleocapsid form and later immersion in ERGIC. In this compartment, the nucleocapsids will join with several other structural proteins and form small saccular vesicles that will be exported out of the cell by exocytosis (Fehr and Perlman 2015; Masters 2006; Indwiani and Ysrafil 2020).

4.1.3.6 Mutations

The mutation is one of the most important mechanisms of viral evolution in nature (Lauring and Andino 2010). The rapid spread of SARS-CoV-2 suggests that its evolution is driven by mutations. Thus, a study was carried out to assess the genetic variation of SARS-CoV-2 in 11 countries (Phan 2020). Eighty-six complete or nearly complete genomes of SARS-CoV-2 from GISAID were collected (https://www.gisaid.org/). These strains of SARS-CoV-2 have been detected in infected patients from China (50), the USA (11), Australia (5), Japan (5), France (4), Singapore (3), England (2), Taiwan (2), South Korea (1), Belgium (1), Germany (1), and Vietnam (1). The alignment of the nucleotide sequence in pairs was performed by ClustalX2, and the sequence of the China strain/WHU01/2020/EPI_ISL_406716 was used as a reference genome (Phan 2020).

Like other beta-coronaviruses, the SARS-CoV-2 genome has a long ORF1ab polyprotein at the 5′ end, followed by four major structural proteins, including the Spike surface glycoprotein, the small envelope protein, the matrix protein, and the nucleocapsid protein (Phan 2020). In this genetic analysis, three deletions were found in the SARS-CoV-2 genomes of Japan (Aichi), the USA (Wisconsin), and Australia (Victoria). Two deletions (3 nucleotides and 24 nucleotides) were on the ORF1ab polyprotein, and one (10 nucleotides) was on the 3′ end of the genome.

This alignment of nucleotide sequences also made it possible to reveal 93 mutations across the SARS-CoV-2 genome. Forty-two missense mutations have been identified in all major nonstructural and structural proteins, except the envelope protein. Twenty-nine missense mutations were in the ORF1ab polyprotein, eight in the Spike glycoprotein, one in the matrix protein, and four in the nucleocapsid protein. It is interesting to note that three mutations (D354, Y364, and F367) were located in the glycoprotein receptor binding domain on the Spike surface. The Spike surface glycoprotein plays an essential role in binding to receptors in the host cell and determines host tropism (Fung and Liu 2019). It is also the main target of neutralizing antibodies (Yu et al. 2020). Mutations in the glycoprotein on the Spike surface can induce its conformational changes, which probably led to the change in antigenicity. Until the present moment, a study on the location of amino acids involved in conformational changes in the structure of the glycoprotein on the peak surface of the SARS-CoV-2 peak is not available. The identification of these amino acids is important and must be investigated by further studies (Phan 2020).

4.1.3.7 Similarities and Genomic and Protein Differences of SARS-CoV and SARS-CoV-2

A Clustal W analysis of N-protein of SARS-CoV and SARS-CoV-2 was carried out by the NCBI amino acid blast that demonstrated more than 90% similarity between the sequences of the two viruses. The amino acid sequence SARS-CoV-2 varies from other coronaviruses exclusively in the regions of 1ab polyprotein and surface glycoprotein or Spike. The Spike protein has two subunits with a subunit link directly to the host receptor, helping the virus to enter cells. The RNA-binding domain of the Spike protein in SARS-CoV-2 has a higher homology with Sars-CoV (Kannan et al. 2020).

4.2 Epidemiology of COVID-19

In the past two decades, coronaviruses (CoVs) have been associated with major disease outbreaks in East Asia and the Middle East. Recently, a new coronavirus, SARS-CoV-02, responsible for systemic morbidity, but with a severe picture of severe acute respiratory syndrome, responsible for coronavirus 2019 (COVID-19), emerged in late 2019 representing a serious threat to global health, culminating in a continuing pandemic in many countries and specific locations on the planet (Rodriguez-Palacios et al. 2020).

In domestic animals, CoV infections are associated with a wide spectrum of pathological conditions. In addition to the infectious bronchitis virus, canine respiratory CoV, and mouse hepatitis virus, CoVs are predominantly associated with gastrointestinal diseases. The emergence of new CoVs may have become possible due to the

maintenance of multiple CoVs in their natural host, which could have favored genetic recombination (Su et al. 2016). Studies suggest a probable zoonotic origin of SARS-CoV-02, given the large number of people infected with the virus were circulating in the market for live animals sold in the city of Wuhan. Initial investigation reports identified two species of snakes that could act as a reservoir for the virus. However, there was insufficient evidence to prove the fact (Ji et al. 2020).

SARS-CoV-2 has a high potential for dissemination. This is measured by the basic reproductive number (R0), which indicates the average number of new infections generated by an infected person in a population that is totally susceptible (Liu et al. 2020a, b). The R0 was measured based on the number of infected in January 2020 in Wuhan, equal to 2.2, that is, an infected person could transmit, on average, to two more susceptible to the virus. However, the detection and increase in the number of domestic cases in other regions outside China suggested that the epidemic would continue to increase in number, and that this risk was probably higher (Liu et al. 2020a, b). This was confirmed with the speed at which the pandemic expanded. In some countries like Indonesia, this index has reached the value of 7.97 (Udomsamuthirun et al. 2020).

The disease continued to spread from Wuhan to the rest of the planet. Also in January 2020, the virus had spread to other countries including Taiwan, Thailand, Vietnam, Malaysia, Nepal, Sri Lanka, Cambodia, Japan, Singapore, Republic of Korea, the United Arab Emirates, the USA, the Philippines, India, Australia, Canada, Finland, France, and Germany (Rothan and Byrareddy 2020). Considering the transmissibility potential of SARS-CoV-2 and the speed at which it reached new territories, in March, the World Health Organization declared a pandemic state and, in just 3 months, the virus was introduced in 216 countries, areas, and territories (WHO 2019). Nowadays, there are currently few areas in the world with no virus circulation.

The transmission of SARS-CoV-2 occurs mainly from person to person, through droplets scattered through the cough or sneeze of an infected individual (Rothan and Byrareddy 2020). However, studies have revealed the presence of viable viruses on several surfaces, including in the home environment of infected people (Han and Yang 2020). It was observed that the virus remained viable for 3 h in aerosols, for up to 72 h in plastic and stainless steel. On cardboard, only after 24 h after the beginning of the experiment the virus became unviable (Van Doremalen et al. 2020) (Fig. 4.10).

Alternative routes of transmission have been investigated and fecal–oral is one of those with potential, as in clinical samples from 73 hospitalized patients with SARS-CoV-2 infection, 39 patients tested positive for SARS-CoV-2 RNA in samples of feces. Besides, 17 patients remained

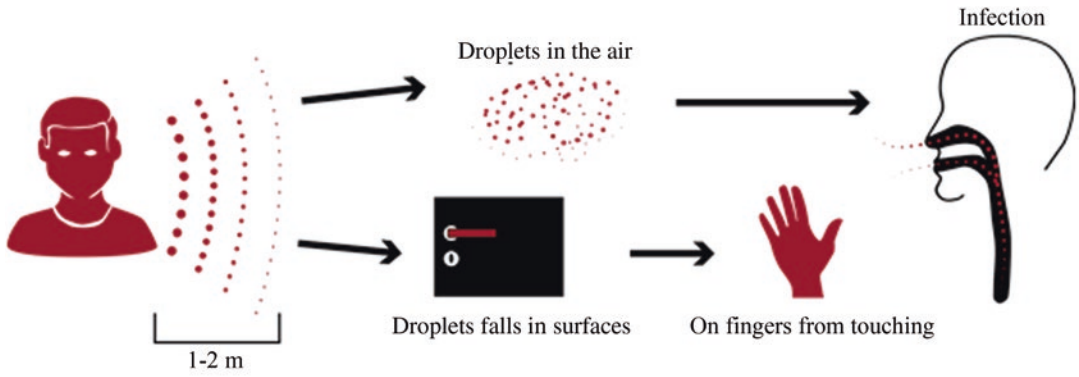

Fig. 4.10 Epidemiological chain of transmission of SARS-Cov-2. (Source: own authorship)

positive for the virus in the stool after becoming negative in respiratory samples (Hindson 2020). The same was observed in children who persistently tested positive on rectal swabs, even after the nasopharyngeal test was negative, corroborating this possibility of transmission (Xu et al. 2020a, b, c). Sexual and vertical transmission has been investigated, but has not yet been confirmed (Song et al. 2020; Chen et al. 2020a, b, c, d, e).

SARS-CoV-2 is seen in people of all ages, but the disease is usually asymptomatic or mild in children (Singhal 2020). Most of the severe cases occurred in individuals who were immunosuppressed or had comorbidities, such as heart disease, nephropathy, blood dyscrasia, hypertension, diabetes, obesity, and over the age of 65 (CDC 2020). The overall fatality rate was estimated at 6.8%, but varies by country (WHO 2019).

There is still no specific treatment, only supportive. The role of antiviral agents has not yet been established, but vaccines are already being distributed even without the completion of the trials. The rate of protection varies from one to the other and the number of doses required as well. In addition, there is no guarantee of protection for all variants of the virus, and vaccination coverage does not occur uniformly in the world, and at the desired rate. Thus, the recommended control measures, such as social distance, the use of masks, and hand hygiene, are still essential for the prevention of the disease.

4.3 Covid-19 Pathogeny

4.3.1 General Considerations

Initial experimental protein modeling assays using virus S protein suggested that SARS-CoV-2 had a relative affinity with angiotensin-converting enzyme 2 (ACE2) receptors in human cells, using them as a mechanism of cell penetration (Shang et al. 2020; Xu et al. 2020a, b, c), later confirmed in January 2020 by independent groups of North American and Chinese researchers (Fig. 4.11). Several studies show that SARS-CoV-2 has a greater affinity for human ACE2 than the original

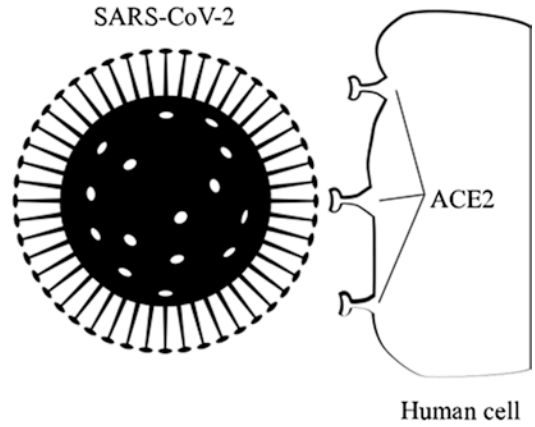

Fig. 4.11 ACE2 cell receiver. (Source: own authorship)

SARS strain (Wrapp et al. 2020; Andersen et al. 2020). Studies show that SARS-CoV-2 can alternatively use the Basigin protein, recognized as CD147, present in erythrocytes, lymphocytes, and elements of spermatogenesis, to penetrate host cells (Wang et al. 2020a, b; Yan et al. 2020). The internalization of SARS-CoV-2 is also facilitated by a priming reaction of the viral S protein by a cellular protein, the transmembrane protease/serine subfamily member 2 (TMPRSS2) (Hoffmann et al. 2020; Sungnak et al. 2020). Scientific investigations still reveal that SARS-CoV-2 determines the appearance of at least three virulence factors that induce the release of virions from host cells that inhibit the immune response (Canrong et al. 2020). All the evidence presented shows a complex pathological picture, revealing that it is a systemic disease and not a simple syncytial viral disease.

The works reveal the presence of the SARS-CoV-2 virus: in the lungs and airways, myocardium, kidney, gastrointestinal tract, and endothelial cells via ACE2 receptor, in hematopoietic cells and spermatogenesis via Basigin protein and central nervous system via connected pathway the synapse to the cardiorespiratory center and also via ACE2 receptor, from the mechanoreceptors and chemoreceptors of the lung and lower respiratory airways, the set being a complex systemic disease (Fig. 4.12).

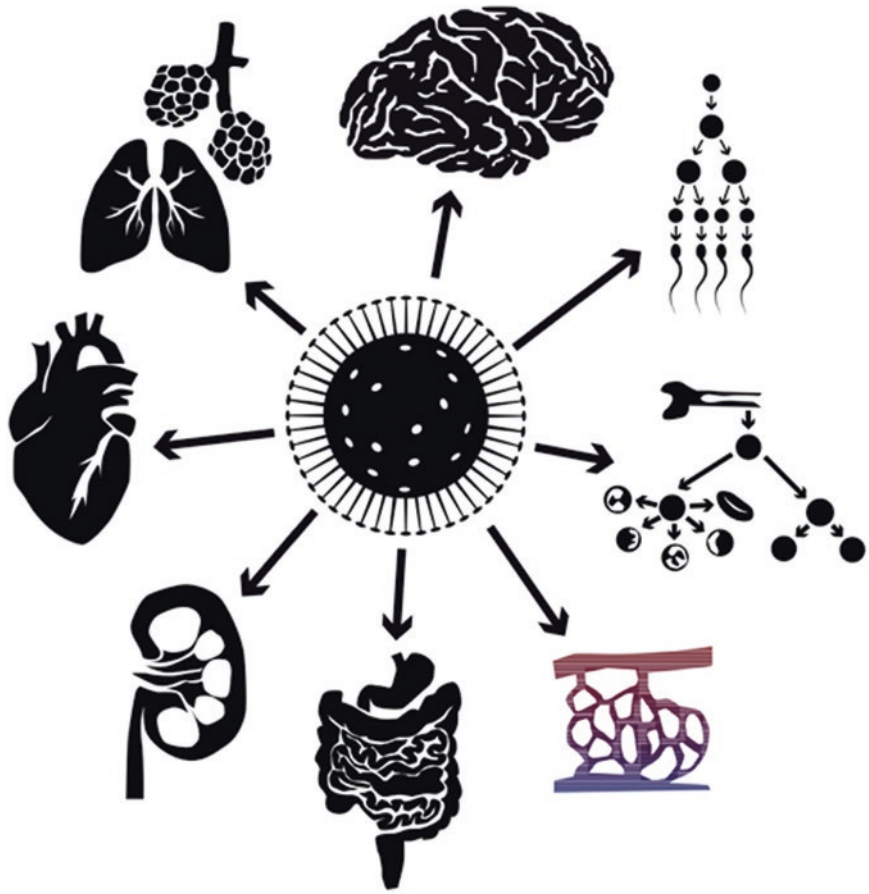

Fig. 4.12 Allocation of SARS-CoV-2 to the various body systems. (Source: own authorship)

4.3.2 Pathogenesis of COVID-19 in the Respiratory System

The process of aggression to the respiratory system determined by the SARS-CoV-2 virus has three distinct and sequential stages. STAGE 01 is characterized by the inhalation of the SARS-CoV-2 virus, adhering to and penetrating the cells of the respiratory epithelium through the receptor of the angiotensin-converting enzyme 2 (ACE2), beginning its replication (Mason 2020). This concept is being revised, considering that *single-cell RNA* reveals low levels of ACE2 expression in *Schneiderian* epithelial cells (Reyfman et al. 2019). In Stage 02, the virus spreads by migrating through the airways toward the alveoli, triggering a more robust innate response. In this moment, the COVID-19 becomes manifest and the expression of CXCL 10, also known as interferon-gamma-induced protein 10 (IP-10) or other cytokines of the innate response, may be predictive for the course of the disease (Tang et al. 2005). It is also known that epithelial cells infected by SARS-CoV-2 are important sources of interferon beta and lambda, and that CXCL 10 is responsive to interferons playing an important role in alveolar type II cell response (Qian et al. 2013; Wang et al. 2011). In about 80% of patients, COVID-19 will be restricted to infection of the upper airways and air conduction system with discrete clinical manifestations (Wu et al. 2020a, b, c). Unfortunately, in about 20% of patients, there is progression to Stage 03. At this stage, the SARS-CoV-2 virus particularly infects type II pneumocytes and macrophages using the ACE2 receptor for entry and the TMPRSS2 serine pro-

tease for protein initiation S (Benvenuto et al. 2020; Mossel et al. 2008). The most compromised pulmonary airways are the peripheral and subpleural (Wu et al. 2020a, b, c). The production of viral particles in type 02 pneumocytes triggers the mechanisms of apoptosis, culminating in the massive death of these cells (Qian et al. 2013). With death, viral particles are released in the quantity that starts to function as a self-replicating pulmonary toxin capable of restarting the viral cycle in adjacent type 2 pneumocytes. The compromised regions will largely lose their type 2 cells and activate the secondary epithelial repair pathway. It is known that, in general, pneumocytes type 02 is the precursor cell for pneumocytes type 01, thus alveolar regeneration will be impaired (Kumar et al. 2011). The morphological result of the pulmonary aggression of the SARS-CoV-2 virus is the diffuse alveolar damage with hyaline membranes rich in fibrin and cytodebris, and in between multinucleated giant cells (Xu et al. 2020a, b, c; Jenkins et al. 2020).

With the repair, redundant healing of the lesions occurs, with more intense interstitial fibrous densification than in other forms of acute respiratory discomfort syndrome. Satisfactory recovery will require a robust primary and secondary immune response in addition to efficient epithelial regeneration (Shield 2020).

4.3.3 Pathogenesis of COVID-19 in the Heart

The myocardial changes determined by SARS-CoV-2 are made through several routes. (1) The direct form is made via binding to the angiotensin-converting enzyme 02 (ACE2), an aminopeptidase bound to the membrane that is highly expressed in the heart and lungs. Evidence shows that SARS-CoV-2 alters ACE2 signaling pathways (Xiong et al. 2020; Li et al. 2020a, b, c, d, e, f, g). (2) The most severe forms of COVID-19 are characterized by an acute systemic inflammatory response, with a cytokine storm, presenting high levels of pro-inflammatory cytokines, resulting in lesions of multiple organs, including the heart (Zhou et al. 2020a, b, c; Huang et al. 2020). (3) The increase in cardiometabolic demand associated with systemic infection and the hypoxia determined by acute respiratory disease impair the myocardial oxygen demand–supply relationship with consequent acute myocardial injury (Bansal 2020). (4) The increase in myocardial stress determined by the increase in the conarian flow can result in myocardial infarction, aggravated by the state of systemic inflammation (Bansal 2020). (5) Various antiviral drugs, the use of corticosteroids, and other therapies used in the treatment of COVID-19 can have deleterious effects on the myocardium (Bansal 2020). (6) Electrolyte disturbances, frequent in severe systemic diseases, precipitating cardiac arrhythmias, can be observed in COVID-19, with particular attention to hypokalemia considering the injection of SARS-CoV-2 in the renin–angiotensin–aldosterone system (Chen et al. 2020a, b, c, d, e).

The changes described above are expressed morphologically as cardiomegaly, with ventricular dilation and *cor pulmonale*; in addition to arrhythmias, left ventricular systolic dysfunction, heart failure, and acute cardiac injury with high levels of troponin, often culminating in death (Fox et al. 2020; Sawicki and Sawicki 1990; Varga et al. 2020). Furthermore, there is a description of myopericarditis with significant diastolic dysfunction (Inciardi et al. 2020).

4.3.4 Pathogenesis of COVID-19 in the Gastrointestinal System

The description of the classic clinical presentation of COVID-19 appears with fever, cough, and an acute respiratory syndrome characterized by dyspnea; some patients have other complaints such as myalgia, headache, sore throat, adynamia, and anosmia. It should be noted that digestive complaints are not uncommon, such as diarrhea, vomiting, and abdominal pain (Cennimo and Bronze 2020; Wong et al. 2020a, b). Previous trials studying severe acute respiratory syndrome (SARS) showed that the coronavirus exerts tropism in the gastrointestinal tract. Studies have identified SARS-CoV-2 RNA in stool samples from infected patients, and a sample of human

tissue from the digestive tract evaluated by electron microscopy revealed intense viral replication in the small and large intestine (Leung et al. 2003). This evidence suggests fecal transmission via fomites, especially when aerosols are generated during flushing the toilet (Yu et al. 2020). Studies reveal that the ACE2 enzyme is redundantly expressed in the epithelial cells of the digestive tract, upper esophagus, liver, small intestine, and colon and that SARS-CoV-2 uses the SARS-CoV ACE2 receptor for entry into the enterocyte and serine protease TMPRSS2 for protein S initiation, with important implications for disease management, transmission, and infection control (Wu et al. 2020a, b, c; Hilton et al. 1986).

In addition to the clinical presentation with gastrointestinal signs and symptoms, the literature shows that patients with COVID-19, around 14.8–53.1% of patients, may have liver damage with serum elevation of the enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the course of the disease, in addition to mild to moderate elevation of serum bilirubin (Guan et al. 2020; Huang et al. 2020; Shi et al. 2020; Yang et al. 2020; Zhao et al. 2020a, b). Liver damage is mild and transient, although severe liver damage can occur, and severe hepatitis can be seen with serum ALT increased by up to 7590 U/L (Chen et al. 2020a, b, c, d, e). Besides, in patients with SARS-CoV-2 infection, changes in the coagulation pathway and fibrinolytic pathway are reported, apparently associated with a significant activation of the innate immune response with excessive and disordered release of cytokines and consequently an overexpressed inflammatory state (Li et al. 2020a, b, c, d, e, f, g).

Currently, studies on the mechanisms of liver injury related to SARS-CoV-2 are limited. However, it is reported that SARS-CoV-2 also uses ACE2 as its input receptor similar to SARS-Cov (Xu et al. 2020a, b, c; Hoffmann et al. 2020). The literature has shown that both hepatocytes and bile duct cells express ACE2. However, the expression of ACE2 in bile duct cells is more robust than in liver cells. Bile duct epithelial cells are known to play an important role in liver regeneration and immune response (Banales et al. 2020). These results suggested that the liver damage that occurred in patients with COVID-19 may be due to damage to the cells of the bile duct, but not to the liver cells due to the virus infection. Besides, the cytokine inflammatory storm has been observed in severe cases of COVID-19 (Tay et al. 2020). Postmortem biopsies were recently performed on a patient dying from COVID-19, and the results showed moderate microvascular steatosis and moderate lobular and portal activity, indicating that the injury could have been caused by SARS-CoV-2 infection or liver damage drug-induced (Tian et al. 2020). Similar to the situation in SARS, antibiotics, antivirals, and steroids are widely used in the treatment of COVID-19 (Tian et al. 2020). These drugs are potential causes of liver damage during COVID-19, but they are not yet evident (Boeckmans et al. 2020). In fact, a recent study reported that liver damage seen in patients with COVID-19 may be caused by lopinavir/litonavir, which is used as antivirals to treat SARS-CoV-2 infection (Cao et al. 2020). So far, reports are lacking that liver failure occurs in patients with COVID-19 with chronic liver diseases, such as chronic hepatitis B or C (Sun et al. 2020).

4.3.5 Pathogenesis of COVID-19 in the Circulatory System

SARS-CoV-2 infects the host using the angiotensin-converting enzyme receptor 2 (ACE2), which is expressed in various organs, such as the lung, heart, kidney, and gastrointestinal tract. ACE2 receptors are also expressed diffusely and widely in endothelial cells. Despite the various existing scientific tests, it is not known whether the vascular disorders observed in COVID-19 are consequences of infection of endothelial cells by the SARS CoV-2 virus. However, interestingly, SARS-CoV-2 can directly infect human blood vessel structures manipulated *in vitro* (Monteil et al. 2020).

The studies by Varga et al. 2020 in a series of three cases clearly show the presence of viruses in endothelial cells and accumulations of inflam-

matory elements, with evidence of endothelial cell death and formation of pyocytes, suggesting that infection by SARS-CoV- 2 facilitates the induction of endothelium in several organs as a direct consequence of viral infection and a concomitant inflammatory response from the host. They also report the importance of the role of apoptosis and pyroptosis in endothelial cell damage in patients with COVID-19. Thus, COVID-19 endothelium would explain the systemic microcirculatory damage dysfunction in different vascular beds and their clinical sequelae in patients with COVID-19. This evidence reinforces the need to maintain endothelial stability while coping with viral replication, particularly with anti-inflammatory anticytokine drugs, ACE inhibitors, and statins (Bansal 2020; Anderson et al. 1995; Feldmann et al. 2020).

4.3.6 Pathogenesis of COVID-19 in the Hematological System

SARS-CoV-2 is approximately 80% similar to SARS-CoV and invades human host cells by binding to the angiotensin-converting enzyme 2 (ACE2) receptor (Zhu et al. 2020). Although it is well documented that SARS-CoV-2 manifests itself mainly as a respiratory tract infection, outcrop data suggest that COVID-19 should be considered a systemic disease involving multiple systems, including the cardiovascular, respiratory, gastrointestinal, neurological systems, immunological, and even hematological (Rothan and Byrareddy 2020). COVID-19's mortality is lower than SARS and Middle East Respiratory Syndrome (MERS) (Wu et al. 2020a, b, c); nevertheless, COVID-19 is more lethal than seasonal flu. Data in the literature demonstrate that COVID-19 is a systemic infection with a significant impact on the hematopoietic system and hemostasis. Lymphopenia can be considered an interesting laboratory discovery, with a potential prognosis. The neutrophil–lymphocyte ratio and the platelet-to-lymphocyte ratio may also have a valuable prognostic in determining severe cases (Henry 2020; Qu et al. 2020).

During the disease, assessing lymphocyte counts and inflammatory markers, including LDH, CRP, and IL-6, can help identify cases with a dismal prognosis and prompt intervention to improve patient recovery (Kermali et al. 2020). Biomarkers, such as procalcitonin and serum ferritin elevated, have also emerged as poor prognostic factors. Besides, blood hypercoagulability is common among patients hospitalized with COVID-19 (Zhou et al. 2020a, b, c). Elevated levels of D-dimer are reported in the literature, relating its gradual increase in the course of the disease with its worsening. Other coagulation changes, such as exacerbation of PT and APTT, increase fibrin degradation products, with severe thrombocytopenia, leading to life-threatening disseminated intravascular coagulation (DIC), which requires continuous surveillance and immediate intervention (Marietta et al. 2020). Patients infected with COVID-19, hospitalized or on an outpatient basis, are at high risk for venous thromboembolism, and early and prolonged pharmacological thromboprophylaxis with low molecular weight heparin is recommended (Terpos et al. 2020).

Although the development of autoimmune antibodies or immune complexes triggered by COVID-19 can play a significant role in hematological changes, especially the induction of thrombocytopenia, SARS-CoV can also directly infect stem cells/hematopoietic progenitors, megakaryocytes, and platelets, inducing its growth inhibition and apoptosis (Yang et al. 2005).

It should be noted that studies of protein modeling and molecular docking revealed that some structural and nonstructural viral proteins of SARS-CoV-2 can bind to porphyrin and, simultaneously, to the orf1ab, ORF10, and ORF3a proteins, carrying out a chemical reaction along the beta-chain 1 of hemoglobin dissociating iron. This reaction, in turn, reduces the volume of hemoglobin available to carry oxygen and carbon dioxide, determining symptoms of respiratory distress with significant dyspnea (Wenzhong and Hualan 2020). Last but not least, studies of 2173 patients with COVID-19 in China, evaluating the ABO blood system, showed that patients in blood

group A were associated with a higher risk of acquiring COVID-19 compared to patients of blood groups non-A. On the other hand, patients of blood group O were associated with a lower risk of infection compared to patients of blood groups, non-O (Zhao et al. 2020a, b).

4.3.7 Pathogenesis of COVID-19 in the Urinary System

Most patients with SARS-CoV infection who progressed to kidney failure ended up dying (Chu et al. 2005). A retrospective study of 201 patients with confirmed COVID-19 pneumonia in China showed that 41.8% developed acute respiratory distress syndrome and 4.5% developed kidney injury (Wu et al. 2020a, b, c).

After lung infection, the virus can enter in the blood, accumulate in the kidneys, and cause damage to resident kidney cells. Potential mechanisms of kidney injury in patients with COVID-19 may involve damage to cytokines, organ crosstalk, and systemic effects (Ronco and Reis 2020). In patients with cytokine release syndrome, the injury can occur as a result of intrarenal inflammation, increased vascular permeability, volume depletion, and cardiomyopathy, which can lead to cardiorenal syndrome type 1. The syndrome includes systemic endothelial injury, which is clinically manifested such as pleural effusions, edema, hypertension, ascites, loss of fluid in the third space, depletion of intravascular fluid, and hypotension (Wu et al. 2020a, b, c; Huang et al. 2020; Ronco and Reis 2020).

In the kidney, ACE2 is present in podocytes, mesangial cells, parietal epithelium of Bowman's capsule, proximal cells brush the border and collecting ducts (Hamming et al. 2004). Several models of nephropathy have shown that ACE2 is implicated in the reduction of glomerular and tubular damage, as well as in fibrosis (Mizuiri 2015; Ye et al. 2006). The difference between the higher renal tropism of SARS-CoV-2 and SARS-CoV can be explained by the increased affinity of SARS-CoV-2 for ACE2, allowing a higher viral load in various organs and, especially, in the kidneys. It can act as a viral reservoir (Perico et al. 2020). An additional study of 26 autopsies found viral particles characteristic of SARS-CoV-2 in the proximal tubular epithelium and podocytes by electron microscopy (Su et al. 2020).

The most frequent finding of renal dysfunction in patients with COVID-19 is mild to moderate proteinuria (Cheng et al. 2020). If a pathological process increases glomerular levels of angiotensin II, podocytes acquire a dysfunctional phenotype mediated by cellular responses, involving calcium signaling, restructuring of the cytoskeleton, and internalization of nephrin, resulting in single nephron hyperfiltration and the manifestation of proteinuria (Königshausen et al. 2016; Srivastava et al. 2018). In patients with RSC, AKI can occur as a result of intrarenal inflammation, increased vascular permeability, and volume depletion, which is reflected in the findings of autopsies of erythrocyte aggregates that obstruct the capillary lumen without platelet or fibrinoid material. Pro-inflammatory IL-6 is considered the most important cytokine in RSC. Among patients with COVID-19, the plasma concentration of IL-6 is increased in those with acute respiratory distress syndrome (Wu et al. 2020a, b, c).

A prospective study involving 701 patients with SARS-CoV-2 infection, with moderate or severe disease, revealed that 43.9% exhibited proteinuria and 26.7% hematuria at hospital admission, while about 13% had elevated serum creatinine levels, serum BUN in the blood, or both, with acute kidney injury occurring in only 5.1% of patients during hospitalization (Cheng et al. 2020). At the intensive care unit in Wuhan (China), evidence proved that in 52 critical patients infected with COVID-19, acute kidney injury was the most common extrapulmonary complication seen in 29.0% of them, followed by cardiac injury (23%) and liver dysfunction (23%). Among patients with acute kidney injury, 25% underwent continuous renal replacement therapy. Eighty percent died after a hospitalization period of around seven days (Yang et al. 2020).

4.3.8 Pathogenesis of COVID-19 in the Nervous System

Human coronaviruses (HCoV) are respiratory pathogens that have already been associated with the development of neurological diseases due to their neuroinvasive and neotropical properties. The viral virus (S) glycoprotein appears to be associated with these neurological characteristics and is an important virulence factor for several coronavirus species, including HCoV-OC43 (Le Coupanec et al. 2015). Recent reports suggest that brain infection in patients with COVID-19 is being seriously considered due to many reports of neurological impairments, such as stroke, epilepsy, and encephalitis (Harberts et al. 2011), as well as changes in mental health, showing symptoms such as anosmia and ageusia, thus indicating a neuroinvasive nature of the virus (Baig et al. 2020; DAS et al. 2020). In this sense, it must be considered that the long-term effects of the neuroinvasive nature of the virus may increase the risk of neurodegenerative diseases with the involvement of neurological disorders such as Parkinson's disease or multiple sclerosis (Toljan, 2020).

The neurological manifestations of SARS-CoV-2 were recognized from computed tomography and magnetic resonance imaging of the brain of a patient who contracted COVID-19 and showed symptoms of necrotizing hemorrhagic encephalopathy (Poyiadji et al. 2020), characterized by lesions symmetrical multifocal in the brain, affecting the brain stem, thalamus, cerebellum, and brain white matter. This disease causes neuroinflammation resulting from a cytokine storm characterized mainly by the production of interleukin-6 (IL-6), secreted by macrophages, which in turn were activated by the granulocyte-macrophage colony-stimulating factor (GM-CSF) produced by cells auxiliary T (Mehta et al. 2020; Toljan 2020). In the magnetic resonance images of the patient with necrotizing hemorrhagic encephalopathy, infected with SARS-CoV-2, hemorrhage can be seen through the intensity of the hypointense signal in the images weighted in susceptibility and increased border in the post-contrast images (Poyiadji et al. 2020).

ACE2 expression in glia and neurons in the brain is low and the specific sites where SARS-CoV2 enters the brain are not clearly identified (Harberts et al. 2011). Studies suggest that the SARS-CoV-2 virus must first invade non-neuronal OE cells (olfactory lining cells) expressing high ACE2 and then move on to mature ORNs with low ACE2 expression to finally be transported along olfactory axons to the brain (Baig et al. 2020). Although SARS-CoV-2 is not yet detected in cerebrospinal fluid, SARS-CoV with similar structural and functional characteristics has been detected in patients' cerebrospinal fluid, indicating the virus's ability to break through the extremely rigid blood–brain barrier. As SARS-CoV-2 reports appear reaching the blood–brain barrier through circulating blood and violating it by attacking the endothelial layer to gain access to the CNS, the virus may be just using an alternating route in the shape of the olfactory bulb of the hematological pathway common (Baig et al. 2020). The olfactory mucosa is connected to the olfactory bulb through the cribriform plaque, found at the very base of the frontal lobes of the brain, a fact that may explain the neurological symptoms presented by patients with COVID-19. Researchers reported the infiltration of the SARS-CoV and MERS-CoV virus, when administered intranasally, in the brains of transgenic mice, mainly affecting the thalamus and the brain stem (Li et al. 2020a, b, c, d, e, f, g). Furthermore, reports of infection of the gastrointestinal tract by SARS-CoV-2 suggest that the virus could even use the enteric nervous system and its sympathetic neurons to reach the CNS (Toljan 2020).

Patients who report early neurological symptoms, such as loss of taste or smell or even seizures, should be tested for COVID-19 and kept under constant observation by neurologists as the latency period may be sufficient for the virus to completely annihilate the medullary neurons and threaten the patient's life (Li et al. 2020a, b, c, d, e, f, g). In a retrospective study of 214 patients admitted with COVID-19 to a hospital in Wuhan, 36.4% had some type of neurological manifestation, categorized as CNS involvement (24.8%), peripheral (10.7%), and musculoskeletal (10.7%),

noting that the most common symptoms were dizziness, headache, hypogeusia, and hyposmia (Mao et al. 2020). In China, studies have reported that headache is the most common symptom when in a sample of more than 1000 patients with COVID-19 (Guan et al. 2020), 13.6% reported headache (15% in severe forms), and 15% had myalgia, and 13.7% high levels of creatine kinase (19% in severe cases) and two cases of rhabdomyolysis (0.2%).

Encephalopathy is a transitory syndrome of cerebral dysfunction that manifests itself as an acute or subacute impairment of the level of consciousness. The risk of having an altered mental state associated with COVID-19 is higher in people with advanced age or with previous cognitive deterioration, as well as in those with vascular risk factors (hypertension) and previous comorbidities (Mao et al. 2020; Filatov et al. 2020). Elderly patients with vascular risk factors appear to be at a higher risk of developing cerebrovascular complications when developing COVID-19 than younger people without comorbidities (Li et al. 2020a, b, c, d, e, f, g). In a retrospective study of 221 patients with Wuhan COVID-19, 11 (5%) had an ischemic stroke, 1 (0.5%), cerebral thrombosis of the venous sinuses, and 1 (0.5%), a cerebral hemorrhage. Cerebral edema was detected in autopsies of patients who died of COVID-19 (Xu et al. 2020a, b, c). Some authors postulate that acute necrotizing encephalopathy is related to the cytokine storm syndrome described by COVID-19 (Mehta et al. 2020). Magnetic resonance imaging of a patient with COVID-19, presenting cough and altered mental status, revealed hemorrhagic lesions of multifocal and symmetrical disposition, in the annular form in the thalamus, in the insula, and in the medial region of the temporal lobes (Poyiadji et al. 2020).

The diagnosis of encephalitis was confirmed in a patient (56 years old) with a decrease in the level of consciousness through the isolation of SARS-CoV-2 in the cerebrospinal fluid using genomic sequencing techniques (Xiang et al. 2020). Similarly, SARS-CoV-2 RNA was detected in cerebrospinal fluid, using RT-PCR, in a patient (24 years old) who had generalized epileptic seizures and decreased level of consciousness, in addition to hyperintense areas in the right lateral ventricle, in the mesial region of the temporal lobe and the hippocampus of the brain (Moriguchi et al. 2020).

The exact route by which SARS-CoV-2 could enter the CNS is currently unknown (Li et al. 2020a, b, c, d, e, f, g). Coronaviruses can cause disorders of the nasal epithelium and, in certain circumstances not yet well known, can cross the epithelial barrier and reach the bloodstream or lymphatic system and spread to other tissues, including the CNS. SARS-CoV-2 is known to bind to ACE2 receptors on endothelial cells, which can lead to an increase in blood pressure. The increase in blood pressure, together with the presence of thrombocytopenia and hemorrhagic disorders, is a factor that can contribute to the increased risk of ischemic and hemorrhagic stroke in patients with COVID-19 (Carod-Artal 2020). The predominant health units that care for patients infected with COVID-19 should include neurologists to gain more perspective on the nature of the infections, which have a great chance of becoming neurological. At the same time, it is important to perform autopsies on the brains of those with neurological symptoms, to define a neuroinfection range of the disease, and adopt appropriate control measures (Das et al. 2020).

4.3.9 Pathogenesis of COVID-19 in the Reproductive System

Recently, the potential pathogenicity of SARS-CoV-2 to testicular tissues has also been proposed by doctors, implying fertility concerns in young patients (Fan et al. 2020). Since ACE2 is located on the X chromosome, there may be alleles that confer resistance to COVID-19, explaining the lower mortality rate in women. Alternatively, the sex hormones estrogen and testosterone have different immunoregulatory functions, which can influence immune protection or the severity of the disease (Taneja 2018).

Several molecular characteristics of SARS-CoV-2 can justify the presence of viruses in the

testis and possible changes in spermatogenesis and endocrine function. The presence of SARS-CoV-2 in the seminal fluid may have sexual and reproductive implications (Aversa and Jannini 2020), giving rise to new information about infection and transmission by COVID-19, despite the lack of investigations at the moment. In particular, the presence of the ACE2 receptor, a binding site for SARS-CoV-2, has been recorded in seminiferous ductal cells, Leydig and Sertoli cells in the testicles, confirming the potential risks associated with SARS-CoV-2 infection in the reproductive system (Wang et al. 2020a, b; Fan et al. 2020). Spermatogonia positive for ACE2 expresses a higher number of genes associated with reproduction and viral transmission and a lower number of genes related to spermatogenesis compared to spermatogonia negative for ACE2. ACE2-positive Leydig and Sertoli cells express higher genes involved in cell–cell junction and immunity and lower genes associated with mitochondria and reproduction (Wang et al. 2020a, b).

Recent studies have shown that patients with COVID-19 showed a significant increase in serum LH level and a dramatic decrease in the serum ratio of testosterone (T) to luteinizing hormone (LH) when compared to healthy men matched with age and with normal fertility (Pozzilli and Lenzi 2020). These findings provide evidence that the human testis is a potential target for SARS-CoV-2 infection, which can have a significant impact on our understanding of the pathophysiology of this rapidly spreading disease (Wang et al. 2020a, b). Currently, there are no data on the clinical significance of ACE-2 receptors on Leydig synthetic cells. However, it is clear that reproductive function needs to be monitored in men who have recovered from COVID-19 (Xu et al. 2006; Gupta and Misra 2020).

4.3.10 Heat Shock Protein in COVID-19

Heat shock proteins (HSPs) are a group of specific proteins produced when cells are subjected to temperatures above their usual growth temperature. The synthesis of HSPs is a universal phenomenon, occurring in all plant and animal species, including humans and prokaryotic cells. Since HSPs can also be induced by oxidants, toxins, heavy metals, free radicals, viruses, and other stressors, they are often called "stress proteins." The highlight members of this group are a class of functionally related proteins involved in the folding and unfolding of other proteins. The main representative of heat shock proteins are chaperones, which often promote the self-assembly of polypeptide chains of proteins recently synthesized in a native spatial structure, as well as the assembly of their complexes and their transport through membranes, further assisting in signal transduction (Matz et al. 1995; De maio 1999; Ritossa 1996).

Several aspects of the interaction of SARS-CoV-02 with heat shock proteins have been described in the literature. One of these interactions is the effect of heat and ambient temperature on the SARS-CoV-02 infection; Hedayati (2020) reports that the HSP72 heat shock protein increases the gene expression of the SARS-CoV-02 virus receptor ACE2 (Matz et al. 1995). Similar work carried out by Elfiky 2020 points out that thermal shock protein A5 (HSPA5) has a possible role in the internalization of the SARS-CoV-02 virus (Matz et al. 1995). Kasperkiewicz 2021 showed the development of autoimmune bullous dermatoses associated with COVID-19, possibly due to the mimicry of SARS-CoV-02 with human proteins, in particular, heat shock proteins 60 and 90 (Matz et al. 1995). Trials show that the risk for fatal outcome in patients with COVID-19, elderly and patients with pre-existing metabolic and cardiovascular diseases have the characteristic of being chronic-degenerative entities of an inflammatory nature associated with a defective response to thermal shock (HSP) (Matz et al. 1995). In December 2020, Rébé, Ghiringhelli, and Garrido describe a possible pathway for mitigating the cytokine storm using heat shock protein 70 (Matz et al. 1995). Successful are the tests that point to heat shock proteins as a pharmacological target in the treatment of COVD-19 (Matz et al. 1995; De maio 1999; Ritossa 1996).

From the above, it is clear that there is a clear antipode effect presented by the heat shock proteins in COVID-19, sometimes participating in viral internalization through various mechanisms, and sometimes as a precipitant of more aggressive clinical conditions, when not suitable for therapeutic targets. What is known seems to be the tip of the iceberg, there is a lot to investigate for a better understanding of the relationship between HSPs and COVID-19.

4.3.11 COVID-19 Reinfection

The infection determined by viruses, in general, gives rise to some degree of immunity to reinfection, with coronaviruses it is no different. This statement is not at all true or at least is questioned, considering that a small number of patients presented COVID-19 for the second time (Wilkinson 2021). A reflection presents itself: are the conditions described in the literature about reinfection or reactivation of the disease?

The literature describes four types of endemic coronavirus (229E, NL63, OC43, and HKU1) that are identified circulating in the human species, associated with respiratory tract infections. Whatever the viral strain, immunity varies in duration, generally ranging from one to two years (Liu et al. 2021). On the other hand, SARS-CoV-2, an entirely new viral strain of coronavirus, the etiological agent of COVID-19, its developed immunity, is still unknown. It is not known to define precisely whether the infection confers immunity to reinfection (Hanrath et al. 2020).

Initially, it was not believed that SARS-CoV-2 could lead to reinfection, at least in the UK, given that the 11,000 health professionals who revealed evidence of infection in the first wave between March and April 2020, there was no report of reinfection between October of November of the same year, leading to believe, at that moment, that the immunity determined by COVD-19 would last at least six months (Stokel-Walker and Journalist 2021).

A study by Wilkinson 2021 found that antibodies, the result of SARS-CoV-02 infection, provide 83% protection against COVID-19 reinfections over 5 months (Wilkinson 2021).

The BNO News website maintains a daily count based on the official country information. The aforementioned website informs that on January 22, 2021, 39 cases had been confirmed and almost 10,000 were under investigation (Pinheiro 2021).

Currently, the reinfection of COVID-19 is well established, as well as that the protection determined by antibodies will not be maintained for many years, perhaps around 5 months. An important study carried out in the UK showed that antibodies resulting from the initial infection with the new coronavirus, SARS-CoV-2, decrease rapidly in people who have recovered from COVID-19, making them vulnerable to new infection (Ries 2020). Although most cases of reinfection for COVID-19 presented mildly, without major complications, the antibody-dependent enhancement phenomenon (Kulkarni 2019), described in the 1960s and observed in infections by the Dengue virus (Dejnirattisai et al. 2010), respiratory syncytial virus (RSV) (Graham 2016), and measles virus (Polack 2007), related to greater effectiveness of reinfections, and often determining death, also evidenced in infectious feline peritonitis (Hohdatsu et al. 1998) and SARS-CoV and MERS-CoV infections (Lee et al. 2020) both in vitro and in vivo, has been a concern for COVID-19, concerning reinfection and in the use of vaccines.

Evidences show that patients who have the mildest symptoms in their initial infection are more likely to reinfect themselves, perhaps because they do not develop an effective immune response in the first infectious condition. The same is true for those who are immunosuppressed and, therefore, would not have mounted an immune response to the first infection, leading to the belief that the second event could be a viral reactivation and not, exactly, reinfection (Wilkinson 2021; Selvaraj et al. 2020). An essay by the Department of Medicine of Nuffield at Oxford University shows that many cases of reinfection may actually be reactivation (Simmonds et al. 2020).

Coronaviruses are responsible for long-lived infections and their prominent genomic structures seem to be implicated in their permanence in the body at low viremic levels to the point of not being detected, but ready to be reactivated (Tay et al. 2020).

The appearance of new variants of SARS-CoV-02, such as B.117 identified in the UK, P1 identified in Manaus, Brazil, and 501.Y.V2 identified in South Africa, may contribute to the appearance of reinfections of COVID-19, but this probability has not yet been estimated. It is assumed that reinfections by COVID-19 may occur more frequently with the new circulating strains due to an absolute increase in the number of infections in general. However, the reinfection is expected to be less aggressive.

The question that remains is about how much variation or genetic alteration should happen in the virus so that the immune system no longer recognizes it and does not show a satisfactory immune response. In the meantime, vaccine companies say that their products are effective against new circulating strains. Even if they are, the message remains for those vaccinated and for those who have recovered from COVID-19: the use of a mask, hand asepsis, and social distance should not be declined.

4.4 Final Considerations

The data suggest that COVID-19 is associated with SAR-CoV-2 infection, with the transmission of contagion by fomites, salivary droplets, and other forms, such as vertical and fecal–oral. The bat and other vertebrates appear to be reservoirs and part of the transmission chain. The virus uses cell receptors to infect human cells, especially ACE2, like other coronaviruses. Heat shock proteins have different roles in infection, sometimes facilitating it, sometimes participating in more severe conditions, when not serving as a therapeutic target.

The available data allow us to conclude that COVID-19 is a pandemic viral disease, behaving as a challenge for public health worldwide, determining aggressive conditions with a high mortality rate in patients with risk factors, without treatment, but with the recent availability of the first vaccines.

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Immunological and Hematological Response in COVID-19

5

Artur Słomka, Gennaro Martucci, Giuseppe Maria Raffa, Pietro Giorgio Malvindi, Ewa Żekanowska, Roberto Lorusso, Piotr Suwalski, and Mariusz Kowalewski

Abstract

Introduction: Infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has recently and rapidly emerged and developed into a global pandemic. Through the renin–angiotensin system, the virus may impact the lung circulation, but the expression on endothelium may conduct to its activation and further systemic damage. While precise mechanisms underlying these phenomena remain to be further clarified, the understanding of the disease, its clinical course, as well as its immunological and hematological implications is of paramount importance in this phase of the pandemic.

Methods: This review summarizes the evidence gathered until 12 June; electronic databases were screened for pertinent reports on coronavirus and inflammatory and hematological changes. Search was conducted by two independent investigators; keywords used were "SARS-CoV-2," "COVID-19," "inflammation," "immunological," and "therapy."

Results: The viral infection is able to trigger an excessive immune response in predisposed individuals, which can result in a "cytokine storm" that presents an hyperinflammation state able to determine tissue damage and vas-

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cular damage. An explosive production of pro-inflammatory cytokines such as TNF-α IL-1β and others occurs, greatly exaggerating the generation of molecule-damaging reactive oxygen species. These changes are often followed by alterations in hematological parameters. Elucidating those changes in SARS-CoV-2-infected patients could help to understand the pathophysiology of disease and may provide early clues to diagnosis. Several studies have shown that hematological parameters are markers of disease severity and suggest that they mediate disease progression. According to the available literature, the primary hematological symptoms-associated COVID-19, and which distinguish patients with severe disease from patients with nonsevere disease, are lymphocytopenia, thrombocytopenia, and a significant increase in D-dimer levels.

Conclusions: SARS-CoV-2 infection triggers a complex response altering inflammatory, hematological, and coagulation parameters. Measuring these alterations at certain time points may help identify patients at high risk of disease progression and monitor the disease severity.

Keywords

Coagulation disorders · COVID-19 · Immune response · Hematological changes · Lab tests · SARS-CoV-2

Abbreviations

ACE2	angiotensin-converting enzyme 2
aPTT	activated partial thromboplastin time
ARDS	acute respiratory distress syndrome
AT	antithrombin
AUC	area under curve
CAC	COVID-19-associated coagulopathy
CRRT	continuous renal replacement therapy
COVID-19	Coronavirus Disease 2019
DIC	disseminated intravascular coagulation
DFPP	double filtration plasmapheresis
ESR	erythrocyte sedimentation rate
FDP	fibrin degradation products
GAGs	glycosaminoglycans
ICU	intensive care unit
MASP-2	mannan-binding lectin-associated serine protease-2
MERS	Middle East respiratory syndrome
NLR	neutrophil to lymphocyte ratio
PAI-1	plasminogen activator inhibitor-1
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
TNF	tumor necrosis factor
TPE	therapeutic plasma exchange
UFH	unfractionated heparin
VA-ECMO	veno-arterial extracorporeal membrane oxygenation
VTE	venous thromboembolism

5.1 Introduction

During this winter season, the novel coronavirus [severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)], an enveloped virus with non-segmented single-stranded positive-sense RNA genome, is causing a pandemic of unprecedented magnitude (Huang et al. 2020; Chen et al. 2020a; Zhu et al. 2020). Many researchers are focusing on elucidating the mechanisms of infection and developing a drug or vaccine; however, much uncertainty still presides over many aspects of disease manifestation (Wang et al. 2020a). The spectrum of disease (called COVID-19) ranges from asymptomatic/mild and self-limiting respiratory tract infections (RTIs) – as far as we know this is the majority of infected people – to severe sepsis and ARDS with an hyperinflammatory

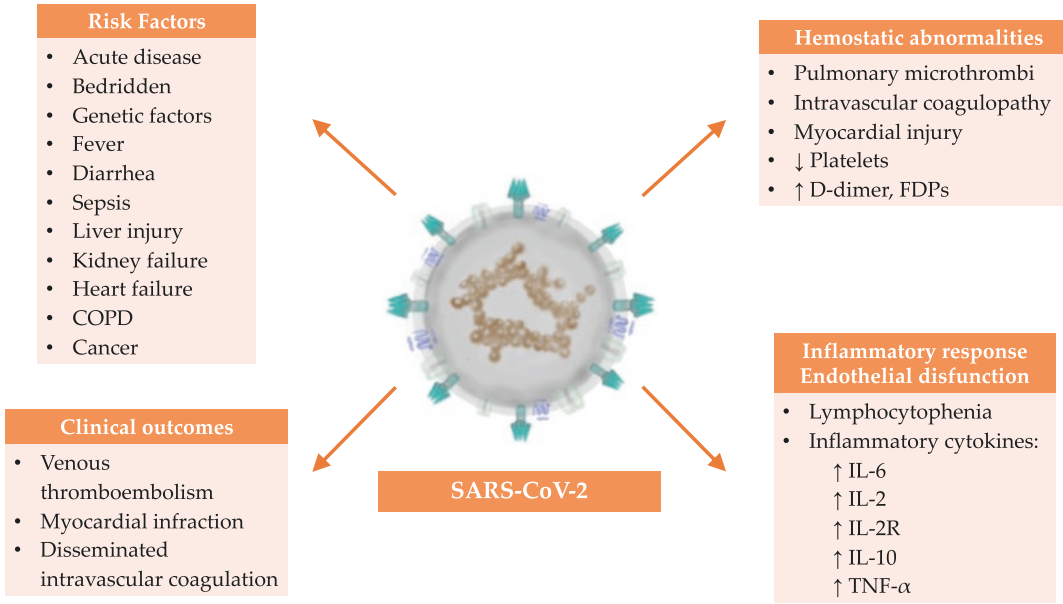

Fig. 5.1 SARS-CoV-2: severe acute respiratory syndrome coronavirus 2

phenotype often associated with multiorgan failure, especially in elderly patients and with comorbidities. SARS-CoV-2 enters target cells via the angiotensin-converting enzyme 2 (ACE2) by a receptor-mediated endocytosis (Fig. 5.1) (Guan et al. 2020).

ACE 2 is a type I integral membrane protein with several physiological functions, well expressed in lungs (overexpressed in smokers), heart, kidney, and gastrointestinal tract (Zhang et al. 2020a; Tang et al. 2020a). Through the renin–angiotensin system (RAS), the virus may impact the lung circulation, but the expression on endothelium may conduct to its activation and further systemic damage (Wang et al. 2020b; Tang et al. 2020b). Increasing evidence suggests that lymphocytopenia, thrombocytopenia, and disturbances in blood coagulation system, such as elevated levels of D-dimer, are the most common hematological abnormalities observed among coronavirus disease 2019 (COVID-19) patients, especially in the severe stage of infection, and may serve as diagnostic and prognostic tools for COVID-19 (Tan et al. 2020a; Li et al. 2004; Ludvigsson 2020; Wu et al. 2020).

While precise mechanisms underlying these phenomena remain to be further clarified, the understanding of the disease, its clinical course, as well as its immunological and hematological implications is paramount in this phase of the pandemic since new trials and new therapeutic approach should be based on the most precise medicine and knowledge (Shrestha et al. 2020).

5.2 Response to Viral Infection

5.2.1 Initial Response in SARS-CoV-2 Infection vs. V-V ECMO

The viral infection is able to trigger an excessive immune response in predisposed individuals, which can result in a "cytokine storm" that presents an hyperinflammation state able to determine tissue damage and vascular damage revealing as fluid leakage and vasodilation responsible of the very profound hemodynamic impairment and also of the exposure of large amounts of tissue factor exposure with coagulation factors activation and consumption (Cummings et al. 2020; Chousterman et al. 2017;

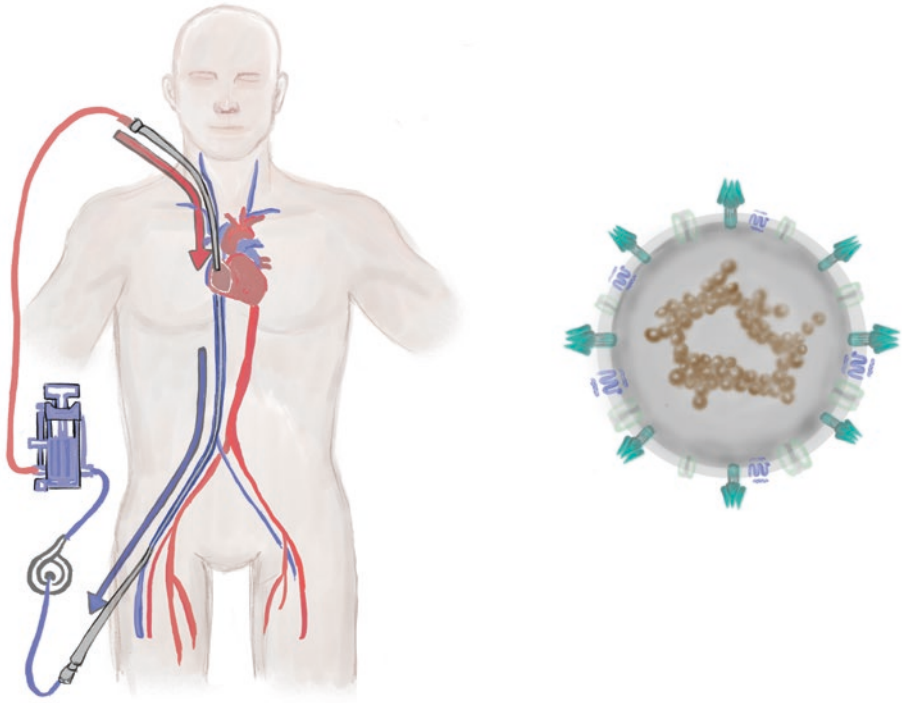

Fig. 5.2 SARS-CoV-2-mediated immunological and hematological changes. COPD: chronic obstructive pulmonary disease; FDP: fibrin degradation products; IL: interleukin

van der Poll and Opal 2008; Mehta et al. 2020) (Fig. 5.2). An explosive production of proinflammatory cytokines such as TNF-α IL-1β and others occurs, greatly exaggerating the generation of molecule-damaging reactive oxygen species (ROS). One of the causes of the hyperinflammatory state is the ability of immune cells to dramatically change their metabolism. Similar to cancer cells in many solid tumors, immune cells such as macrophages/monocytes under inflammatory conditions abandon mitochondrial oxidative phosphorylation for ATP production in favor of cytosolic aerobic glycolysis (also known as the Warburg effect) (Bar-Or et al. 2018). The change to aerobic glycolysis allows immune cells to become highly phagocytic, accelerate ATP production, intensify their oxidative burst, and to provide the abundant metabolic precursors required for enhanced cellular proliferation and increased synthesis and release of cytokines. Melatonin, an endogenous molecule, may be useful in this regard (Reiter et al. 2020). Melatonin has been found produced in mitochondria and consequently in every human cell specifically in lung monocytes/macrophage (Muxel et al. 2012). Melatonin has been proposed to reduce the highly proinflammatory cytokine storm and neutralize the generated ROS, thereby preserving cellular integrity and preventing lung damage (Martín Giménez et al. 2020). A similar and combined role may be hypothesized also for vitamin D (Amrein et al. 2020; Martucci et al. 2019). In the current limited health resources scenario, it would be important to adopt any adjuvant treatment that may contribute to a better outcome if it is inexpensive and with few or unimportant side effects at tested doses: vitamin D, vitamin C, as well as melatonin and other potential adjuvant of innate immune system seems to respond to this need.

As suggested by several authors, the main response to the SARS-CoV-2 is the innate immune system (Nasab et al. 2020; Birra et al. 2020). As a key player in this puzzle, there is for sure the complement system. It has, in fact, a relevant role as a bridge in both coagulation and

inflammatory system by a continuous cross-talk of mediators (Piacente et al. 2020). Early in COVID-19 disease, Diao et al. have recognized complement deposits in case of renal insufficiency (Diao et al. 2020). In a study from Italy, the plasma levels of sC5b-9 and C5a were significantly higher in COVID patients associated with a high level of acute phase protein release. This is relevant to understand the pathogenesis of pulmonary disease and potentially to recognize new therapeutic targets. In fact, it is known that C5a increase is able to promote the lung sequestration of leukocytes and pulmonary dysfunction, and that sC5b-9 has similar effects by causing transendothelial leukocyte migration and vascular leakage (Cugno et al. 2020; Gralinski et al. 2018).

Such involvement of the complement justifies the use of drugs impacting on this system. First of all, immunoglobulins have entered several protocols of treatment worldwide since their role to enhance specific immunity guided by antibodies (not only on the viral infections but also for the prolonged nosocomial and frequent bacterial superinfections) but also for its immunomodulant role able to decrease C5 activation and deposition of the membrane attack complex (Basta and Dalakas 1994). Moreover, following the pathogenesis of immune-mediated diseases with microangiopathy, the block of complement may be obtained by specific drugs targeting C5 like the humanized monoclonal antibody eculizumab or the mannan-binding lectin-associated serine protease-2 (MASP-2) by the human monoclonal antibody narsoplimab (Patriquin and Kuo 2019).

Interleukin-6 (IL-6), as part of the nonspecific innate immune response, is produced by activated leukocytes and endothelial cells and has as effectors many tissues and cells (Kruttgen and Rose-John 2012). In the cytokine release syndrome characterized by fever and multiorgan dysfunction, it plays a relevant pathogenetic role, and in COVID-19 has been recognized (accompanied by low lymphocyte count) to be associated to poor outcomes (Mehta et al. 2020). IL-6 is well known being linked to the trans-signaling pathway, which causes vascular leakage as the first step of a cascade followed by tissue edema, hypoxia, and finally necrosis.

Tocilizumab is a monoclonal antibody against IL-6 mainly used for the treatment of rheumatoid arthritis. It has recently emerged as an alternative treatment for COVID-19 patients with a documented cytokine storm (Fig. 5.3). Reports and single-center experiences have been documented, and its actual efficacy is going to be assessed by dedicated investigations [NCT04317092] (Luo et al. 2020; Michot et al. 2020; Zhang et al. 2020b).

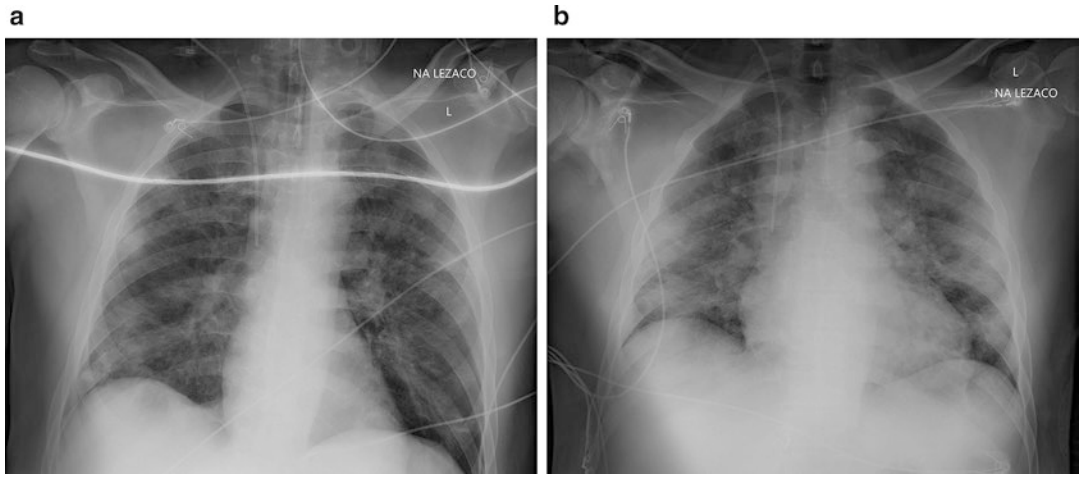

Fig. 5.3 Respiratory distress in cytokine storm with typical ground glass opacities (**a**), resolution of the lesions after treatment with tocilizumab (**b**)

5.2.2 Respiratory Distress vs. ARDS?

The majority of patients with severe manifestations of COVID-19 meet the criteria for the ARDS according to the Berlin definition (Arabi et al. 2020; Bellani et al. 2016; ARDS Definition Task Force et al. 2012). From the physiological side, the ARDS is represented by the so-called baby lung postulated by Gattinoni and collaborators (Gattinoni et al. 1987). Considering that ARDS is a syndrome, and consequently has a specific development even though with different causes (pulmonary and extrapulmonary), the respiratory impairment of COVID-19 probably is a different syndrome or at least should be classified as an atypical ARDS (Gattinoni et al. 2020a, b). In fact, at least initially (so before the ventilator associated lung injury, the self-induced lung injury or severe bacterial pneumonia does not reveal) the disease does not couple with the baby lung theory or classical CT scan images or physiological respiratory dynamics characteristics (Gattinoni et al. 2020c). In particular, the relevant finding since the beginning of the outbreak spread was a relevant hypoxia associated with almost normal compliance (Marini and Gattinoni 2020). In these patients, the hypoxemia is primarily due to the VA/Q maldistribution caused by the loss of the lung perfusion regulation. High tidal volume follows (increased strain) in association with higher transpulmonary pressure (stress) to assure oxygenation (Gattinoni et al. 2020d). If this increase in stress and strain remains without correction, patient self-inflicted lung injury develops, causing overt lung edema, inflammation, and lymphocyte sequestration (Fig. 5.4).

5.2.3 Other Organ Manifestations

Although COVID-19 is a new disease and much of its pathomechanism remains unknown, it is

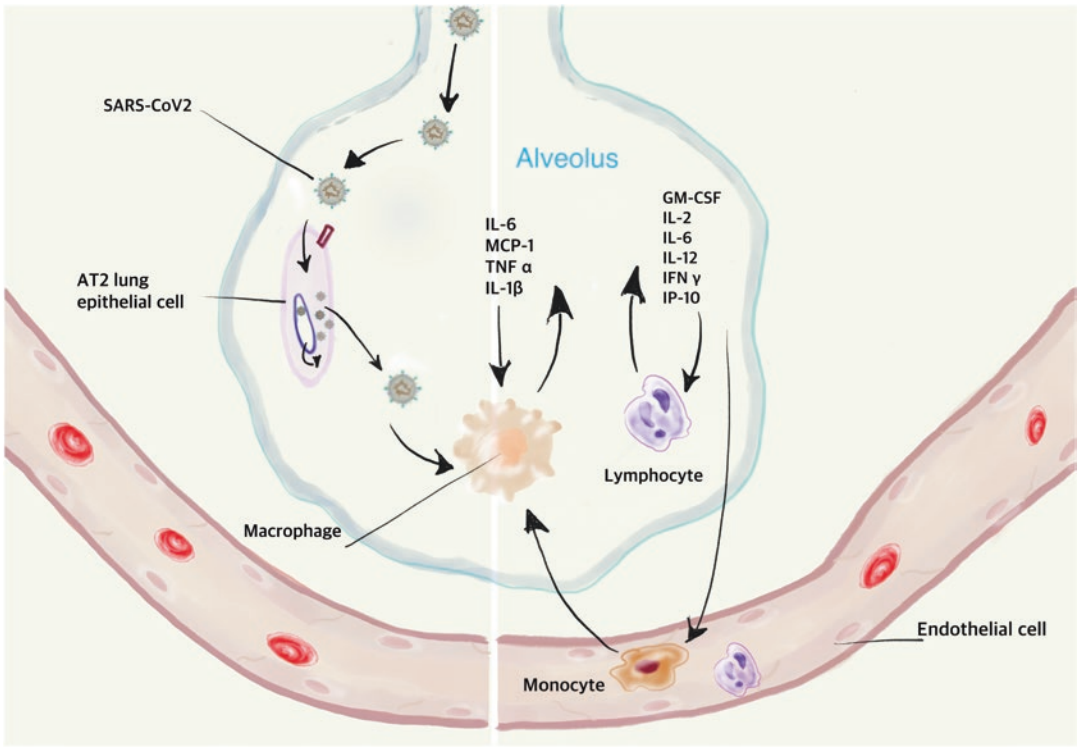

Fig. 5.4 Pathogenesis of respiratory failure in SARS-CoV-2 infection. AT2: angiotensin 2; IL: interleukin; MCP-1: monocyte chemoattractant protein-1; TNF: tumor necrosis factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; IFN: interferon; IP-10: interferon gamma-inducible protein

widely believed that COVID-19 is not a respiratory-only disease, but is a cardinal challenge for many medical disciplines. This is due to the fact that expression of ACE2, a receptor for SARS-CoV-2, is not limited to respiratory track only, but the high ACE2 gene expression is observed in many tissue types (Li et al. 2020a; Nicin et al. 2020; Song et al. 2020). In addition, cytokine storm associated with severe COVID-19 is a systemic disease with serious consequences for patients, including ARDS and multiple-organ failure (Ye et al. 2020). Moreover, an intensified inflammatory reaction, hypercoagulability and endothelial and cardiomyocytes damage are all factors that predispose COVID-19 patients to myocardial infarction (MI) (Atri et al. 2020). SARS-CoV-2-infected patients are at high risk of ischemic stroke; however, the mechanism underlying this phenomenon is unclear, but it has been proposed that high incidence of stroke is associated, similarly to MI, with inflammation, endothelial dysfunction, and blood coagulation imbalance (Oxley et al. 2020; Hess et al. 2020; Avula et al. 2020; Markus and Brainin 2020). Possible role of SARS-CoV-2 in liver failure has also been suggested by the finding that aminotransferases (ALT, AST), lactate dehydrogenase (LDH), creatinine kinase (CK), or myoglobin levels are increased in COVID-19 patients (Zhang et al. 2020c; Bangash et al. 2020). Five sophisticated mechanisms are engaged in liver injury, including inflammation, cytotoxicity of the virus, anoxia, drug-induced liver injury, and reactivation of pre-existing liver disease (Sun et al. 2020). Kidney is another organ that may be damaged in COVID-19 via the cytopathic effects of SARS-CoV-2 on podocytes and proximal straight tubule cells (Cheng et al. 2020; Pan et al. 2020). For the reasons set out above, COVID-19 is a highly contagious disease involving, especially in severe form, many systems, and requiring an interdisciplinary approach.

5.3 COVID and Hematological Changes

5.3.1 Hematological Symptoms of COVID-19

The clinical manifestations of COVID-19 are not consistent and may evolve depending on disease progression. Most cases are asymptomatic or have mild or moderate symptoms, while fewer are characterized by a severe or critical form of SARS-CoV-2 infection (Yuki et al. 2020; Lai et al. 2020; Fung et al. 2020). The purpose of this chapter is to familiarize readers with the primary hematological symptoms associated with SARS-CoV-2 infection and COVID-19 disease manifestation. An increasing number of studies have shown correlations between changes in the blood system and unfavorable rates of disease progression, which are also discussed in this chapter. We focus on discussing the results of original papers, excluding case reports concerning single or small patient presentation. Intentionally, this part has been divided into subsections describing changes in white blood cells (WBCs), red blood cells (RBCs), platelets (PLTs), and plasma coagulation parameters, which have become associated with infection with this novel pathogen.

5.3.2 WBC Count

Due to the nature of inflammatory reactions in response to viral infections, the leukocyte (WBC) system is highly affected in response to SARS-CoV-2 infection. Most of the articles cited here supply patient data relevant at the time of admission to a hospital. Therefore, the long-term effect of infection on the leukocyte population is unknown. Among cases with laboratory-confirmed SARS-CoV-2 infection, the majority of cases present with lymphocytopenia, defined

as lymphocyte counts below 1 × 10^9 cells/L (Huang et al. 2020; Guan et al. 2020; Wang et al. 2020b; Liu et al. 2020a).

In a preliminary report involving 41 COVID-19 patients (median age 49 years) published by Huang et al (2020), it is reported that 45% of patients exhibited a normal number of WBC (4−10 × 10^9 cells/L); however, patients with severe disease were found to have twofold higher WBCs than those with nonsevere disease. Overall, 40% of patients exhibited elevated number of WBCs (above 10 × 10^9 cells/L), whilst 25% subjects exhibited leukopenia (WBC count over 4 × 10^9 cells/L) (Tripodi 2011). Lymphocytopenia was the most common change, observed in 63% of patients. In addition, blood from severely ill patients contains more neutrophils and a lower number of lymphocytes compared with patients with nonsevere disease (Huang et al. 2020). Among the clinical characteristics that the authors identified in 138 infected patients (median age 56 years), elevated WBC and neutrophils counts were more prevalent in patients with severe COVID-19 disease manifestation. In turn, lymphocyte counts in this group were lower (median = 0.8 × 10^9 cells/L) than in nonsevere patients (median = 0.9 × 10^9 cells/L) (Wang et al. 2020b). The authors concluded that nonsurvivors exhibited more advanced lymphocytopenia compared with survivors. Low lymphocyte number was further confirmed as a primary hematological symptom of COVID-19 by a study of 137 patients (median age 57 years), of whom 72.3% had lymphocyte counts lower than 1 × 10^9 cells/L of blood (Tripodi 2011). Finally, Guan et al. reported on a study analyzing 1099 patients (median age 47 years) whereby they demonstrated that 33.7% of these were diagnosed with leukopenia, which inflated to over 61% of patients suffering from severe disease symptoms (Guan et al. 2020). This subgroup was also characterized by lymphocytopenia, which was reported in 96.1% of patients, confirming previous observations by other studies.

The research reviewed thus far concerned patients mostly from Wuhan (China). In contrast to those data, a study involving a small group of 13 Chinese patients (median age 34 years) located outside of Wuhan reported no changes in any of the leukocyte cell types and all the patients recovered (Chang et al. 2020). It may be relevant, however, that these patients were mostly adults without comorbidities and were much younger than those evaluated by other studies.

A further study conducted outside Wuhan reported that of 62 patients (median age 41 years) with mild to moderate clinical symptoms, the majority (62%) had normal WBC counts, while lymphocytopenia was diagnosed in 42% of patients. (Xu et al. 2020) Patient age and stage of the disease appear to be the key factors determining the development of lymphocytopenia and its progression in association with COVID-19. Eosinopenia was reported by Zhang et al. in more than half (52.9%) of 138 cases (median age 57 years) and in 78.8% of 52 COVID-19 patients by Li et al. (Zhang et al. 2020d; Li et al. 2020b).

5.3.3 Lymphocyte Populations

Preliminary studies have investigated the potential impact on lymphocyte subpopulations in response to SARS-CoV-2 infection. A study conducted by Liu et al. involving 40 COVID-19 patients (mean age 48.7 years), concluded that patients with severe disease manifestation had significantly lower numbers of CD3+ and CD8+ T cells both at time of admission and one week post admission. (Liu et al. 2020b) Chen et al. also reported below-normal CD4+ and CD8+ T cells in 21 COVID-19 patients (mean age 56 years), especially in those with severe disease manifestation. In addition, expression of interferon gamma (IFNγ) by CD4+ T cells was reduced in seriously ill patients, which plays a crucial role in the antiviral responses (Chen et al. 2020b). The precise mechanisms responsible for the decrease in lymphocyte number and activity are not known; however, this change seems to be crucial in the pathophysiology of the disease and is directly related to the severity of clinical symptoms. Lymphocyte pathways would therefore be a priority area for further research.

5.3.3.1 Predicting Severity of COVID-19: The Roles of NLR and N8R

Recent studies under review (Liu et al. 2020c; Zhang et al. 2020e) have proposed the possibility of predicting severity of disease based on the neutrophil to lymphocyte ratio (NLR). According to these studies, a high NLR is associated with severe presentation of COVID-19. More specifically, patients over 50 years of age and with an NLR $\geq$ 3.13 were characterized by severe disease symptoms, and these patients should be monitored intensively due to their vulnerability toward unfavorable disease progression (Liu et al. 2020c). In addition to NLR, Liu et al. observed that the neutrophil to CD8+ T cell ratio (N8R) correlated very well with disease severity, with an area under the curve (AUC) equal to 0.94 (Liu et al. 2020b).

5.3.3.2 Mechanism of Lymphocytopenia: Hypothetical Pathways

There appears to be extensive evidence that the majority of adults presenting with severe COVID-19 symptoms exhibit low lymphocyte counts (Cao 2020; Tan et al. 2020b). The diminished number of lymphocytes is also associated with other coronavirus diseases, including SARS and MERS (Li et al. 2004; Ko et al. 2016). Although the mechanisms of lymphocytopenia are not fully understood, three potential hypotheses are beginning to form. The first of these asserts that lymphocytopenia is associated with intensification of the inflammatory process. During COVID-19 disease, progression cytokine storm syndrome may occur, characterized by increased production of potent pro-inflammatory cytokines. The second proposes that SARS-CoV-2 may directly infect lymphocytes and lead to destruction of lymphoid organs (Cao 2020; Tan et al. 2020b; Lin et al. 2020). Third, glucocorticosteroids used to treat COVID-19 patients are known to cause lymphocytopenia (Yao et al. 2008). These are three extremely diverse hypotheses, but all concur that low lymphocyte count is directly related to the severity of COVID-19 disease, highlighting the importance of elucidating the precise mechanisms responsible for this hematological phenomenon for the accurate diagnosis and prognosis of COVID-19.

5.3.4 Red Blood Cells

No analyses performed to date showed any differences in hemoglobin levels between patients exhibiting severe COVID-19 symptoms and patients with mild/moderate disease manifestation (Huang et al. 2020; Li et al. 2020b; Liu et al. 2020c). However, using a systematic review and meta-analytical approach Lippi and Mattiuzzi have suggested that severely ill COVID-19 patients may have decreased hemoglobin levels (Lippi and Mattiuzzi 2020). These conclusions should be tentatively interpreted as only four studies were included in the statistical analysis and the featured high levels of heterogeneity in their reported findings. Notable, however, levels of iron-containing ferritin were elevated in patients with severe COVID-19 symptoms (Liu et al. 2020b; Chen et al. 2020b). This is associated with the intensification of inflammation, rather than with disturbances in iron metabolism (Northrop-Clewes 2008). Furthermore, Zhang et al. reported the interesting observation that erythrocyte sedimentation rate (ESR) is significantly higher in patients with severe disease manifestation. Increased ESR, as a marker to monitor, may be applicable in clinical practice for predicting the severity of COVID-19, with a very high AUC = 0.95 (Zhang et al. 2020f).

5.3.5 Platelets and Coagulation Markers

Most of the research reviewed in this chapter did not observe thrombocytopenia or platelet count differences between patients diagnosed with serious disease and patients with mild disease. One report involving the largest study group (n = 1099) observed reduced platelet counts in more than half (57.5%) of patients in the intensive care unit, with a median of 137,500 platelets/μL. A meta-analysis of nine studies covering

1779 COVID-19 cases showed that low platelet count is associated with increased severity and mortality of SARS-CoV-2-infected patients (Lippi et al. 2020). These diverse findings indicate a need for further investigation into the involvement of thrombocytopenia and the role of platelets in COVID-19 disease progression.

5.4 Coagulation Disorders in SARS-CoV-2 Infection

Coagulopathy, and more precisely hypercoagulability, is one of the most significant prognostic factors in COVID-19 and a number of definitions have flourished starting from the evaluation of the altered coagulation parameters. COVID-19-associated coagulopathy (CAC) has been proposed as well as the definition of MicroCLOTS (microvascular COVID-19 lung vessels obstructive thromboinflammatory syndrome) for the severe cases of pulmonary disease to underline the role in the pathogenesis of the most severe cases of the microvascular pulmonary thrombosis (Ciceri et al. 2020). The thromboinflammatory syndrome initially localized to the lungs, giving the widely availability of the ACE-2 receptors, in case of viremia, may involve other organs like the brain and the liver with very severe consequences largely illustrated by a number of series published on COVID-19 that highlighted the high rate of ischemic stroke and liver impairment (Gandhi and Goerlinger n.d.).

A relevant potential pathogenetic mechanism is the upregulation of tissue factor expression to circulating monocytes, thrombopoietin, and fibrinogen, as well as the downregulation of plasminogen activator inhibitor type 1 (PAI-1) by IL-6. Downregulation of PAI-1 is responsible of more stable and diffused resistance of thrombi with clot formation also in undamaged vessels contributing, in particular in the small pulmonary vessels to the characteristics of the early stage disease that has the pulmonary shunt as the main cause of hypoxia associated to a high elevated compliance (Gattinoni et al. 2020b, d)

Interestingly, the hypercoagulable state has been confirmed by viscoelastic tests exploration. In a thorough evaluation of coagulation parameters in 24 COVID-19 patients comparing standard but wide results associated with thromboelastography, Panigada and collaborators have demonstrated a decreased (considering the mean reference range value) R and K value [respectively 6.3 (3.0–11.9) and 1.5 (0.8–2.9)] and an increase of angle K and maximal amplitude MA [respectively 69.4 (51.1–78.5), and 79.1 (58.0–92.0)]. Such values are not deranged in absolute way and are accompanied by a mild reduction in platelets and antithrombin and probably should be interpreted in the light of the multiorgan impairment and population differences. We are far away from the understanding of the contribution of the endothelial activation, factor consumptions, and liver impairment in the single patient (Panigada et al. 2020).

Fibrinogen may be increased in case of mild and in the early phase of severe disease since it is an acute response protein and may be a contributing marker of hyperinflammation. But it should be considered that in late disease (usually after 7–10 days in the ICU) fibrinogen may be reduced due to large consumption and degradation as well as due to lower production.

Coagulation parameters used in routine practice appeared to be within normal range, although prolonged prothrombin time (PT) (Huang et al. 2020) and shortening of activated partial thromboplastin time (aPTT) (Wu et al. 2020) in severely ill COVID-19 patients were also described. Changes in D-dimer levels are the most commonly observed anomaly of the hemostasis system in patients with COVID-19. Most studies have shown a significant increase in D-dimer levels in patients with severe disease manifestation (Huang et al. 2020; Guan et al. 2020; Wang et al. 2020b; Zhang et al. 2020d, f; Liu et al. 2020b; Chen et al. 2020b). D-dimers are very sensitive and very specific laboratory indicator of the activation of coagulation and fibrinolysis. It is also well known that they are helpful in early diagnosis of acute disseminated intravascular coagulation (DIC) (Tripodi 2011; Bates 2012). Patients with SARS-CoV-2 infection are at high risk of developing this complication. Tang et al. observed that over 71% of nonsurvivor cases met the criteria of DIC (Tang et al. 2020c). It has also been speculated that these patients

have an elevation in blood plasmin(ogen) activity, which may enhance the virulence of SARS-CoV-2 and play a cardinal role in hyperfibrinolysis during DIC (Ji et al. 2020). This evidence indicates that special attention must be directed toward thrombotic and hemorrhagic complications in patients with COVID-19.

A state of acute disseminated intravascular coagulation (DIC) very similar to the hemostasis derangement observed in sepsis is frequently described in COVID-19. Using the several available scores to evaluate such conditions all are able to contribute to establish the prognosis in COVID-19 (Taylor Jr et al. 2001).

All this is also associated to a higher frequency of pulmonary embolism that probably was the initial cause of the sudden deaths seen in Chinese outbreak and also the cause of the frequent initial secondary cardiac involvements like ischemia and arrhythmias as well as VA-ECMO need.

5.5 Recommendation from Scientific Societies

The extent to which blood cells and coagulation system represent important diagnostic and prognostic markers for the severity of COVID-19 disease manifestation has led to international and national scientific societies to recommend that these be evaluated in clinical practice. These recommendations endorse, above all, monitoring of patients with particular attention to changes in D-dimer levels to assess the risk of pulmonary embolism and DIC (Thachil et al. 2020; Flisiak et al. 2020).

5.6 Conclusions

Infection with SARS-CoV-2 has presently become a rapidly spreading and devastating global pandemic Although most COVID-19 patients have moderate symptoms and recover quickly, some patients develop severe respiratory failure and acute respiratory compromise often requiring intensive care unit admission and mechanical ventilation. The above is often a result of immunological and hematological response rather than virus infiltration of human cells itself. According to the available literature, the primary hematological symptoms associated with COVID-19, and which distinguish patients with severe disease from patients with nonsevere disease, are lymphocytopenia, thrombocytopenia, and a significant increase in D-dimer levels. In this context, however, there is a shortage of research that would explain the mechanisms responsible for the observed changes.

Conflicts of Interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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6 Toll-Like Receptors (TLRs) as Therapeutic Targets for Treating SARS-CoV-2: An Immunobiological Perspective

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Abstract

Introduction: Coronavirus disease-19 (COVID-19) caused by SARS-CoV-2 is presently the biggest threat to mankind throughout the globe. Increasing reports on deaths, cases of new infection, and socioeconomic losses are continuously coming from all parts of the world. Developing an efficacious drug and/or vaccine is currently the major goal to the scientific communities. In this context, toll-like receptors (TLRs) could be the useful targets in adopting effective therapeutic approaches.

Methods: This chapter has been written by incorporating the findings on TLR-based therapies against SARS-CoV-2 demonstrated in the recently published research papers/reviews.

Results: TLRs are the essential components of host immunity and play critical roles in deciding the fate of SARS-CoV-2 by influencing the immunoregulatory circuits governing human immune response to this pathogen. Hitherto, a number of multi-subunit peptide-based vaccines and pharmacological agents developed against SARS-CoV-2 have been found to manipulate TLR function. Therefore, circumventing overt immunopathology of COVID-19 applying TLR-antagonists can effectively reduce the morality caused from "cytokine storm"-induced multiorgan failure. Similarly, pre-administration of TLR- agonists may be used as a prophylaxis to sensitize the immune system of the individuals having risk of infection. A lot of collaborative efforts are required for bench-to-bench transformation of these knowledges.

Conclusion: This chapter enlightens the potentials and promises of TLR-guided therapeutic strategies against COVID-19 by reviewing the major findings and achievements depicted in the literatures published till date.

Keywords

COVID-19 · Immunity · Immunotherapeutic intervention · SARS-CoV-2 · Toll-like receptors · Vaccine

Abbreviations

ACE2	Human angiotensin-converting enzyme 2
AGPs	Amino-alkyl glucosaminide phosphates
AP-1s	Activator protein-1

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CCL2	Chemokine ligand 2
CDC	Centre for Disease Control and Measures
COVID-19	Coronavirus disease-19
CTL	Cytotoxic T lymphocyte
DAMPs	Damage-associated molecular patterns
FP	Fusion peptide
HCS	Highly conserved segment
HTL	Helper T lymphocyte
IFN- γ	Interferon- γ
IkB	Inhibitory subunit of NF-κβ
IKK	Inhibitor of κβ kinase
IL	Interleukin
IRAK	Interleukin-1 receptor (IL-1R)-associated kinase
ISG	IFN inducible genes
LRR	Leucine-rich repeats
MAL	MyD88 adapter-like
MALP-2	Macrophage-activating lipopeptide-2
MAPKs	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MERS	Middle east respiratory syndrome
MIP-1α	Macrophage inflammatory protein-1α
MNA	Microneedle array
MPL	Monophosphoryl lipid A
MyD88	Myeloid differentiation primary response protein 88
NF-κβ	Nuclear factor-κβ
NiRAN	N-terminal nidovirus RdRp-associated nucleotidyl-transferase
NK	Natural killer
nsp	Non-structural protein
OEA	Oleoylethanolamide
ORF	Open reading frame
OxPL	Oxidized phospholipid
PAMPs	Pathogen-associated molecular patterns
Poly IC	Polyinosinic–polycytidylic acid
Poly IC: LC	Polyinosinic- polycytidylic acid, poly L-lysine and carboxymethylcellulose
PRRs	Pattern-recognition receptors
QFPD	Qingfei Paidu Decoction
RBD	Receptor binding domain
RdRp	RNA-dependent RNA polymerase
RIP1	Receptor-interacting protein 1
RSV	Respiratory syncytial virus
SARS	Severe acute respiratory syndrome
TAB1	TAK1 binding protein
TAK1	TGF-β activating kinase
TCM	Traditional Chinese medicine
TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adapter protein
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TRAF6	Tumor necrosis factor receptor (TNFR)-associated factor 6
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain–containing adapter-inducing interferon-β
Ubc13	E2 ubiquitin-conjugating enzyme 13
Uev1A	E2 ubiquitin-conjugating enzyme 13 variant 1A
VS	Variable segment
WHO	World Health Organization

6.1 Introduction

Corona, the term is now the utmost talkative word and most of the researchers have currently turned their focus toward this concerned area. In late December of 2019, novel coronavirus disease (COVID-19) outbreak caused by severe acute respiratory syndrome- coronavirus-2 (SARS-CoV-2) was firstly reported from Wuhan city of Hubei province of China (Li et al. 2020a). An increasing number of infections and death due to this viral infection consequently led to the declaration of this outbreak as a global pandemic by the World Health Organization (WHO) on 9 January 2020. Till June 2020, the epidemiological map comprises more than 215 countries worldwide with 6,057,853 confirmed cases and a death toll of more than 428,100 (World Health

Organization 2020). Despite tremendous efforts from the medical, scientific, and sociopolitical communities, this virus is continually threatening the world with an increasing number of new cases and mortalities.

The potential threat of coronavirus has come into the picture through two previous outbreaks, viz., Middle East respiratory syndrome (MERS) and severe acute respiratory syndrome (SARS) during the early phase of the twenty-first century (Opie 2013). Regarding clinical features, MERS is more lethal than SARS-CoV and SARS-CoV-2, and even also less closely related to the other two in pathogenesis and phylogenetic features (Petrosillo et al. 2020). Phylogenetic studies have demonstrated that SARS-CoV-2 belongs to the subfamily Orthocoronavirinae in the family of Coronaviridae under the order Nidovirales (Hui et al. 2020; Xie and Chen 2020).

Several hypotheses based on the phylogenetic analysis have suggested bat and/or pangolin to be the most probable animal reservoir(s) of this virus (Andersen et al. 2020). A recent *in silico* study by Choudhury and Mukherjee (Choudhury and Mukherjee 2020) revealed bat as the most possible origin of SARS-CoV-2. However, considerable debate is still going on the origin of the virus and the possible role of any intermediate host. Alike SARS-CoV and MERS, SARS-CoV-2 is also having the zoonotic transmission properties and later subsequently adapted in human (Choudhury and Mukherjee 2020). In humans, the transmission of this disease primarily occurs via direct contact between man to man and secondarily through droplets from an infected one (Lu et al. 2020). Such adaptation and ability of rapid human to human transmission of SARS-CoV-2 eventually made this virus so challenging to control (Rothan and Byrareddy 2020). The Centre for Disease Control and Measures (CDC) has advised four precautionary measures such as frequent washing of hands, social distancing, quarantine, and isolation (Centers for Disease Control and Prevention 2020).

Among the active cases, most of the patients are belonging to the age group of 35–55 years while fewer are from children and infants groups (Hamid et al. 2020). From several studies, it has become transparent that the median age of patients is 59 while in the case of death it is 75 (Li et al. 2020a; Wang et al. 2020a; Chen et al. 2020). In addition, the maximum number of positive cases (approximately 59%) belongs to males (Li et al. 2020a; Wang et al. 2020a; Chen et al. 2020). Healthy as well as immunosuppressed people have been found highly susceptible to COVID-19 (Li et al. 2020a). Intriguingly, people with hypo-active immune function (majorly old people) and those who are already suffering from diseases like pulmonary, kidney, and hepatic dysfunction are considered to be a high-risk group (Li et al. 2020a).

Several studies have concluded SARS-CoV-2 as a highly infectious virus that primarily targets the respiratory tract and gastrointestinal tract to some extent for its entry inside the human cells (Xie and Chen 2020). In general, viral spike protein binds to human angiotensin-converting enzyme 2 (ACE2) and with the help of another human protein namely TMPRSS2 (Cao and Li 2020). After its entry, the viral replicase/RNA-dependent RNA polymerase (RdRp) is activated with the help of a protease and resulting in transcription of viral RNA following synthesis of the viral proteins for the reconstruction of new viral particles (Cao 2020). The major pathological manifestations like breathing difficulties, accumulation of fluid in the lungs, respiratory and multiorgan failure are majorly resulted from "cytokine storm" (Cao 2020; Astuti and Ysrafil 2020). This overt proinflammatory milieu majorly resulted from the hyperactivation of various immune cells of the body wherein interactions between viral antigen(s)/molecular pattern(s) and toll-like receptors (TLRs) are considered as the key mediators (Choudhury and Mukherjee 2020; Kalita et al. 2020). This chapter has been written with an objective to present a comprehensive overview on the role of human TLRs in the immunopathology of COVID-19 and the prospect of TLR-guided immunotherapeutic intervention strategies to combat this global pandemic.

6.2 Pathogenesis of COVID-19

6.2.1 Structure and Genome of SARS CoV-2

SARS-CoV-2 is a β-coronavirus belonging to the order Nidovirales of Coronaviridae family (Hui et al. 2020). It is a spherical enveloped, nonsegmented virus containing a positive-sense single-stranded-RNA genome of 26–32 kb having a close homology to SARS-CoV (~79% similarity) and MERS-CoV (~50% similarity) (Lu et al. 2020). The structure of the virus is composed of four principal proteins along with the nucleocapsid made up of phosphorylated nucleocapsid (N) protein harboring the viral genome (Mousavizadeh and Ghasemi 2020). The surface covering consists of a phospholipid bilayer, envelope (E) protein, and type III transmembrane glycoprotein or membrane (M) protein. A club-like projection made up of highly glycosylated spike (S) protein is present throughout the surface of the envelope (Cao and Li 2020).

The viral genome comprises a large number of overlapping open reading frame (ORF) encoding the nonstructural proteins for the viral replicase complex consisting of various proteases, RNA-dependent RNA polymerase (RdRp), and several helicases and primases. The other part of the genome is the interspersed ORF for synthesizing the structural and accessory proteins (Bergmann and Silverman 2020). The spike protein is the main component for the attachment of the virus with the host cell (Choudhury and Mukherjee 2020). Spike protein is a homotrimeric protein having two subunits, namely S1 and S2. The S1 subunit comprises an amino-terminal domain and a receptor-binding domain (RBD) that cleaves and is inserted within the host membrane, whereas the S2 subunit has a fusion peptide (FP) region and two heptad repeat regions, viz., HR1 and HR2, thereafter bring its heptad region together leading to fusion and release of viral packages inside the host (Tay et al. 2020). The replication and the transcription of the viral genome depend on the RdRp complex (Ogando et al. 2020). It consists of a catalytic subunit, nonstructural protein (nsp) 12 having N-terminal nidovirus RdRp-associated nucleotidyltransferase (NiRAN) domain, an interface domain, and a C-terminal RdRp domain, and other two accessory subunits called (nsp8) and (nsp7) (Hillen et al. 2020). The RdRp domain resembles a right hand, having fingers, palm, and thumb subdomain, where the nsp7 binds to the thumb and the two copies of nsp8 bind to fingers and thumb subdomains (Gao et al. 2020).

6.2.2 Transmission and Pathogenesis

According to the current update by the WHO, it is mainly transmitted by droplets of various sizes amongst the people through the respiratory droplets and contact routes with high risk within the 1-meter range (Lu et al. 2020). The incubation period of the virus is one of the key aspects in the knowledge of COVID-19 biology as it reflects the information of when the infected person will be symptomatic and most likely to spread the disease. According to Yang et al. 2020 (Yang et al. 2020a), the mean incubation period of COVID-19 is 4.75 days. However, it has been changed to 5.2 days, and currently, it seems to be fixed to a mean value of about 3 days ranging from 0 to 24 days (Li et al. 2020a; Xie and Chen 2020; Yang et al. 2020a). The incubation period of SARS-CoV-2 is therefore much longer than SARS (4 days) and MERS (4.5–5.2 days) (Li et al. 2020a; Xie and Chen 2020; Yang et al. 2020a; Lessler et al. 2009; Park et al. 2018). However, estimation of the mean serial incubation period of 2019-nCoV, which is about 7.5 days, also reflected a much lesser value than SARS and MERS (Li et al. 2020a). On the other side, the reproductive number (R_0) indicates the transmissibility of the virus and represents the average number of new infections propagated by a single infected person in a naïve population. According to the researchers, the mean R_0 for SARS-CoV-2 was found to be 3.28 that is quite similar to SARS (around 3) but highly varied with MERS due to poor transmission history (Liu et al. 2020; Bauch et al. 2005; Bauch and Oraby 2013). The diagnostic data along with electron

microscopy have collectively revealed that the viral particle mainly damages the bronchial and alveolar epithelial cells of lungs forming hyaline membrane along with the accumulation of mononuclear cells and macrophages, leading to the infiltration of air spaces and thickening of the cell wall (Xu et al. 2020). The mechanistic insights of the pathogenesis of SARS-CoV-2 are presented in Fig. 6.1a.

6.2.3 Immunobiology of SARS-CoV-2

The binding of the viral spike protein with the human ACE2 receptor results in the impairment of the renin–angiotensin system and disrupting the blood pressure and electrolyte balance (Choudhury and Mukherjee 2020; Astuti and Ysrafil 2020; Shang et al. 2020). Release of the virus particle and active replication inside the host induces pyroptosis and release of the damage-associated molecular patterns (DAMPs), which are recognized by host epithelial and endothelial cells and alveolar macrophages to trigger inflammatory responses and release of proinflammatory cytokines and chemokines including tumor necrosis factor (TNF)-α, interleukin-1β (IL-1β), IL-6, IL-2, IL-7, IL-8, IL-9, IL-17, granulocyte-colony stimulating factor, interferon gamma-induced protein-10, macrophage inflammatory proteins 1-α and chemokine ligand 2 (CCL2), CCL3, and CCL4, resulting in a cytokine storm (Cao 2020; Bonam et al. 2020). In the peripheral blood, a decrease in the count of monocytes, basophils, and T cells, especially CD4+ and $CD8^+$ T cells, is usually found due to the translocation of these cells to the site of infection (Bonam et al. 2020). In addition, an increase in neutrophil counts in the blood is also observed (Bonam et al. 2020). The overproduction of various innate and adaptive immune cells poses feedback regulation on natural killer (NK) cell and T-cell activation. The $CD4^+$ T cells stimulate B cells for the production of virus-specific antibodies, while the CD8+ T cells are cytotoxic and directly kill virus-infected cells, and finally, the alveolar macrophages recognize them and clear them by phagocytosis (Tay et al. 2020). In the meantime of replication, double-stranded RNA (dsRNA)-mediated immune response is sensitized by TLR3, leading to the IRFs and NF-κB activation for the production of type I interferons (IFNs) and proinflammatory cytokines to amplify the release of antiviral proteins for protecting the uninfected cells (Li et al. 2020b). The mechanistic insights of the immunopathogenesis of SARS-CoV-2 are presented in Fig. 6.1b.

6.3 Regulatory Roles of Toll-Like Receptors (TLRs) in the Human Immune System

6.3.1 Immunobiology of TLRs

Innate host defense response against the pathogenic microorganisms is generally activated within a few minutes of their invasion into the human body. This process mostly depends on the TLRs, the major class of pattern-recognition receptors (PRRs) in humans that are known to act as innate immune sensors recognizing conserved pathogen-associated molecular patterns (PAMPs) (Mukherjee et al. 2019a). After recognizing distinct PAMP, TLR activation results in the expression of the proinflammatory cytokine to activate different immune cells and to amplify the immune response against the invading pathogen (Mukherjee et al. 2016). Till date, 11 different TLR genes have been identified in humans amongst which 10 (TLR1 to TLR10) are usually expressed as receptors because human TLR11 contains stop codon (Ishii et al. 2008). TLRs are categorized into two subgroups such as transmembrane TLRs (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10) and intracellular TLRs (TLR3, TLR7, TLR8, TLR9) (Kawasaki and Kawai 2014). Intracellular TLRs are found in the endoplasmic reticulum (ER), endosome, lysosome, or endolysosomal compartments (Kawasaki and Kawai 2014). TLRs are predominantly expressed in the innate immune cells, especially in the antigen-presenting cells like dendritic cells and macrophages and also some-

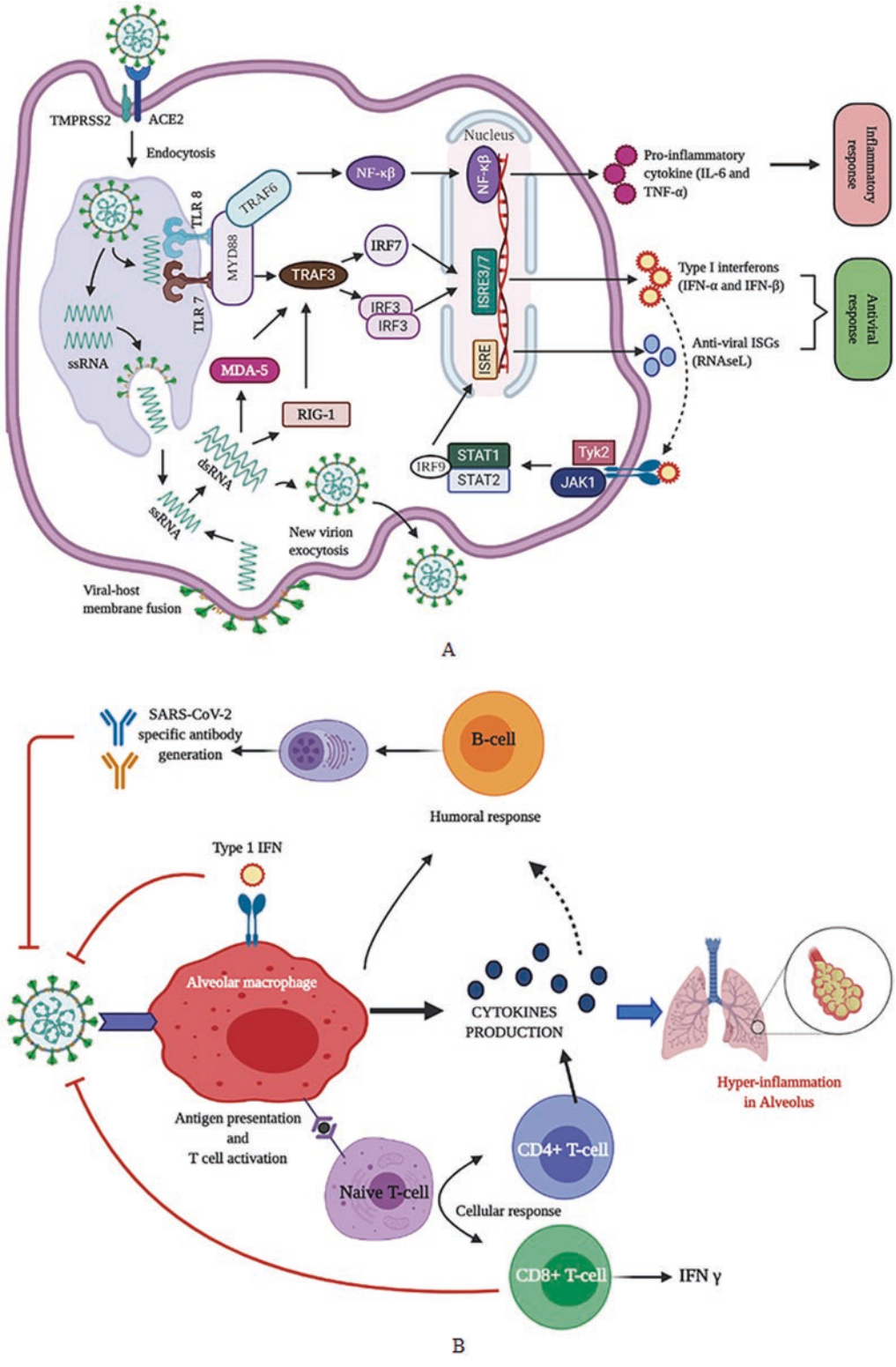

Fig. 6.1 Molecular mechanism of pathogenesis in COVID-19. (**a**) Viral genome components induce inflammatory and antiviral response-dependent or independent of TLR. NF-κβ is responsible for proinflammatory response while IRF3 and IRF7 are engaged in releasing type I interferon. (**b**) Excessive cytokines in the presence

times in nonimmune cells such as fibroblast and epithelial cells (Mukherjee et al. 2017, 2019b).

Each trans-membrane TLR comprises an extracellular leucine-rich repeats (LRR) domain that contributes recognition of PAMPs such as lipid, protein, lipoprotein, and nucleic acid and a cytoplasmic toll/IL-1 receptor (TIR) domain that induce downstream signaling to trigger inflammatory responses (Farhat et al. 2008). Highly conserved segment (HCS) and variable segment (VS) are the two parts of LRR. The HCS part consists of a distinct peptide stretch containing either 11 (LxxLxLxxNxL) or 12 amino acids (LxxLxLxxCxxL), in which "L" is substituted with Leu, Ile, Val, and Phe, "N" is Asn, Ser, Thr, and Cys and "C" is Cys, Ser, and Thr while "x" is an amino acid (Matsushima et al. 2007; Werling et al. 2009; Kajava and Kobe 2002). In human, TLRs are composed of 19–25 LRRs including "typical (T)" (LxxLxLxxNxLxxLxxxxFxxLxx) and "bacterial (B)" (LxxLxLxxNxLxxLPx(x) LPxx) LRRs (Matsushima et al. 2007). Species-specific co-evolution is known to cause variation in structural as well as functional variation in TLRs (Mukherjee et al. 2019a).

TLR2 functions in combination with TLR1 and TLR6 for recognizing lipoprotein, lipoteichoic acid, peptidoglycan, zymosan, mannan, and trypomastigote-derived glycosylphosphatidylinositol (tGPI)-anchored -mucin (Mukherjee et al. 2016). On the other side, cell surface TLR4 selectively recognizes LPS, whereas TLR5 binds to bacterial flagellin (Mukherjee et al. 2016). Intracellular TLR3 senses viral dsRNA while nucleic acids and heme motifs are known to be recognized by TLR7, 8, and 9 for (Roach et al. 2005).

6.3.2 TLR Signaling Pathway

Exposure to a variety of PAMPs resulting in ligand-induced dimerization of TLRs that facilitates the recruitment of adaptor protein(s) to the cytoplasmic TIR domain required for initiating the intracellular signaling cascade (demonstrated in the subsequent section). TIR domain has been identified as a conserved domain across the different TLRs as well as species (Werling et al. 2009). Several adaptor molecules are required for transmitting the signals originated from the TLRs. This includes myeloid differentiation primary response protein 88 (MyD88), TIR-domain–containing adapter-inducing interferon-β (TRIF), TIR domain-containing adapter protein (TIRAP)/MyD88 adapter-like (MAL), and TRIF-related adaptor molecule (TRAM), which dock at the TIR domain and transmit inflammatory signal (Mukherjee et al. 2019a). TIRAP helps in the recruitment of MyD88 with TLR4 and TLR2, which in turn activates nuclear factor-κβ (NF-κβ) and/or mitogen-activated protein kinase (MAPKs) (Mukherjee et al. 2019a). Similarly, TRIF binds at the TIR domain of TLR4 and TLR3 guiding the activation of interferon regulatory factor-3(IRF-3), NF-κβ, and MAPKs while TRAM functions as a linker between TRIF and TLR4 (Kawasaki and Kawai 2014). For example, successful binding of LPS to TLR4 induces a molecular signal that transmits through either MyD88-dependent or -independent (also called TRIF-dependent pathway) pathways (Mukherjee et al. 2019a; Kawasaki and Kawai 2014). In MyD88-dependent pathway, MyD88 interacts with the interleukin-1 receptor (IL-1R)-associated kinase (IRAK) family to form a myddosome structure (Lin et al. 2010), where actuated IRAK4 induces IRAK1 (Jiang et al. 2002; Kollewe et al. 2004). After autophosphorylation-

Fig. 6.1 (continued) of type I interferon leads to inflammation in lung alveoli and in turn induce B cell to produce SARS CoV2-specific antibodies. Abbreviations: TMPRSS2, transmembrane serine protease 2; ACE2, angiotensin-converting enzyme 2; TLR, Toll-like receptor; MyD88, myeloid differentiation primary response protein 88; TRAF6, TNFR-associated factor 6; NF-κβ, nuclear factor kappa B; IRF, interferon regulatory factor-3; ISRE3, interferon-stimulated response element; MDA-5, melanoma differentiation-associated protein 5; RIG-I, retinoic acid-inducible gene I; STAT, signal transducer and activator of transcription; JAK, janus kinase; Tyk, tyrosine kinase 2

mediated activation, the activated IRAK1 gets released from the myddosome (Jiang et al. 2002; Kollewe et al. 2004). IRAK1 eventually links with an E3 ubiquitin-protein ligase, namely tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) that in conjugation with E2 ubiquitin-conjugating enzyme (Ubc13 and Uev1A) as TRAF6-Ubc13 complex mediates K63-linked polyubiquitination of TRAF6 itself as well as of a complex comprising TGF-β-activating kinase (TAK1; members of MAPKKK family), TAK1 binding protein (TAB1), TAB2, and TAB3 (Clark et al. 2011; Xia et al. 2009). TAK1 further induces activation of the inhibitor of κβ kinase (IKK) complex (comprising IKKα, IKKβ, and NEMO or IKKγ) to facilitate NF-κβ-mediated signaling ways (Adcock and Caramori 2001; Ip and Davis 1998). IKK complex catalyzes the phosphorylation of the inhibitory subunit of NF-κβ (IkB) and allows the activated NF-κβ to translocate into the nucleus triggering the expression of the genes encoding the proinflammatory cytokines. TAK1 can also phosphorylate MAPK to maintain another arm of the inflammatory pathway wherein phosphorylated MAPK activates activator protein-1 (AP-1s) to regulate inflammatory responses (Adcock and Caramori 2001; Ip and Davis 1998).

In the case of MyD88-independent pathways, the adaptor molecule TRIF recruits both TRAF6 and TRAF3 (Dunne and O'Neill 2005). TRAF6 interacts with receptor-interacting protein 1 (RIP1) that subsequently activates the TAK1 complex, leading to NF-κβ and MAPKs actuation following induction of the proinflammatory cytokines (Dunne and O'Neill 2005). On the other hand, TRAF3 interacts with IKK-related kinase, namely TABK1, IKKi, and NEMO, to phosphorylate IRF-3, which is further translocated to the nucleus by forming a dimer and induces the expression of IFN-γ and IFN-inducible genes (Clark et al. 2011). The generalized view on the signal transduction pathways originating from different TLRs is presented in Fig. 6.2.

6.3.3 TLR as a Target for Immunotherapy

TLRs play extremely critical roles in maintaining the immune-homeostasis of the human body. Hypoactivation or impairment of TLR signaling results in increased vulnerability to infectious disease while hyperactivation leads to the development of a high proinflammatory milieu due to the massive release of proinflammatory cytokines and chemokines (Mukherjee et al. 2019a). Dysregulation of the TLR signaling pathway is linked with a wide range of human diseases that include various infectious diseases, atherosclerosis, asthma, cardiac disease, liver disease, renal disease, inflammatory bowel disease, obesity, diabetes (types I and II), rheumatoid arthritis, Alzheimer's disease, Parkinson's disease, and multiple sclerosis (Mukherjee et al. 2016; O'Neill et al. 2009; Achek et al. 2016; Mifsud et al. 2014). The strategies for immunotherapy majorly include situation-specific switching of the TLR responses. For example, an individual having a chronic infection due to hypo-activated immunity caused from downregulated response of distinct TLR can be treated with specific TLR agonists to restore the immune homeostasis. On the other side, individuals with hyperactivated immune response can be treated by downmodulating the activation of TLR using various antagonists that block the binding of a specific ligand to the receptors and/or interfere with the functioning of the intracellular domain to subdue the TLR signaling cascade. Therefore, immunotherapeutic/immunopharmacological intervention of human diseases by the use of TLR agonists (homologs of TLR ligand that enhance the immune response against the infectious agent) and antagonists (compounds that reduce the inflammatory consequences) are now being considered as effective therapeutic means (Xiang et al. 2010).

Formation of a heterodimer of TLR2 with TLR1 and TLR6 as well as binding of the ligands has been utilized for targeting TLR2 (Mifsud et al. 2014; Hennessy et al. 2010). TLR2 agonist, namely macrophage-activating lipopeptide-2 (MALP-2) isolated from *Mycoplasma fermen-*

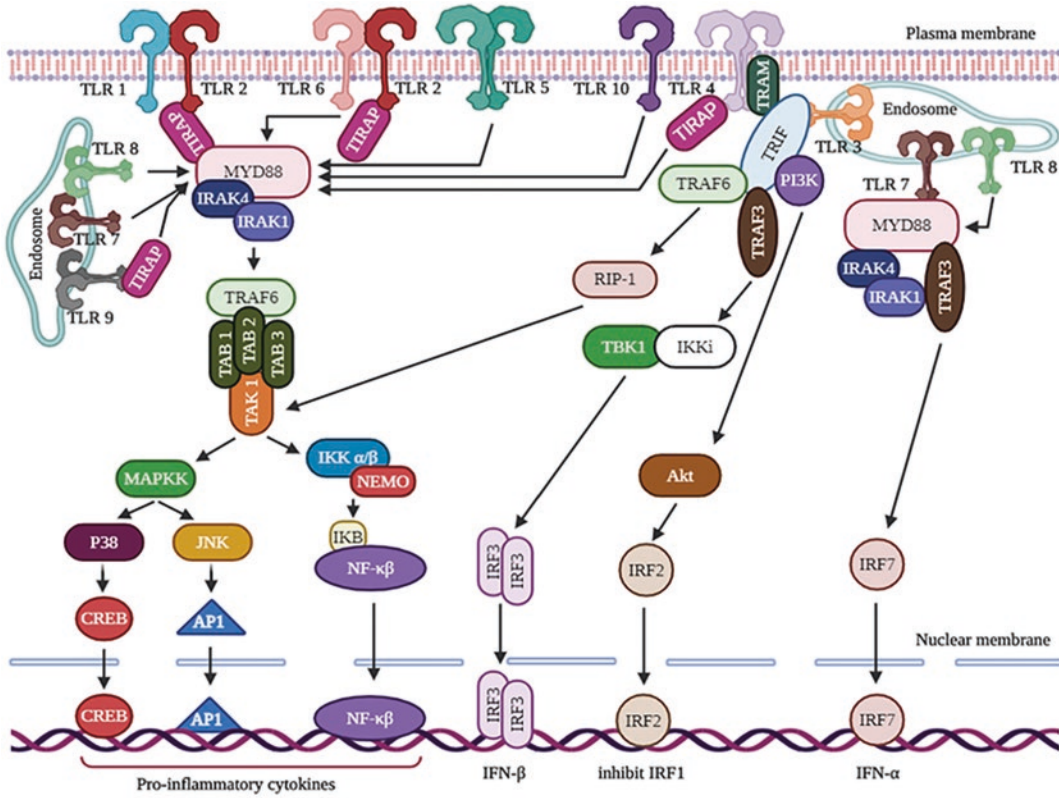

Fig. 6.2 Mechanistic insights of TLR signaling pathways. Except for TLR3, all the TLRs participate in MyD88-mediated signaling transduction. TLR2 in complex with TLR1 or TLR6, TLR4, and TLR9 interact with MYD88 via TIRAP while others show a direct connection with MyD88. Subsequently, MyD88 interacts with IRAK1 and IRAK4 to induce the activation of TRAF6 and TRAF3. Later K63-linked polyubiquitination on TRAF6 and TAK1 leads to the activation of IKK complex and MAPKK. MAPKK induces activation of AP1 and CREB transcription factor activation, whereas IKK complex activates NF-κβ. TRAF3 induces the IRF7-mediated gene expression. On the other hand, Myd88-independent signaling is originated from TLR4 and TLR3 inducing the activation of TRIF and later followed by the recruitment of TRAF6 and TRAF3. TRAF6 in engagement with RIP-1 activates TAK1 and follows the NF-κβ and AP1 activation. While TRAF3 recruits TBK1 and IKKi, which subsequently phosphorylates IRF3 and leads to results in IRF3-mediated transcription. Abbreviations: AP1, activator protein 1; IFN, interferon; IKK, IκB kinase; IRAK, IL-1R-associated kinase; IRF-3, interferon regulatory factor-3; JNK, c-Jun N-terminal Kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinases; MyD88, myeloid differentiation primary response protein 88; NF-κB, nuclear factor kappa B; RIP1, receptor-interacting protein1; TAB1, TAK1-binding protein 1; TAK1, TGF-β-activated kinase 1; TIR, Toll-interleukin-1 receptor domain; TIRAP, TIR-associated protein; TLR, Toll-like receptor; TRAF6, TNFR-associated factor 6; TRAM, TRIF-related adaptor molecules; TRIF, TIR-domain-containing adapter-inducing interferon β; PI3K, phosphoinositide 3-kinase; CREB, c-AMP response element-binding protein; TBK1, TANK-binding kinase 1; IKB, inhibitor of nuclear factor kappa B; Akt, protein kinase B

tans, elevates the production of monocyte chemoattractant protein-1 (MCP-1), interleukin-8 (IL-8), macrophage inflammatory protein-1α (MIP-1α), MIP-1β, IL-6, and tumor necrosis factor-α (TNF-α) (Reppe et al. 2009). Another agonist SMP-105, a mixture of cell-wall skeleton components (mycolic acid and peptidoglycan) of *Mycobacterium bovis*, was found to act as a strong adjuvant and antitumor agent for the treatment of bladder cancer by triggering the downstream inflammatory responses originating from TLR2 (Hennessy et al. 2010). On the other side, TLR2 antagonists render the inflammatory effect of the inflammatory cytokine production. Mistry

et al. (Mistry et al. 2015) had developed a small molecule ($C_{16}H_{15}NO_4$) that blocks the dimerization of TLR2 and TIR domain to inhibit TLR2 activation (Mistry et al. 2015). In mice, it inhibits the TLR2-induced secretion of IL-12 p40 and TNF-α in serum. Interestingly, a human-specific anti-TLR2 antibody, namely OPN-305, has been reported to inhibit the signaling of TLR2-mediated inflammatory production of cytokine (Arslan et al. 2008). Binding of viral dsRNA with TLR3 dimer resulting in the production of type 1 IFNs is majorly targeted for manipulating the TLR3 function (Hennessy et al. 2010). PIKA is a synthetic TLR3 agonist structurally analog to dsRNA that has been reported to significantly reduce the viral infestation in lung tissues caused due to various influenza virus including A/teal/HK/W312/97 (H6N1), A/rhea/NC/93 (H7N1), A/HK/1073/99 (H9N2), and A/Vietnam/1203/2004 (H5N1) (Lau et al. 2010). This molecule is also a potent adjuvant that enhances the cellular and humoral immune response (Lau et al. 2010). Another synthetic dsRNA, namely polyinosinic–polycytidylic acid (Poly IC) condensed with poly L-lysine and carboxymethylcellulose (Poly IC: LC), was also reported to activate TLR3 for antiviral activity by stimulating the production of IFN-γ, α, and β (Mifsud et al. 2014). Binding of ligand, receptor dimerization, and activation of the TIR domain are the major targets of the immunomodulators that target TLR4 (Lu et al. 2008). Monophosphoryl lipid A (MPL) isolated from *Salmonella minnesota* R59 has been reported to act as an adjuvant for a vaccine for hepatitis B inducing mucosal and systemic immune responses (Mifsud et al. 2014; Cluff et al. 2005). Synthetic mimetics like amino-alkyl glucosaminide phosphates (AGPs) that occupy TLR4 and induce production of the proinflammatory mediators, viz., MIP-2, TNF-α, and IFN-γ (Mifsud et al. 2014). On the other hand, Eritoran (E5564) derived from *R. sphaeroides* is a synthetic lipid A analog that inhibits the binding of LPS to the MD2 pocket, leading to hindering the activation of TLR4 (Achek et al. 2016). A peptide called SPA4 interfering with the surfactant protein A and TLR4 interface has been reported for inhibiting the TLR4 signaling pathway (Ramani et al. 2013). It also evokes an anti-inflammatory response and alleviates the endotoxic shock-like symptoms in mice (Ramani et al. 2013). A recent study by Debnath et al. (2019) demonstrated that synthetic aryl quinolinyl hydrazone derivatives can act as potential inhibitors of TLR4 and the downstream signaling pathway in macrophages to display strong anti-inflammatory activity (Debnath et al. 2019). Flagellin isolated from Gram-positive and Gram-negative bacteria has been reported to act as an agonist for TLR5 to induce the production of proinflammatory cytokines and enhancing the immunogenicity of therapeutic preparations (Chakraborty et al. 2020). Several vaccine adjuvants have been found to activate TLR5; for example, VAX-102 has been reported as a TLR5 agonist having a protective effect against influenza virus in mice model and it is currently under preclinical trial (Huleatt et al. 2008). Similarly, pyrimidine triazole thioether derivatives have been screened as an inhibitor of flagellin binding to TLR5 to suppress the TLR5-flagellin complex formation as well as the downstream expression of TNF-α signaling pathways (Yan et al. 2016).

Intracellular TLRs (TLR 3, 7, 8, and 9) play the most important role in recognizing viral patterns and inducing proinflammatory responses to counteract the pathogenesis of the virus. Therefore, ligand binding and the downstream signaling are considered as effective targets for developing agonists or antagonists. 1-(2-methylpropyl)-1H-imidazo(4,5-c) quinoline-4-amine (Imiquimod) has been described as a potent TLR-7 agonist that enhances the production of endogenous proinflammatory cytokines to heighten both innate and adaptive immunity (Schiller et al. 2006). In this context, resiquimod (R-848), which is an agonist of both TLR7 and TLR8, is currently used for the treatment of hepatitis C and other viral (Pockros et al. 2007). This compound induces activation of natural killer (NK) cells, expression of IFNα, IL-12, and TNF-α, and also promotes antigen-specific cell-mediated immune responses (Pockros et al. 2007; Caron et al. 2005). Furthermore, IMO-2125 has been reported as a TLR9 agonist that activates innate immune cells to secrete TNF-α

and also stimulates T-cells and NK cells in non-human primates (Caron et al. 2005). CpG DNA-based TLR9 agonists along with specific allergen immunotherapies are presently used for treating various viral respiratory infections (Hennessy et al. 2010). CpG-ODN c41 isolated from *Pseudomonas aeruginosa* has been found to act as an antagonist of TLR9 inhibiting proinflammatory responses in murine macrophage and human monocyte (Li et al. 2011). IMO-3100 (NCT01622348), a synthetic compound, has also been developed to target TLR7 and TLR9 for inhibiting the secretion of the proinflammatory cytokines and is used for the treatment of several autoimmune and inflammatory diseases (Suárez-Fariñas et al. 2013). Lists of natural and synthetic molecules/compounds that target different human TLR and modulates their functions are presented in Table 6.1.

6.4 Role of Toll-Like Receptors (TLRs) in the Conception of Therapeutic Strategies Against SARS-CoV-2

As discussed in the earlier section, the host–pathogen interactions involving SARS-CoV-2 and the innate immune receptors, especially the TLRs, determine the fate of the invading virus particles and disease display. Generalized picture of pathogenesis of the flu-causing viruses including SARS suggests that viral surface antigens (proteins) interact with the host macrophages and dendritic cells present in the lungs tissue and these interactions play a key role in virus-induced immune response producing a large amount of type-1 IFN and a wide array of immunomodulatory cytokines from the stimulated antigen-presenting cells (Ito et al. 2005; Colonna et al. 2004; Laiosa et al. 2006). The type 1 IFN acts as an initiator of the early innate immune response to block the viral pathogenesis (Takaoka and Yanai 2006). It upregulates several antiviral effectors molecules like PKR and RNaseL to limit the viral replication and also stimulate the expression of the IFN inducible genes (ISGs) (Takaoka and Yanai 2006). All these responses are primarily dependent on the innate immune sensing of the virus-associated molecular patterns by the cell surface and intracellular TLRs (Frieman et al. 2008; Li et al. 2020c). Particularly TLR3, TLR7, TLR8, and TLR9 can detect viral entry to the endosomal compartments while the viral RNA in the cytoplasm is sensed by the cytoplasmic CARD domain-containing RNA helicases, RIG-I and Mda5 (Frieman et al. 2008). Regarding this, it has been found that the intracellular TLRs, viz., TLR 3, 7, 8, and 9, can comparatively sense the mRNA of NSP10, S2, and E proteins of SARS-CoV-2 and can be strategically used as therapeutic target by downregulating the TLR responses (Choudhury et al. 2021). Targeting TLRs with TLR-antagonist and agonist can be used as a therapeutic strategy against SARS-CoV-2 (Patra et al. 2021). In this section, we have discussed the progress and therapeutic relevance of TLR-based intervention strategies for combating the COVID-19 pandemic.

6.4.1 TLRs as Potential Immunotherapeutic Therapeutic for Treating COVID-19

Considering the immense impact of the TLRs in human immunity as well as the pathogenesis of several human diseases, many researchers have aimed to explore the key roles of different TLRs in COVID-19 (Patra et al. 2021). Immunopathological manifestations of COVID infection are primarily associated with a very high level of proinflammatory response due to the increased expression of proinflammatory cytokines, viz., interleukin-6 (IL-6), IL-1β, and tumor necrosis factor-alpha (Gautret et al. 2020). Expression of all these cytokines is primarily regulated by the transcription factor NF-κB and activation of NF-κB is signaled by the TLRs (Mukherjee et al. 2019a; Mukherjee et al. 2016). These facts have indeed encouraged scientists to examine the involvement of TLRs in COVID-19 pathology and immunity. In a recent study by Choudhury and Mukherjee (Choudhury and Mukherjee, 2020), spike protein of SARS-CoV-2

Table 6.1 Potential TLR agonists and antagonists for treating infectious and inflammatory diseases of humans

TLR	Ligands	Agonists			Antagonists			Reference
		Agent	Target	Immune response	Agent	Target	Immune response	
TLR2	Mycoplasmalipoproteins, lipoteichoic acid, peptidoglycan (bacteria), zymosan, heat-shock proteins, HMGB1, versican, hyaluronic acid	Diacyl lipopeptides	Bacterial peptidoglycan layer	Elevate production of MCP-1, IL-8, MIP-1α, MIP-1β, IL-6, and TNF-α; strong adjuvant and antitumor agent	OPN-305,	Autoimmune disease	Block TLR2 dimerization; antibody-specific inhibitor	Mifsud et al. (2014), Hennessy et al. (2010), Mistry et al. (2015), Arslan et al. (2008) and Tan et al. (2012)
		Pam2Cys	Influenza A virus		Small molecule "C29" of $C_{16}H_{15}NO_4$	Liver cytokine mRNA		
		MALP-2	*S. pneumoniae*					
TLR3	Viral dsRNA, mRNA	Poly (I:C)	*L. major*,HSV-2	Structural analog to viral dsRNA; potent adjuvant; stimulate production of IFN-γ, α, and β	CNTO4685; CNTO5429	Monoclonal antibody	Inhibit poly(I:C)-induced activation of HEK293T cells transfected with mTLR3	Hennessy et al. (al. 2010), Lau et al. (2010) and Bunting et al. (2011)
		Poly IC:LC and LE Poly IC:LC	Influenza A virus, yellow fever virus, rabies					
		PIKA	Influenza A virus					
TLR4	LPS, respiratory syncytial virus HSP, HMGB1, β-defensin 2, fibronectin extra domain, hyaluronic acid, heparan sulfate, oxidized phospholipids	Lipid A mimetics	Respiratory syncytial virus	Immunomodulator; adjuvant for vaccine; induce production of MIP-2, TNF-α, and IFN-γ	Eritoran (E5564)	Sepsis	Inhibits binding of LPS to the MD2; anti-inflammatory activities; TLR4 inactivators	Achek et al. (2016), Mifsud et al. (2014), Lu et al. (2008), Cluff et al. (2005), Ramani et al. (2013) and Debnath et al. (2019)
		Monophosphoryl lipid A	*L. monocytogenes,* Influenza A virus		Aryl quinolinyl hydrazone derivatives	Macrophage inactivation		
		Aminoalkyl glucosamine 4-phosphate	*F. novicida*		NI-0101	Acute and chronic inflammation		
		FimH	Influenza A virus		SPA4	Lungs inflammation		
TLR5	Flagellin	Flagellin protein,	*S. pneumoniae*	Activation of proinflammatory cytokines production and enhanced immunogenicity	Pyrimidine triazole thioether derivatives	Flagellin binding inhibitor to TLR5	Regulate TNF-α signaling	Huleatt et al. (2008) and Yan et al. (2016)
		VAX-102	*Salmonella typhimurium,* Influenza A virus					

TLR7	ssRNA, imidazoquinolines, guanosine analogs, immune complex	AZD8848 (DSP-3025)	Allergy, asthma	Anti-inflammatory effect, enhances the production of endogenous proinflammatory cytokines	IMO-8400	Psoriasis	Inhibits the secretion of proinflammatory cytokine	Hennessy et al. (2010), Schiller et al. (2006), Pockros et al. (2007), Caron et al. (2005) and Suárez-Fariñas et al. (2013)
		Resiquimod	HSV-2 infection		IMO-3100	SLE, rheumatoid arthritis, multiple sclerosis		
		ANA773	Cancer, Hepatitis C					
TLR8	Viral ssRNA, immune complex	Resiquimod	HSV-2 infection, Hepatitis C	Therapeutic treatment	IMO-8400	Psoriasis	Inhibit activation and immune response	McCluskie et al. (2006) and Jiang et al. (2012)
TLR9	Unmethylated CpG Motifs, Chromatin IgG complex	CpG oligodeoxynucleotides	*L. major,* Influenza A virus, HSV-2	Activate the INFα and also stimulate the activation of T-cell and NK cell; stimulate TLR9 for the potential treatment	IMO-3100	SLE, rheumatoid arthritis, multiple sclerosis	Inhibits the secretion of proinflammatory cytokine and TLR9-mediated response	Achek et al. (2016), Hennessy et al. (2010), Li et al. (2011), Suárez-Fariñas et al. (2013) and Barry and Cooper (2007)
		SD-101	Hepatitis C infection		CpG-ODN	*Pseudomonas aeruginosa*		
		IMO-2125	Hepatitis C infection		IRS-954 (DV-1079)	SLE progression		
		HEPLISAV	Hepatitis B					

was found to interact with the extracellular domain of the cell surface TLRs including TLR1, TLR4, and TLR6. Intriguingly, the highest binding affinity and strength were evident in the spike protein-TLR4 complex. The protein–protein interaction between spike protein and TLR4 is majorly consisting of hydrogen bonds and hydrophilic interactions (Choudhury and Mukherjee 2020). Therefore, targeting the interaction between spike protein and the TLR domain or its signaling pathways might be a meaningful therapeutic strategy to fight against SARS-CoV-2. In response to SARS-CoV-2, the dying cells or activated innate immune cells release on the extracellular HMGB1 that form complexes with the DNA, RNA, and DAMP (Cao and Li 2020; Bonam et al. 2020). These complexes separately bind to RAGE and translocated to the lung's endolysosomal system to trigger TLR4 activation for initiating an intense proinflammatory response (Cao and Li 2020; Yang et al. 2020a; Bonam et al. 2020). The use of pharmaceutical compounds to inhibit TLR4 activation and/or downstream signaling pathway may be an effective strategy to treat COVID-19. Till date, acetylcholine, heparin, statins, dexmedetomidine, and ketamine have been documented for inhibiting the RAGE-HMGB1-mediated activities (Andersson et al. 2020). Moreover, all these compounds along with resveratrol were found to downregulate TLR4-HMGB1 activation (Andersson et al. 2020)

The pathogenesis of COVID-19 is mainly due to consequent release of proinflammatory cytokines including interleukin (IL)-1β and IL-6. The binding of spike protein to the surface TLRs activates the pro-IL-1β, cleaved using caspase-1 followed by inflammasome activation and production of active mature IL-1β (Conti et al. 2020). Suppressing the response of IL-6 may be an effective therapeutic choice to counteract these proinflammatory responses. In this connection, cytokine IL-37 has been found to display immunosuppressive activity by acting on mTOR and increasing AMP kinase to inhibit the inflammatory response by inhibiting the MHC molecules and by suppressing IL-1β, IL-6, TNF, and CCL2 (Conti et al. 2019). Similarly, IL-38 produced by various immune cells like B-cells and macrophages inhibits IL-1β and other proinflammatory IL-family members (van de Veerdonk et al. 2018). Importantly, the use of IL-37 and IL-38 also appears to be an effective therapeutic choice for minimizing the proinflammatory response caused by the IL-1 family members in COVID-19 (Conti et al. 2020).

SARS-CoV-2 is an RNA virus and therefore the functional roles of the intracellular TLRs (TLR-3, 7, 8, and 9) cannot be ignored. A recent study by Choudhury et al. (Choudhury et al. 2021) confirms the sensing of the mRNA of NSP10, S2, and E proteins of SARS-CoV-2 with the intracellular TLRs including TLR 3, 7, 8, and 9. It is noteworthy to mention that TLR3 triggers the activation and production of IFN, which plays an important role in the defense against the coronavirus disease in the human body (Kumaki et al. 2017; Mosaddeghi et al. 2020). Previous studies have demonstrated the use of TLR3 agonists such as poly IC:LC and poly IC inhibiting the replication of CoV and compensating the inhibitory effects on IFN signaling pathways in mice model (Kumaki et al. 2017). Thus, proper employment of TLR3 agonists in the early stages of SARS-CoV-2 infection and also for use as an adjuvant for vaccine development may emerge as an efficient therapeutic strategy (Mosaddeghi et al. 2020).

Advancement of nanotechnology is also showing a hope in the accurate and rapid delivery of antiviral therapeutics for fast recovery from viral diseases. A recent investigation in mice model revealed that the use of gold nanorod fused with an immunogenic viral protein of respiratory syncytial virus (RSV) can eliminate the disease by inducing the activation of TLR signaling pathways, leading to the generation of CD8+ T-cells an natural killer cells as well as secretion of TNF-α and IFN-γ in the lung and bronchiolar tissue (Sivasankarapillai et al. 2020). In addition, peptide-based nanoparticles, inorganic, and polymeric nanoparticles also possess the viricidal activity and these nanomaterials can be used as therapeutics against SARS-CoV-2 (Sivasankarapillai et al. 2020).

6.4.2 TLRs as Targets for Anti-SARS-CoV-2 Vaccine

Considering the alarming situation of COVID-19, the design, and development of vaccines against SARS-CoV-2 are currently the major thrust areas for the scientific communities. Designing a multi-peptide subunit-based epitope vaccine against SARS-CoV-2 using advanced bioinformatics approaches has been demonstrated as a powerful and useful tools (Kalita et al. 2020; Bhattacharya et al. 2020; Abdelmageed et al. 2020). Recently, Kalita et al. (2020) have developed a multi-epitope subunit vaccine against the three key viral proteins, viz., nucleocapsid protein, membrane glycoprotein, and spike glycoprotein along with an adjuvant and epitopes corresponding to helper T-lymphocyte (HTL), cytotoxic T lymphocyte (CTL), and B-cell, connected by a suitable linker. This *in silico* designed vaccine was found to interact with the human TLR3 and regulating the signaling pathway of TLR3 (Kalita et al. 2020). Moreover, good thermodynamic stability, antigenicity, and nonallergic nature of the vaccine determined through molecular docking and molecular dynamics stimulation promises for its future application in field trials (Das et al. 2021). Another immunoinformatic study by Bhattacharya et al. (2020) showed the use of various B-cell and T-cell epitopes combined with MHC-I and MHC-II to develop a peptide-based vaccine against SARS-CoV-2. In contrast to the earlier study conducted by Kalita et al. (2020), the vaccine developed by Bhattacharya et al. (2020) was found to bind with human TLR5 as determined through molecular docking analysis. The TLR5-targeted vaccine was reported for displaying significant effects of the component epitopes, which is an ideal vaccine character while the proper binding with TLR5 is expected to shape the immune response for demolishing the virus (Bhattacharya et al. 2020). Rahman et al. (2020) have also developed a chimeric vaccine called CoV-RMEN using six peptides, viz., PADRE (13 aa), MBE (20 aa), NTD (139 aa), RBD (200 aa), EBE (15 aa), and Invasin (16 aa) fused with certain linker and epitope for increasing the efficiency. Molecular docking analysis revealed that CoV-RMEN shows significant binding and stability against both TLR3 and TLR4 and leads to the activation of dendritic cells for subsequent antigen processing and presentation to CD4+ and CD8+ T-cells via MHC-II and MHC-1 to fight against COVID-19 (Rahman et al. 2020). The generation of coronaviruses S1-subunit vaccine fused with the trimeric domain and engineered with immune stimulants including TLR4 and TLR5 agonists such as RS09 and Flagellin, respectively (Kim et al. 2020). This vaccine was also tested preclinically in mice models using microneedle array (MNA) that showed strong and everlasting antigen-specific antibody response that promises to be an active immunization strategy against the COVID-19 pandemic (Kim et al. 2020). TLR5 is expressed in various immune cells including dendritic cells, monocytes, respiratory epithelium, and pneumonocytes, and plays an important role in inducing innate immune response (Cao et al. 2017). In the early stage of infection, immunomodulation and activation of TLR5 using agonists like flagellin could counteract the effect of SARS-CoV-2 by inhibiting the type I interferon via the production of cytokines (IL-22, IL-18) through TLR5 signaling pathway (Golonka et al. 2020). A list of currently designed/developed vaccines that target human TLRs is given in Table 6.2.

6.4.3 Immunopharmacological Targeting of TLRs for Intervention of SARS-CoV-2 COVID-19

Functions of each TLR can be manipulated by using different pharmacological activators and inhibitors, as we have discussed in the earlier section. In this context, both natural and synthetic compounds having immunopharmacological background could be effective in restoring the immune-homeostasis of SARS-CoV-2-infected patients. Recently, the Chinese government has approved the use of traditional Chinese medicine (TCM) as the therapeutic option for the treatment of COVID-19 and this has been described in the third version of COVID-19 treatment guidelines

Table 6.2 Multi-subunit vaccine targeting TLRs for immunotherapeutic intervention of SARS-CoV-2 infection

Multi-subunit vaccine components	Target TLR binding	Advantages	Reference
Spike protein, adjuvants, HTL, CTL, and B-cell epitopes	TLR3	Thermodynamic stability, antigenic, and non-allergic	Kalita et al. (2020)
B-cell and T-cell epitope combined with MHC-I and MHC-II	TLR5	Activation of immune response	Bhattacharya et al. (2020)
Coronaviruses S1-subunit vaccine fused with immune stimulants	TLR4, TLR5	Preclinically tested using MNA and showed active immunization strategy	Kim et al. (2020)
CTL, HTL, and B-cell epitope	TLR3	Elicits cellular and humoral immune response	Srivastava et al. (2020)
Multi-epitope chimera vaccine-CoV-RMEN	TLR3 and TLR4	Cellular and humoral immune response and cheap cost	Rahman et al. (2020)

published on January 23, 2020 (Jin et al. 2020). The efficacy of natural compounds on SARS-CoV-2 has been supported by a study conducted by Yang et al. (2020b), which has demonstrated that Qingfei Paidu Decoction (QFPD), a natural formulation consisting of 21 components including both herbs and mineral drugs. QFPD directly interferes with TLR4 pathway activation by negatively regulating its downstream signaling pathways including both NF-κB and MAPK signaling pathways and thus inhibiting the release of proinflammatory cytokines (Yang et al. 2020b). In addition, QFPD also regulates the activity of PI3K, AKT, and CASP8, without interfering with TLR2/3 and also impact the INF-α response caused in response to the invasion of viral RNA. All these evidences suggest that QFPD strongly interferes with COVID-19 and possesses the potential to be an effective anti-corona therapeutic (Yang et al. 2020b).

Oleoylethanolamide (OEA), an endocannabinoid-like lipid belonging to N-acylethanolamine family, is usually derived from omega-9 monounsaturated fatty acid. This lipid (OEA) has been reported to be an endogenous ligand for peroxisome proliferator-activated receptor-a (PPAR-a) to mediate anti-inflammatory response by increasing the expression of the anti-inflammatory cytokines (e.g., IL-10) (Tutunchi et al. 2019). Such a strong anti-inflammatory response attenuates the expression and inflammatory response induced by the activation of the TLR4 pathway and also interferes with the ERK1/2/AP-1/STAT3 signaling cascade (Yang et al. 2016). Recently, OEA has been found to induce a synergistic effect against SARS-CoV-2 infection by inhibiting the inflammatory responses caused by TLR activation upon binding of viral ligands (Ghaffari et al. 2020). Therefore, this lipid has been projected as a potential therapeutic to cure COVID-19 (Ghaffari et al. 2020). However, many lipid-derived agents can also activate proinflammatory pathways. In influenza A virus (IAV)-infected mice, it was found that oxidized phospholipids (OxPL) activate the alveolar macrophages to produce cytokines and chemokines via TLR4-TRIF signaling causing acute lung injury similar to that of SARS-CoV-2 infection (Imai et al. 2008). Interestingly, the use of Eritoran (a TLR4 antagonist) resulted in a decrease in the level of OxPL and inflammatory cytokines/chemokines in the mice model (Shirey et al. 2013). Thus, TLR4 antagonists could be a potential therapeutic option for preventing SARS-CoV-2-induced lung injury (Sun et al. 2020).

6.5 Current Status, Prospects, and Challenges

SARS-CoV-2 proteins involved in the pathogenesis, as well as the pathway of inflammatory pathology of COVID-19, are currently considered as the major targets for combating the pandemic. All the proposed specific vaccines and drugs for treating COVID-19 are either in clinical/preclinical trials or at the developing stage. In absence of specified target(s), various pre-established drugs like hydroxychloroquine,

azithromycin, remdesivir, lopinavir/ritonavir, and oseltamivir used for various infectious diseases have been recommended to handle the current situation. However, these drugs are giving variable results in different groups of patients especially those who are from different demographic regions. In this context, the conception of therapeutic strategy by targeting human TLRs is expected to be a hope for all. TLRs are pivotal in human innate immunity as these receptors can selectively sense a diversified group of pathogens including SARS-CoV-2 and governs the fate of the infection. Thus, TLRs can be the specified targets for developing therapeutics against COVID-19. Interestingly, the use of various TLR agonists and antagonists has already been proven as an efficient treatment approach in many human diseases (as described in the earlier section). Therefore, existing TLR-targeting therapeutics as well as newly developed compounds having an influence over TLR function seems to be a useful strategy. TLR-antagonists are expected to reduce the inflammatory pathology/cytokine storm in infected patients while TLR-agonists are expected to act as prophylactic agents in uninfected individuals (Patra et al. 2021). However, the targeted manipulation of TLRs with TLR-antagonists or agonists might lead to unexpected perturbations in the immune homeostasis of humans. TLR-antagonists can efficiently downregulate the IFN-related responses, reduce NF-κβ activity, and suppress TNF-α secretion, which may cause immunosuppression and increase of viral load within the body. On the other hand, the overdose of TLR-agonists may induce uncontrolled proinflammatory responses that can uplift the severity of the disease. Regarding this, the appropriate dose optimization and duration of treatment is very much essential for the TLR-mediated therapeutic strategy (Patra et al. 2021). Moreover, a monoclonal antibody developed against a specific TLR can also be a useful choice. Many of the studies are particularly suggesting to target ACE2, but this receptor does play an important role in regulating blood pressure via the renin–angiotensin axis. So, blockage of ACE2 can create several physiological abnormalities. In this context, TLR is a good choice. SARS-CoV-2 is an RNA virus, and therefore, the influence of the intracellular TLRs (TLR-3, 7, 8, and 9) cannot be overlooked (Choudhury et al. 2021). However, the major route of immunopathological consequences has primarily resulted from host–virus interaction wherein surface spike protein interacts with the cell surface TLRs (TLR1, 2, 4, 5, and 6) present in the immune cells in the human lungs (Choudhury and Mukherjee 2020; Li et al. 2020c). The major issue in adopting TLR-based strategy is that most of the studies on the development of TLR-targeted vaccine candidates or therapeutics have been conducted *in silico,* and therefore, the studies still require few months to present conclusive results. An overview of the therapeutic potential of TLR-guided therapeutic strategies against COVID-19 is summarized in Fig. 6.3.

6.6 Conclusion and Future Direction

Neither a specific antiviral drug nor vaccine is available to date for treating COVID-19 patients. Currently, few broad-spectrum antiviral drugs (remdesivir, lopinavir/ritonavir, and oseltamivir), anti-protozoan drug (hydroxychloroquine), and antibiotic (azithromycin) are the limited nonspecific therapeutic agents for combating COVID-19 (Gautret et al. 2020; Grein et al. 2020; Wu et al. 2020). In addition, the use of neuraminidase inhibitors, RNA synthesis inhibitor, as well as HIV-protease inhibitor has also been exploited to attenuate SARS-CoV-2 infection (Rothan and Byrareddy, 2020). From various *in silico* studies, it is evident that most of the researchers are aiming toward developing effective multi-peptide-based vaccine(s) more specifically epitope-based for rapid results (Kalita et al. 2020; Bhattacharya et al. 2020; Abdelmageed et al. 2020). Intriguingly, all these studies have indicated the involvement of TLRs as the vaccine target to heighten the immune response up to the required level (Kalita et al. 2020; Bhattacharya et al. 2020; Abdelmageed et al. 2020). *In silico* TLR-targeted vaccine development approaches are particularly less time and labor consuming as well as devoid

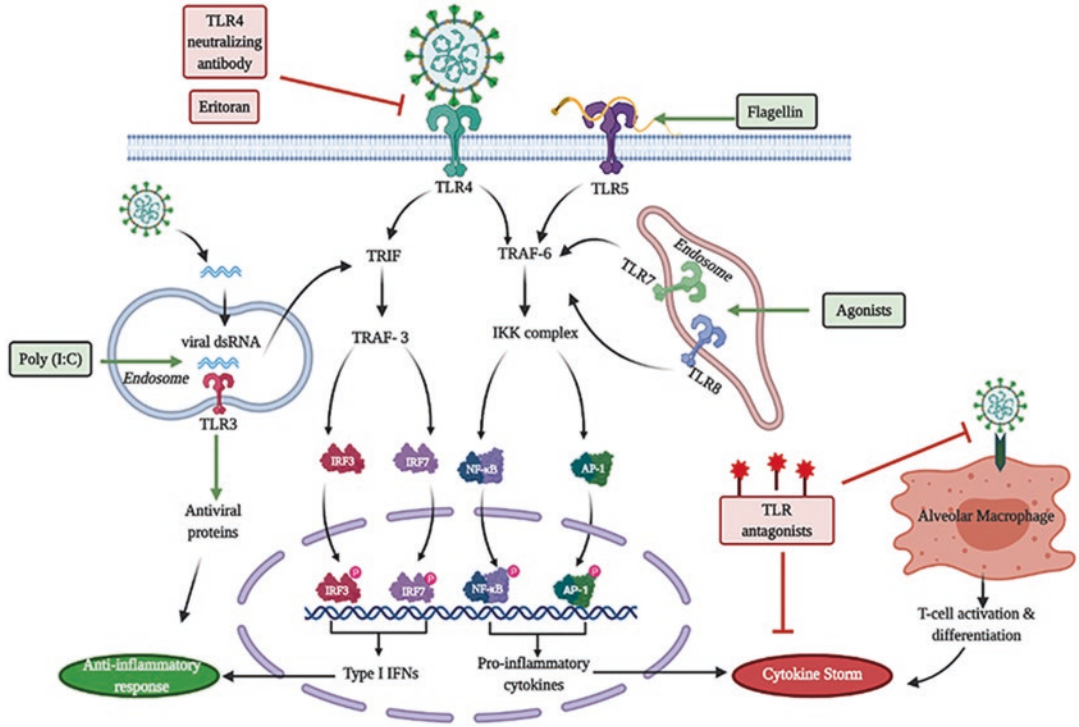

Fig. 6.3 TLR-guided therapeutic strategy against SARS-CoV-2. TLR4 antagonists like Eritoron and neutralizing antibody inhibit the binding and activation of SARS-CoV-2 with TLR4. An immune response to dsRNA can be partially generated during SARS-CoV-2 replication sensitized by TLR3 leading to cascades of signaling pathways (IRFs and NF-κB activation, respectively) are activated to produce type I IFNs and proinflammatory cytokines. In the early stage of infection, SARS-CoV-2 is capable of inhibiting host type I interferon (IFN) antiviral immune defenses that can be counteracted by utilizing flagellin to activate TLR5 inducing the production of cytokines (i.e., IL-22, IL-18) and IFN, which may restore the impaired immune responses. It is suggested that the use of TLR antagonists may inhibit the macrophage-activated cytokine storm and may act as therapeutic against SARS-CoV-2. The production of type I IFNs is important to enhance the release of antiviral proteins for the protection of uninfected cells

of excessive use of *in vitro* culture to check the biological efficacy (Kalita et al. 2020; Bhattacharya et al. 2020; Abdelmageed et al. 2020). Although an mRNA-based vaccine candidate, mRNA-1273 has already entered in the first phase of the human trial (Wang et al. 2020b), TLR-targeted therapeutic strategies are also ushered to be a hope for all of us. Particularly, clues obtained from the dry-lab experiments are needed to validate in biological systems including both *in vitro* and *in vivo* exploration. Considering urgent stipulation, synchronization amongst the laboratories and industries are particularly required to check and validate the efficacy of TLR-based vaccine and/or drugs for speedy development intervention strategy. Moreover, parallel studies on the fundamental biology of SARS-CoV-2 and immunity are also required to enlighten the pathway for developing new therapeutics.

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Compliance with Ethical Standards

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Ethical Approval for Studies involving Humans This chapter does not contain any studies with human participants performed by any of the authors

Ethical Approval for Studies involving Animals This chapter does not contain any studies with animals performed by any of the authors.

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Potential Drug Strategies to Target Coronaviruses

7

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Abstract

Introduction: As the world has witnessed three severe coronavirus outbreaks in the past two decades, including the recent pandemic COVID19, caused by SARS-CoV2, it has become of utmost importance to develop drugs and vaccines against coronaviruses. The previous two outbreaks, severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) emerged in China and Saudi Arabia in 2003 and 2012, respectively. COVID19 is considered the worst of all and has taken more than 4 million lives so far and crippled the socioeconomic life of human beings in the entire world. Extensive research is being carried out to find out a solution that will not only help us to fight the current situation but also prepare us to prevent further intervention by similar viruses in the future. Here, we aim to highlight potential drug target sites in coronavirus infection or life cycle in general.

Methods: We have gone through the research papers published on coronavirus, with special emphasis on SARS-CoV, MERS-CoV, and SARS-CoV2, in peer-reviewed journals and tried to identify the possible sites in the coronavirus life cycle which can be used as potential drug targets.

Results: Studies showed that there are several unique enzymes and mechanisms involved in the coronavirus life cycle which can be manipulated to develop drugs against it. However, it has been always a challenge to develop drugs or vaccines against viruses as they utilize the host cell machinery and more difficult against RNA viruses because of their high mutation rate.

Conclusions: Effective control of the current (2020) pandemic necessarily depends on the development of either a vaccine or an effective therapeutic agent. In the past, many attempts were taken to develop vaccines after the outbreak of SARS-CoV and MERS-CoV, though no successful vaccine reached to the market as the situation came under control. In the current scenario, many laboratories have developed effective vaccines against SARS-CoV2, which have reduced both the severity of the infection and the rate of mortality considerably. However, world needs to be prepared for similar viral outbreaks in future and research must be continued to develop more effective vaccines and therapeutics against coronaviruses.

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Keywords

Coronavirus · Severe acute respiratory syndrome · SARS · Innate and adaptive immune response · Neutralizing antibody · Cytokine storm · Vaccination

Abbreviations

$3CL^{pro}$	Chymotrypsin-like protease
ACE2	Angiotensin converting enzyme 2
Ang II	Angiotensin II
COVID19	Coronavirus disease of 2019
CREB1	CAMP responsive element binding protein 1
CTL	Cytotoxic T lymphocytes
DMVs	Double-membrane vesicles
DPP4	Dipeptidyl-peptidase 4
ERGIC	Endoplasmic reticulum Golgi intermediate compartment
G-CSF	Granulocyte colony-stimulating factor
HCoV-229E	Human coronavirus 229E
HKU1	Human coronavirus HKU1
HMG CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
IL-10	Interleukin 10
IMP α/β	Importin α/β
IP-10	Interferon gamma-induced protein *10* (also known as CXC motif chemokine *10* or CXCL10)
MCP-1	The monocyte chemoattractant protein-*1*
MERS	Middle East respiratory syndrome
MIP-1A	Macrophage Inflammatory Proteins 1A
M^{pro}	Main protease
mTOR	Mammalian target of rapamycin
MβCD	Methyl-β-cyclodextrin
NL63	Human coronavirus NL63
NNIs	Non-nucleoside inhibitors
Nsp	Non-structural proteins
OC43	Human coronavirus OC43
ORF1a	Open reading frame 1a
p53	Tumor suppressor p53
PL^{pro}	Papain-like protease
RaTG13	Bat coronavirus *RaTG13*
RBD	Receptor binding domain
RdRp	RNA dependent RNA polymerase
RTC	Replicase–transcriptase complex
SARS	Severe acute respiratory syndrome
SMAD4	Mothers against decapentaplegic homolog 4
STST1	Signal transducer and activator of *transcription* 1
TLR	Toll like receptor
TMPRSS11D	Transmembrane protease, serine 11D
TMPRSS2	transmembrane protease serine 2

7.1 Introduction to Coronavirus

The family *Coronaviridae* is composed of several groups such as α-CoVs, β-CoVs, γ-CoVs, and δ-CoVs, which vary in their genetic content and variation in their antigenic properties. Human α and β coronaviruses such as 229E, NL63, OC43, and HKU1 are common and cause COVID19 like symptoms (Li and Luk 2019). The first human coronavirus strain, 229E, was isolated by Dorothy Hamre in the 1960s. SARS-CoV2 bears about 79% and 50% sequence similarity with SARS-CoV, a lineage B beta-coronavirus, and MERS-CoV, a lineage C beta-coronavirus, respectively (Lu et al. 2020; Mousavizadeh et al. 2020). SARS-CoV2 has almost 96% similarity with Bat-CoV RaTG13 and 88% identity to Bat-SL-CoVZC45 and Bat-SL-CoVZXC21 (SARS-like coronaviruses in bat), collected in 2018 in Zhoushan, eastern China (Lu et al. 2020). These viruses can cross the species barriers and mainly infect mammalian and avian species.

The members of *Coronaviridae* are positive-sense, single-stranded RNA viruses with envelope. The genome length varies between 27,000–32,000 bases among different coronaviruses. The envelope derived from host cell membranes carries three viral structural proteins, namely, membrane protein (M), spike protein (S), and envelope protein (E). The RNA genome is wrapped by N proteins to form the nucleocapsid inside the envelope (Siu et al. 2008). Trimeric S proteins form a crown-like spike on the viral envelope and responsible for initiating the infection (Fang Li, 2016). M protein is abundant in the viral envelope, while E protein is the smallest and is found in a small proportion in the envelope (Walls et al. 2020; Schoeman et al., 2019).

7.2 Infection Cycle of Coronavirus

Coronaviruses enter the human system through upper respiratory tract, i.e., nose, mouth, and mucous membrane of eyes and travel down through the throat and bronchial tubes toward the lungs. The initial symptoms are mild and like common flu or cold, which include cough, sore throat and runny nose, difficulty in breathing, headache, and fever. Sometimes fewer common symptoms like diarrhea, conjunctivitis, loss of smell or taste, a rash on skin, or discoloration of fingers or toes may appear. Later, extensive inflammation in the mucous membranes of airways leads to the damage of the air sacs. The sacs are filled up with fluids, pus, and dead cells leading to pneumonia (Lee et al. 2020), and patients find difficulty in breathing and require ventilator support. In case of SARS-CoV2 infection, patients with diabetes and heart diseases are at a high risk of mortality as total lung function collapses. SARS-CoV2 can infect the gastrointestinal tract and enter the bloodstream too and infect other organs like the heart, kidneys, liver, etc. (Wang et al. 2020; Naicker et al. 2020). As the endothelial cells under the epithelial cells of airway get damaged, small blood clots appear leading to stroke as a second attack. Hence, SARS-CoV2 is associated with venous and arterial thrombosis and acute cerebrovascular diseases too (Chen et al. 2020).

Trimeric S proteins present on the viral envelope are responsible for initiating infection by binding to specific receptor (Gui et al, 2017). SARS-CoV and SARS-CoV2 utilize the transmembrane protein, angiotensin converting enzyme 2 (ACE2) to enter the cell, while MERS-CoV binds to an 110KDa glycoprotein, an exopeptidase called Dipeptidyl-peptidase 4 (DPP4). The length of the S proteins varies between 1160–1400 amino acids among coronaviruses. The protein is cleaved by host proteases at a multibasic cleavage site between S1 and S2 domains (S1/S2) (Du et al. 2009). S1 subunit has the receptor binding domain (RBD) which recognizes specific receptors, and the hydrophobic fusion loop or fusion peptide in S2 subunit helps in the host-viral membrane fusion (Wong et al. 2004). The nature of the protease that cleaves the S glycoprotein varies with the strain of the coronavirus. The S proteins of SARS-CoV and SARS-CoV2 are cleaved by transmembrane protease serine 2 and 11D (TMPRSS2 and TMPRSS11D) just after recognizing the receptor. However, the S protein of MERS-CoV is processed intracellularly by furin proteases before exit from the infected cell (Shulla et al. 2010; Hoffmann et al. 2020). Thus, newly released MERS-CoV particles are ready to enter new cells. In contrast, S proteins of SARS-CoV remain in an uncleaved condition upon virus release from cells. Interestingly, SARS-CoV2 has been found to contain the furin-like cleavage site at S1/S2, which is not present in the same clade of coronavirus though present in human coronaviruses OC43, HKU1 (Coutard et al. 2020). However, whether the S protein of SARS-CoV2 is cleaved or not inside the host cell and how does it depend on the TMPRSS2 mediated cleavage before entry into a cell need more studies (Hoffmann et al. 2020).

Inside the endosome, the S2 domain is further cleaved into S2' by another protease, cathepsin, and activates membranes fusion within endosomes (Miao Gui, 2017). Following endosomal membrane fusion, the viral RNA enters the cytoplasm and is translated. Two open reading frames

1a and 1ab (ORF1a and ORF1ab) are translated to produce polyproteins pp1a and pp1ab. Two proteases, M^{pro} (Main protease) or $3CL^{pro}$ (picornavirus 3C protease like cleavage-site specificity) and PL^{pro} (papain like protease) encoded by ORF1a cleave the polyproteins in at least 11 sites to produce non-structural proteins (nsp) (Gorbalenya et al. 1989). These proteins join to form the RNA replicase–transcriptase complex (RTC) which produces the complementary –ve sense RNAs of the +ve sense genome during replication. Discontinuous transcription gives rise to multiple sub-genomic RNAs encoding the structural proteins.

The N proteins encapsidate the genomic RNA and make the nucleocapsids in the cytoplasm. The nucleocapsids then travel through the lumen of the endoplasmic reticulum Golgi intermediate compartment (ERGIC) or vesicular-tubular cluster and acquire their membrane envelope (Ujike et al. 2015). The M proteins interact among themselves and take part in the formation of the envelope. The M proteins also help in retention of S proteins in the ERGIC and mediate its incorporation into new virions. The E proteins are abundantly produced and localized to the ERGIC where they participate in the assembly of complete virus particles.

The newly formed infectious virions are then released through the secretory pathway of the infected host cell and are ready to attack new host cells. The infection induces a cellular stress condition known as the unfolded protein response mediated primarily by viral S protein.

7.3 Role of Our Immune System

The duration of infection by SARS-CoV2 can be divided into broadly three phases. Stage I or early infection phase is called the viral response phase. In this stage, upon entry of the virus particle, the phagocytic cells of our innate immune system such as macrophages, neutrophils, dendritic cells, natural killer cells try to engulf and remove it. These cells release several cytokines, chemokines and initiate the process of inflammation. At the same time, peptide fragments generated from the virus particle are presented by the antigen presenting cells (macrophages, dendritic cells, and B-lymphocytes) to the T-lymphocytes, both T helper and T-cytotoxic. Within 7–10 days after infection, activation of B- lymphocytes and cytotoxic T lymphocytes (CTL) lead to the production of antibodies and memory cells. Neutralizing antibodies prevent the attachment of newly produced virus particles to host cells and effector Tc cells kill the viral antigen presenting cells. Now, the immune response works with both innate and adaptive branches and slowly leads to the recovery of the patient. This is the second phase, viral load decreases and most of the people recover on their own without showing much symptoms. Activation of adaptive response clears the virus more quickly and efficiently than innate response and thus the recovery time of an infected person depends on his immune system. It has been found in case of older people and people with co-morbidities, adaptive immune response is weaker, and the virus persists for long. It generates huge amount of cytokines and chemokines which ultimately lead to 'Cytokine storm' and multi-organ failure. This is the third phase of the infection also called the hyperinflammation phase. Patients need the help of ventilators and some succumb to death.

7.4 Difficulty in Designing Drugs Against Viruses

Designing of drugs against viruses is very difficult as they use host cell surface proteins having specific physiological roles as the receptors to enter the cells. Upon entry into the cell, the viruses capture the host cell machinery to make progeny virions. Hence, targeting any intermediate step(s) in the viral life cycle would interfere with the host machinery leading to severe side-effects. The only way to prevent viral infection without interfering with the host system is through vaccination in which neutralizing antibodies developed against the viral surface proteins inhibit the attachment of the virus to its receptor. Moreover, high mutation rate of RNA-dependent RNA polymerase makes it more difficult to design drugs against RNA viruses.

7.5 Different Approaches to Prevent and Cure Coronavirus Infection

1. ***Prevention of initiation of an infection by blocking entry of coronavirus into the target cells.***

Development of Vaccines Against Coronaviruses Literature survey shows that serious attempts were made to develop vaccines against SARS-CoV and MERS-CoV after the outbreaks in 2003 and 2012, respectively. Several groups have reported their findings on development of vaccines against SARS-CoV between the year 2003 and 2007. The strategies include use of attenuated vesicular stomatitis virus expressing spike protein of SARS-CoV, live attenuated SARS-CoV with deletion of E and other proteins, inactivated SARS-CoV, recombinant S2 fragment, B cell epitopes of S2 spike protein, trimeric recombinant spike protein, recombinant adenovirus with N terminal segment of S1 gene, DNA vaccine, etc. (Bukreyev et al. 2004; Kapadia et al. 2005; Netland et al. 2010; Tsunetsugu-Yokota Y 2008; Zhao et al. 2005). Similarly, there are reports on development of vaccines against MERS-CoV between the year 2012 and 2020, which include use of inactivated MERS-CoV, S1 subunit vaccines, recombinant adenovirus encoding the S1 subunit, DNA vaccine, virus-like particles, etc. (Folegatti et al. 2020; Muthumani et al. 2015; Kato et al. 2019).

Vaccine development strategy against SARS-CoV2, includes inactivated virus vaccine, recombinant viral vectored vaccine, protein subunit vaccine, mRNA vaccine, adenoviral vector vaccine, and recombinant influenza viral vector vaccine are prominent (Sharpe et al. 2020).

Vaccination generates neutralizing antibodies against viral surface proteins along with other protective mechanisms like activation of T cells. The antibodies attach to the viral surface and facilitate its phagocytosis and consequent removal from the host system.

Blocking of ACE2 Receptors to Prevent Viral Entry As SARS-CoV attach to the membrane bound protein ACE2 present on many cell types, blocking of this receptor can be a way to prevent the viral entry. ACE2 plays an important role in Renin angiotensin system (RAS) and its main function is to cleave Angiotensin II (Ang II) to angiotensin 1–7 which has opposite actions of Ang II. The level of Ang II has been found to be doubled in ACE2 deficient mice, while the levels of Ang 1–7 are almost undetectable. ACE2 has beneficial and protective role against tissue injury, cardiovascular diseases, hypertension, etc. Thus, blocking of ACE2 might have severe side effects and can increase susceptibility to inflammation, cell death, and eventual organ failure. Some small molecules have been identified by researchers which can act as inhibitors of ACE2, like lividomycin, which is an aminoglycoside antibiotic, burixafor, a chemokine receptor type 4 antagonist, quisinostat, a histone deacetylase inhibitor, fluprofylline, spirofylline, and diniprofylline as phosphodiesterase inhibitors, pemetrexed, an antifolate, edotecarin, a topoisomerase I inhibitor, N-(2-aminoethyl)-1 aziridine-ethanamine, etc. (Terali K et al. 2020; Markus H et al. 2020; Huentelman et al. 2004). These molecules are engaged with ACE2 through ionic interactions and can block the interaction with SARS-CoV.

7.6 Inhibition of Host Cell Proteases

Inhibition of TMPRSS2 The protease, TMPRSS2, that cleave the S protein of CoVs, is a 492 amino acid Type II transmembrane serine protease. It is abundant throughout the human respiratory tract cells and can be a potential drug target (Hoffmann et al. 2020). Though the cleavage of S protein of MERS-CoV takes place inside the host cell, it still depends on TMPRSS2 mediated cleavage and addition of trypsin with MERS-CoV increases its infectivity (Shirato et al. 2013). Therefore, inhibitors of serine proteases could be

employed for blocking the entry of coronavirus, though the major question that remains is the bioavailability of the drug in the lungs. One such serine protease inhibitor is Camostat mesilate, which blocks TMPRSS2 activity and has been approved in Japan for human use for a long time against chronic pancreatitis, reflux esophagitis, etc. (Uno Y 2020) (Fig. 7.1).

Inhibition of Cathepsin Fusion between viral envelope and endosomal membrane is dependent on endosomal protease, Cathepsin-mediated cleavage of S2 into S'. Hence, use of inhibitors of Cathepsin can block the membrane fusion and release of viral RNA into cytosol. Several protease inhibitors have been analyzed and identified which can inhibit this cysteine protease inside the endosomes (Simmons et al. 2005) (Fig. 7.1).

Inhibition of Furin and Furin-Like Proteases The protease furin is a cellular endopeptidase which is involved in proteolytic activation of many proproteins in the secretory pathway. Furin cleaves at multibasic consensus sequences having arginine residues. Many bacterial and viral proteins having furin-like cleavage sites are processed by this protease inside the cell which leads to virus propagation. Among these viral proteins, hemagglutinins of highly pathogenic avian influenza virus, surface glycoproteins of the HIV, Ebola, Marburg, and measles virus are important. Thus, blocking the furin enzyme can be a potential target in the control of many viral life cycles. The first furin inhibitor was chloromethyl ketones and many more inhibitors were developed later like α_1-antitrypsin Portland, mutated forms of Eglin c, 83-mer prodomain of furin produced synthetically, various types of oligopeptides, and small molecule inhibitors, etc. Nona-D-arginine, a polyarginine, inhibits furin with high efficiency (Becker et al. 2012) (Fig. 7.2).

7.7 Prevention of Endosomal Membrane Fusion and Release of Viral Genome into the Cytosol

Chloroquine or hydroxychloroquine, an antimalarial agent is known to increase the endosomal pH and prevent virus-host cell membrane fusion leading to blocking of release of viral RNA into the cytosol (Wang et al. 2020). As endosomes are involved in protein trafficking and glycosylation, increase in pH interferes with the glycosylation process of ACE2 inside them. Improperly glycosylated ACE2 does not act as receptors and prevent the interaction between virus and host cell (Vincent et al. 2005) (Fig. 7.1).

7.8 Inhibition of Viral RNA Polymerase

The activity of the RNA-dependent RNA polymerase (RdRp, nsp12) enzyme can be blocked by using nucleoside analogs which are structurally similar to dNTPs or rNTPs except the 3′ carbon -OH group. Incorporation of dNTPs terminates chain elongation during replication or transcription. One such antiviral drug is Remdesivir**,** a nucleotide analog whose active form is incorporated into the growing RNA chain by RdRp, escapes the proofreading activity by viral exoribonuclease (ExoN) and prevents viral propagation by terminating chain elongation. Remdesivir and its other derivatives have been found to be useful against many RNA viruses like influenza, Nile virus, yellow fever virus, etc., though several various side effects like occasional myopathy, neuropathy, pancreatitis, nephrotoxicity, etc. (Khungar et al. 2010) are associated with their use (Fig. 7.2).

RdRp can be inhibited by non-nucleoside inhibitors (NNIs)**,** which bind to conserved sequences of RdRp and change its conformation required for polymerase activity. These mole-

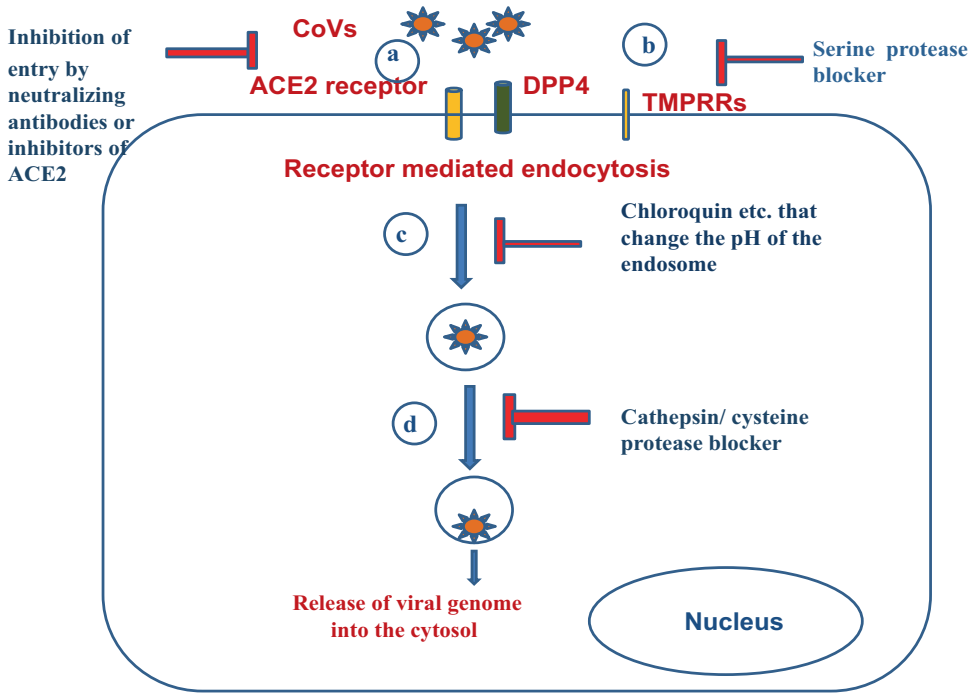

Fig. 7.1 Early stages in viral life cycle can be targeted for drug development
Different sites in coronavirus life cycle have been shown where drugs can be administered. **(a)** The entry of the virus can be prevented by generating antibodies through vaccination or by using inhibitors of ACE2; **(b)** entry can be further blocked by targeting the cell surface co-receptor DPP4 and protease TMPRSS; **(c)** after receptor mediated endocytosis of the virus particle, changing of endosomal pH can prevent the release of the viral RNA into the cytosol; **(d)** action of another protease cathepsin required for membrane fusion can be blocked by suitable agents inside the endosome

cules are not competitive inhibitors of nucleotides and have least side effects on the host. The disadvantage is that a single mutation in the NNI binding site on RdRp can lead to drug resistance. The NNIs used for hepatitis C virus treatment include benzimidazole, indole derivatives, benzothiadiazine, thiophene-2-carboxylic acids, dihydropyranones, etc. (Chan et al. 2004). An anti-influenza virus drug, Avigan, which selectively inhibits the RNA polymerase is under phase III clinical trial against SARS-CoV2.

Replication of MERS-CoV has been found to be inhibited by Saracatinib, a 5, 7-substituted anilinoquinazoline, inhibitor of protein tyrosine kinases. It interferes with early events in the viral life cycle as found in in vitro studies (Shin et al., 2018a, b). Saracatinib also exhibits synergistic effect with anticancer drug, Gemcitabine, having antiviral activity against RNA viruses.

7.9 Inhibition of Viral Protease

The main protease, M^{pro} or nsp5 can be a potential target for drug development against coronaviruses with minimal toxic effects on host as similar cleavage specificity is not found in human proteases. M^{pro} shares spatial/structural similarity with the active site of HIV protease and is inhibited by anti-HIV drugs Lopinavir/Ritonavir. Many groups have proposed ligands based on in silico studies, which can interact with the protease and stop its action (Zhang et al. 2020). Some of the compounds include antibiotic Colistin, antitumor drugs like Valrubicin, Epirubicin, Vapreotide, Aprepitant, antirhinitis drug Bepostatine, etc. (Liu and Wang 2020). Nitazoxanide, an antiprotozoal agent has shown inhibitory activity against human and animal coronaviruses, at a low-micromolar concentration (Fig. 7.2).

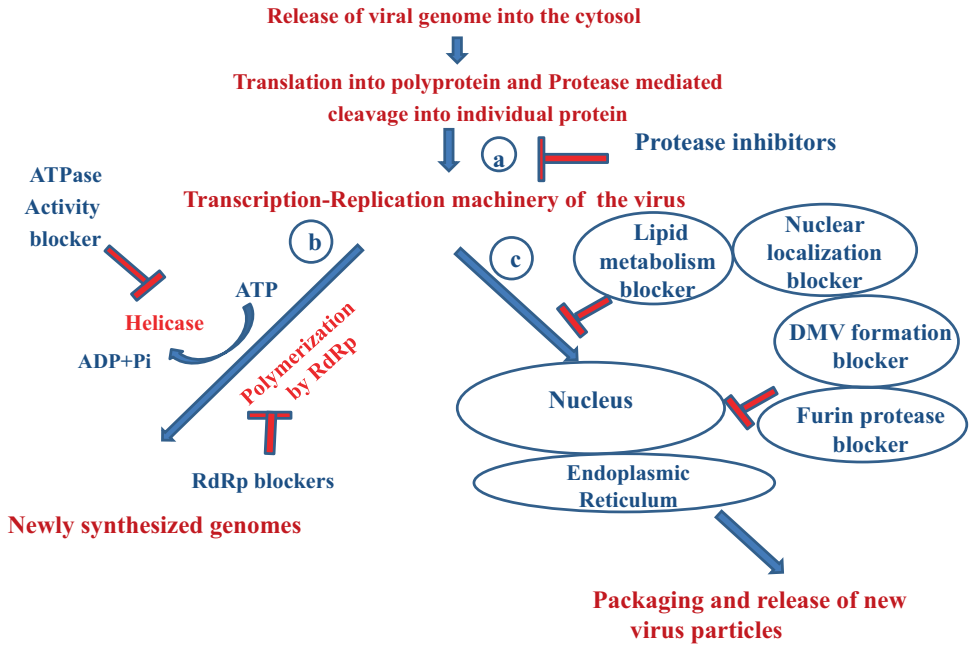

Fig. 7.2 Inhibition of replication cycle of coronavirus inside the host
(a) The viral genome is translated into polyproteins and proteases after entry into the cytosol. The viral main protease cleaves the polyprotein into many non-structural proteins. Inhibition of the main protease can be a way to prevent viral replication inside the host; **(b)** the transcription replication machinery (composed of RdRp, helicase) which produces newly synthesized +/-RNA strands can also be targeted for drug development; **(c)** other molecular events in the host cell utilized by the coronavirus e.g., lipid metabolism, nuclear localization, transport through double membrane vesicles (DMVs), can also be exploited for drug development

7.10 Inhibition of Viral Helicase

The enzyme helicase, nsp13 has been found to be conserved among different coronaviruses. This enzyme catalyzes NTP-dependent unwinding of the duplex RNA into single strands from 5′ to 3′ direction (Singleton et al. 2007; Jia et al. 2019). The unwinding activity has been postulated for domain 1A (Jia et al. 2019). It has been found that RdRp interacts directly with helicase and increases the overall helicase activity (Jia et al. 2019; Adedeji et al. 2012). Inhibition of the activity of nsp13 can be achieved by targeting NTP binding site or nucleic acid binding site or directly blocking the NTPase activity or helicase translocation, etc. (Habtemariam et al. 2020). Some potential inhibitors have been identified for MERS-CoV helicase based on in silico molecular docking experiments (Zaher et al. 2020). Some of them are benzotriazole, imidazodiazepine, quinoline, anthracycline, triphenylmethane, pyrrole, small peptide, bananin derivatives (Briguglio et al. 2011), etc. Bananins, an antiviral compound with trioxa admantane moiety have been shown to inhibit coronavirus helicase activity at a very low concentration (Tanner et al. 2005) (Fig. 7.2).

7.11 Modulation of Host Cell Lipid Metabolism and Inhibition of Viral Double Membrane Vesicle Formations

The lipid metabolism pathway is hijacked by most of the enveloped viruses and biosynthesis of lipid molecules is enhanced to produce viral envelope. An agent that interferes with cholesterol depletion in cells is Methyl-β-cyclodextrin (MβCD). Pretreatment with MβCD has been found to inhibit the production and release of SARS-CoV particles in Vero E6 cells. The nor-

mal viral life cycle was restored by the addition of cholesterol to the culture medium indicating the role of MβCD mediated loss of cholesterol (Li et al. 2007). The interaction between S protein and ACE2 reduces in cells treated with MβCD in a dose-dependent manner leading to reduced viral replication. MβCD treatment successfully inhibited poliovirus entry into in vitro cell culture system (Pranav and Marie 2004).

Similarly, use of phytosterols reduce membrane cholesterol and destabilize the membrane structure and inhibit viral infectivity significantly (Abu-Farha et al. 2020). The cholesterol biosynthesis pathway is reduced by Statins, which inhibit the activity of HMG-CoA reductase. They exert pleiotropic effects on inflammation and oxidative stress, modulate the immune response and restore the vascular redox balance by reducing reactive oxygen species and increasing antioxidants (Castiglione et al. 2020).

Another potential target site is sphingolipid biosynthesis pathway as lipid rafts involved in coronavirus life cycle are enriched in sphingolipids, cholesterol, and many other proteins. Sphingolipids are very important for lungs as they protect the lungs from pulmonary leak and injury. Hence, this pathway can be modulated for therapeutic intervention strategies (Pratelli and Colao 2015).

Double-membrane vesicles (DMVs) formed from ER during the replication of coronavirus can be another target for drugs. DMVs have been found to carry out the replication-transcription complexes or RTCs composed of non-structural proteins 3, 4, and 6 (Angelini et al. 2013). Drugs targeting DMV formation can prevent viral infection by impairing viral RNA synthesis. K22 is one such compound which interacts with nsp6 and inhibits replication of many animal and human coronavirus (Lundin et al. 2014). K22 resistance has been found in nsp6 mutants suggesting the inhibitory role of this molecule. K22 is also active against other viruses like Nidoviruses and Flaviviruses, suggesting its involvement in a critical and conserved step during viral replication (Rappe et al. 2018). Hence, DMVs can be targeted to develop broad-spectrum antivirals and more studies are needed to fully elucidate their roles in viral life cycle (Shahmohamadnejad et al. 2020) (Fig. 7.2).

7.12 Attenuating the Inflammatory Response

Extensive replication of coronavirus in the epithelial cells and pneumocytes of the respiratory system results in acute inflammation. This process starts when the virus binds to the Toll like receptors (TLR) on macrophages and dendritic cells and unlocks a cascade of signaling pathway leading to activation of IL-1, IL-6, IL-8, tumor necrosis factor-α, and decrease in CD4+ and CD8+ T cells. Other cytokines like IL-2, IL-7, IL-10, G-CSF, IP-10, MCP-1, and MIP-1A have been confirmed at increased levels in many respiratory viral infections, including COVID-19 patients (Huang et al. 2020). The levels of these cytokines correlate with the rate of replication of SARS-CoV-2 (Conti et al. 2020) and lead to cytokine storm. This situation was more visible in elderly patients (over 60 years old) with comorbidities like diabetes, hypertension, etc. (Huang et al. 2005; Chen et al. 2013; Channappanavar and Perlman 2017). Hence, reducing the cytokine storm can be a way of treating the infection. Use of antibodies against inflammatory cytokine IL-6 can be another approach as blocking of the JAK-STAT pathway prevents the cytokine storms in the immune system, one of the reasons behind organ failure and death in many patients. We also have other cytokines such as IL-37 and IL-38 that trigger anti-inflammatory mechanism through diverse mechanisms. For example, IL-37 can increase the levels of AMP kinase and decrease mTOR signaling leading to the inhibition of expression of pro-inflammatory cytokines (Fig. 7.3).

The mTOR or mammalian target of rapamycin, a serine/threonine kinase, is an important signaling protein with crucial functions in cell growth, metabolism, and proliferation (Polivka and Janku, 2014). This protein is also involved in the inflammatory process. Many DNA and RNA viruses utilize this signaling pathway for their replication in mammalian host cells (Cooray

2004; Wang et al. 2006; Shin et al. 2007; Buchkovich et al. 2008; Qin et al. 2011) and inhibitors of mTOR, everolimus and sirolimus, have shown antiviral effects against MERS-CoV (Kindrachuk et al. 2015). In another study, the essential role of mTOR has been shown on antigen-specific humoral immune responses in rapamycin-treated mice (Ye et al. 2017). Furthermore, mTOR inhibitors enhance the production of CD8+ T cells during vaccination (Turner et al. 2011) and could suppress B cell production in germinal centers. Inhibitors of mTOR have been found to successfully prevent severe pneumonia and acute respiratory failure caused by H1N1 (Wang et al. 2014). There were some studies suggesting the positive effect of mTOR inhibitors on early stages of SARS-CoV2 infection, especially in the high-risk groups of patients (Liu et al. 2020; Zheng and Liu 2020; Zhou et al. 2020). However, there are some restrictions regarding the use of mTOR inhibitors along with other drugs. For example, lopinavir/ritonavir used in the treatment of HIV should not be co-administered with sirolimus (rapamycin) and everolimus (Boettler et al. 2020; Guillen et al. 2020). With this limitation in mind, further studies on mTOR inhibitors as a target for SARS-CoV-2 can be done.

In a randomized controlled trial started in March 2020 in UK (RECOVERY), against coronavirus treatment, many potential therapeutic agents were tested. The findings revealed in a press release suggest that Dexamethasone is effective on terminally ill patients who are in ventilator or oxygen support. It showed no effect on the patients with mild symptoms as the steroid acts on and prevents the cytokine storm, the ultimate cause of death, generated in advanced stages in COVID19 patients (Ledford H, 2020). (Fig. 7.3).

7.12.1 Other Strategies

Apart from these above-mentioned strategies, there are more sites in the viral life cycle which can be targeted for drug development. Coronavirus accessory protein 6 encoded by ORF6 has been found to interfere with nuclear import factors or karyopherin-dependent transcription factors of host cells. ORF6, located in the endoplasmic reticulum of the infected cells, has been found to antagonize many transcription factors like STST1, VDR, CREB1, SMAD4, p53, etc., which are responsible for mediating antiviral responses and other important cellular functions. Thus, blocking ORF6 can reduce the inhibition of antiviral mechanism (Frieman et al. 2007; Hussain et al. 2010). Also, inhibition of nuclear import proteins IMP a/b, which transport proteins to nucleus through nuclear pore complex, can be targeted. One such compound identified is Ivermectin, which acts against SARS-CoV2 in vitro by preventing IMP from binding to viral proteins (Heidary et al. 2020).

Detailed research on the viral life cycle and its interaction with the host immune system will bring out more possible and effective target sites and effective treatment in future.

7.13 Conclusion

Since there is no available treatment for COVID19 at present, palliative care is the only way to relieve some symptoms in the sufferers. Repurposing of some drugs and convalescent plasma therapy, i.e., administration of serum from an individual successfully recovered from the infection, are the two ways of treatment. However, because of the rapid spread of this virus, vaccination seems to be the only efficient method to prevent and control the COVID-19 pandemic. More research should be conducted on coronavirus so that the world remains ready with proper therapeutic interventions in future.

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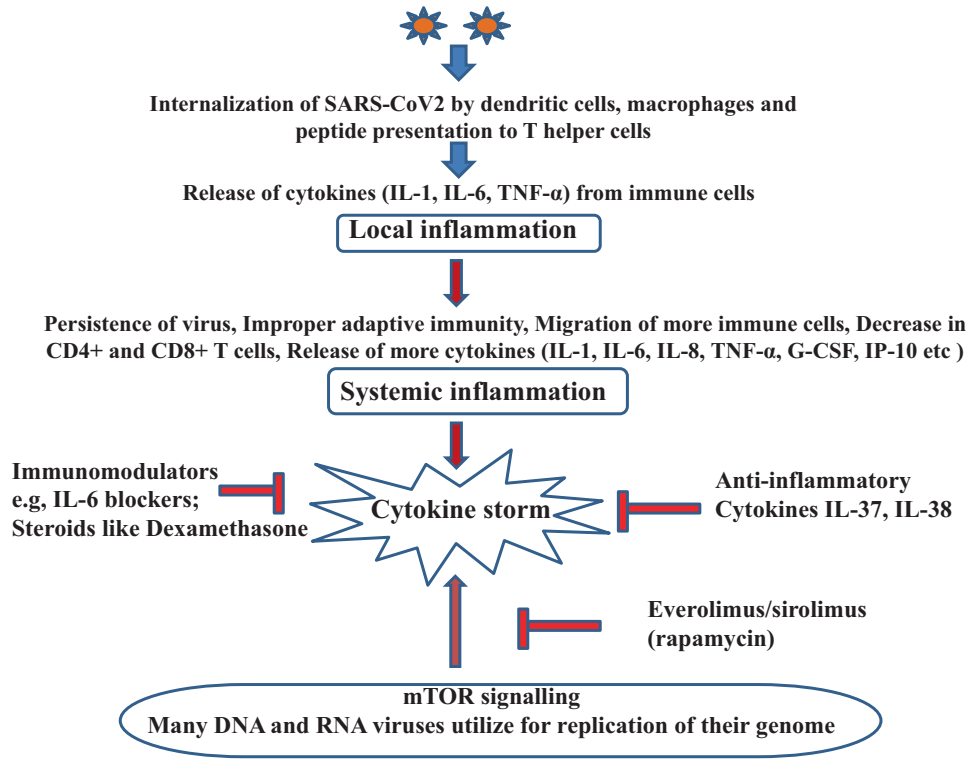

Fig. 7.3 Blocking of cytokine storm can prevent death from COVID19
Cytokine storm, produced by release of excess cytokines and chemokines by immune cells, is the cause of death in many patients in COVID19. Inhibition of cytokine storm using steroids like dexamethasone (Ledford H, 2020), antibodies against IL6, mTOR blocker like Everolimus have been found to be effective

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8 Possible Therapeutic Intervention Strategies for COVID-19 by Manipulating the Cellular Proteostasis Network

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Abstract

Introduction: The recent outbreak of coronavirus infection by SARS-CoV-2 that started from the Wuhan Province of China in 2019 has spread to most parts of the world infecting millions of people. Although the case fatality rate of SARS-CoV-2 infection is less than the previous epidemics by other closely related coronaviruses, due to its high infectivity, the total number of SARS-CoV-2 infection-associated disease, called Covid-19, is a matter of global concern. Despite drastic preventive measures, the number of Covid-19 cases are steadily increasing, and the future course of this pandemic is highly unpredictable. The most concerning fact about Covid-19 is the absence of specific and effective preventive or therapeutic agents against the disease. Finding an immediate intervention against Covid-19 is the need of the hour. In this chapter, we have discussed the role of different branches of the cellular proteostasis network, represented by Hsp70-Hsp40 chaperone system, Ubiquitin-Proteasome System (UPS), autophagy, and endoplasmic reticulum-Unfolded Protein Response (ER-UPR) pathway in the pathogenesis of coronavirus infections and in the host antiviral defense mechanisms.

Results: Based on scientific literature, we present that pharmacological manipulation of proteostasis network can alter the fate of coronavirus infections and may help to prevent the resulting pathologies like Covid-19.

Keywords

Autophagy · Covid-19 · ER-UPR · Molecular chaperones · Hsp70 · Proteostasis · SARS-CoV-2

Abbreviations

ARDS	Acute Respiratory Distress Syndrome
Covid-19	Coronavirus Disease of 2019
DMV	Double Membraned Vesicles
ERAD	Endoplasmic Reticulum Associated Protein Degradation
ER-UPR	Endoplasmic Reticulum Unfolded Protein Response
Hsp	Heat Shock Protein
HSF1	Heat Shock Factor 1

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MERS-CoV	Middle East Respiratory Syndrome Coronavirus
RdRp	RNA-dependent RNA polymerase
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
UPS	Ubiquitin Proteasome System

8.1 Introduction

Several emerging infectious diseases have claimed millions of human lives in the last two decades and caused a significant jeopardy to the world economy. Among these emerging infectious diseases, repeated episodes of infections by different coronaviruses have escalated the search for specific preventive and therapeutic agents against the coronaviruses. Coronaviruses are enveloped, single-stranded, positive sense RNA viruses that potentially can infect a multitude of vertebrate hosts. Back in 2002, the emergence of SARS (Severe Acute Respiratory Syndrome) caused by SARS-CoV-1 (SARS coronavirus-1) describes a major epidemic due to viral infections. However, the impact of SARS-CoV-1 was much less severe in terms of number of infected individuals and its geographical distribution, compared to the current pandemic caused by SARS-CoV-2 (Coronaviridae Study Group of the International Committee on Taxonomy of, 2020). The SARS viruses are the etiological agent of viral pathogenesis which starts with non-specific symptoms like fever, headache, fatigue, cough and often progresses to severe symptoms like dyspnea, hemoptysis, lymphopenia and can affect the lower respiratory system leading to ALI (Acute Lung Injury) and ARDS (Acute Respiratory Distress Syndrome) (Donnelly et al. 2003). Although there are earlier pieces of evidence of coronavirus infections in humans and other mammalian hosts, the year 2002 has been earmarked as the beginning of the emergence of severely pathogenic coronaviruses capable of causing epidemics resulting in significant morbidity and mortality of the infected individuals (Kahn and McIntosh 2005). After a few years of SARS epidemic, the next epidemic by coronaviruses emerged in the form of MERS (Middle East Respiratory Syndrome) and the causative virus was termed as MERS coronavirus (MERS-CoV). MERS-CoV primarily affected the lower respiratory tract causing ALI and ARDS, thus the severity of pathogenesis and the mortality associated with MERS-CoV were significantly higher than SARS-coV-1 (Memish et al. 2013a, b). The primary reservoirs of the SARS and MERS viruses are reported to be bats and dromedary camels, respectively. The MERS-CoV is phylogenetically different from SARS-CoV-1 and the viral tropism for cell surface receptors is also significantly different between these two coronaviruses and they have caused two consecutive epidemics within a decade. This is a fact of immense concern, as it indicates that two phylogenetically dissimilar coronaviruses evolved in parallel to infect human hosts within a short time with the potential to cause epidemics. The impact of the SARS and MERS epidemics on human health was severe as both the epidemics claimed approximately 9500–10,000 lives in total where the case fatality rates were ~ 10% and ~ 34%, respectively (Mahase 2020; Ramadan and Shaib 2019).

In 2019, nearly seven years after the MERS epidemic, a third wave of epidemic caused by a variant of coronavirus termed as Novel Coronavirus 19 (nCoV-19 or SARS-CoV-2) has emerged (Coronaviridae Study Group of the International Committee on Taxonomy of, 2020). This epidemic started from Wuhan Province in China and within the next six months has spread to most of the countries of the world affecting approximately nine million people globally. Due to its spread across the globe, WHO has declared the current epidemic by SARS-CoV-2 (Severe Acute Respiratory Syndrome—coronavirus-2) as a pandemic. So far, the case fatality rate of the SARS-CoV-2 remains well below 10%, yet owing to its much wider spread affecting millions of people, the total mortality associated with the SARS-CoV-2 pandemic is significantly higher than the previous epidemics by coronaviruses. Like its predecessors, SARS-CoV-1 and MERS-

CoV, SARS-CoV-2 causes primarily respiratory illness to the infected people which occasionally progresses to pneumonia and ARDS. Most infected people with SARS-CoV-2 infection commonly present with non-specific symptoms such as cough, sore throat fever, malaise, fatigue, shortness of breath, body ache, nausea, vomiting, and gastro-intestinal symptoms like diarrhea (Esakandari et al. 2020). Some distinctive symptoms of SARS-CoV-2 infection that have been reported, are loss of sensation of smell or anosmia and altered taste sensation or dysgeusia (also known as parageusia) (Esakandari et al. 2020). Patients presented with these non-specific symptoms or with more severe symptoms like ARDS who are tested positive by RT-PCR (Reverse Transcription-Polymerase Chain Reaction) test for viral RNA isolated from nasopharyngeal swabs or other equivalent diagnostic tests are designated as positive cases Covid-19. In the majority of infected people, the initial mild non-specific symptoms do not advance to severe complication although in a small but significant fraction of Covid-19 patients, the disease progresses to severe complication like pneumonia leading to acute lung injury requiring respiratory assistance. In critically ill Covid-19 cases, renal failure and associated multi-organ failure have also been reported (Yuan et al. 2020). The outcome of Covid-19 in affected people with various comorbidities like hypertension, diabetes, cardiac diseases, or chronic obstructive pulmonary disease (COPD) and compromised immunities are bleak and of great concern currently. Notably, in the age groups of above 60 years, the mortality rate of Covid-19 cases is significantly higher due to comorbid conditions and declined immunity associated with aging (Yang et al. 2020).

8.2 Molecular Basis of Pathogenesis by SARS-CoV-2

The Coronaviridae are a family of viruses that are divided into two subfamilies. Among these, Orthocoronavirinea is the most studied one. Orthocoronavirinea is further divided into four genera—alpha, beta, gamma, and delta coronaviruses. Alpha and beta coronaviruses exclusively infect mammalian hosts such as humans. SARS-CoV-1, MERS-CoV, and SARS-CoV-2 are beta-coronaviruses. Apart from these epidemic-causing coronaviruses, several other Human Coronaviruses (HCoVs) have been discovered such as the HCoV-229E, HCoV-NL63, HCoV-OC43, and HCoV-HKU1 (Killerby et al. 2018).

The SARS-CoV-2 possesses an approximately 30 kb long, unsegmented RNA (Ribonucleic acid) genome, which is single stranded, positive sense and contains 11 open reading frames (ORFs). The genomic RNA of SARS-CoV-2 is processed by the replicase/transcriptase complex (RTC) and host translation machinery to give rise to protein products (Fig. 8.1). The genomic RNA may be replicated to make a negative strand template, it can translate ORF 1ab, or transcribe negative sense subgenomic template RNA, which can be used for transcription of sub genomic mRNA. The SARS-CoV-2 Spike 1 protein binds to the human Angiotensin 1 converting enzyme 2 (ACE2) receptor through its Receptor Binding Domain (RBD) (Hoffmann et al. 2020; Lan et al. 2020). The entry of the virus into host cells depends on priming of the spike protein (S) by host cell transmembrane protease serine 2 (TMPRSS2) by cleavage into S1 and S2 (Spike 1 and Spike2) proteins (Hoffmann et al. 2020) (Fig. 8.2). After TMPRSS2 mediated cleavage, viral S2 spike protein can mediate viral entry through the endosomal pathway (Belouzard et al. 2012; Hoffmann et al. 2020; Zhou et al. 2020). The endosome releases the viral contents into the cytoplasm, which initiate the process of viral pathogenesis. Upon entry into the cell, SARS-CoV-2 genomic RNA serves as a template for direct translation into the polyprotein in ORF1a and ORF1b (Peng et al. 2020). The first ORF encodes a polyprotein which must be processed by proteases such as virally encoded chymotrypsin-like protease (3CL-protease), main protease (M-protease), and papain-like proteases (PLpro). The processed polypeptides give rise to 16 non-structural proteins known as nsps1–16 (Figs. 8.1 and 8.2) (Nakagawa et al.

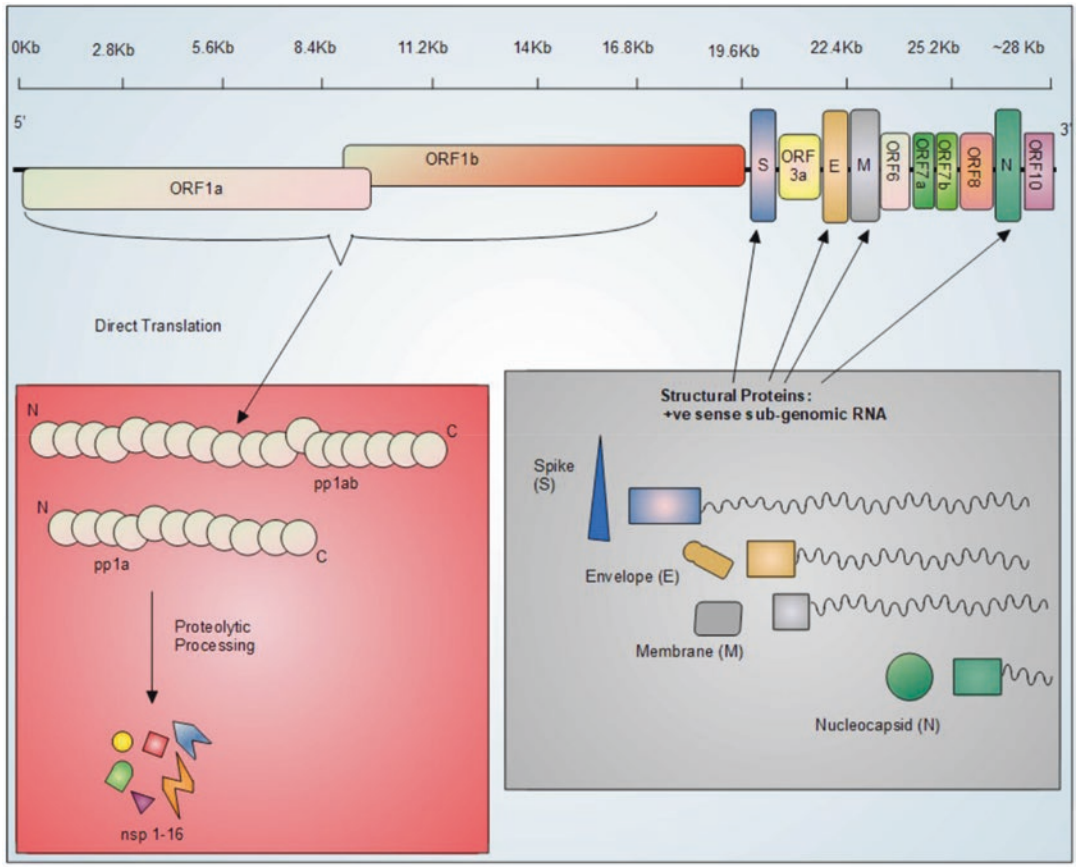

Fig. 8.1 Schematic picture of SARS-CoV-2 RNA genome and the protein products translated from the RNA genome

The SARS-CoV-2 RNA genome is approximately 30 kb long and it encodes different open reading frames (ORF) which are subsequently translated to the non-structural and structural proteins of the virus to make new virions. The ORF1a and ORF1b are translated to polyproteins pp1a and pp1ab (shown in the red box) which are subsequently proteolyticallyprocessed to non-structural proteins (nsps), 1 -16 (as described in the text and Table 8.1). The protein products (structural proteins of the virus) of other ORFs encoded by the genome are shown in the grey box

2016). These nsps have important functions such as the formation of the replicase/transcriptase complex (RTC), modification of the genomic RNA and interaction with host proteins. Assembly of the replicase/transcriptase complex (RTC) allows the viral genomic RNA transcription of nested subgenomic mRNAs as well as the negative template of the positive sense genomic RNA. The structural proteins of virus, namely, spike(S) proteins, envelope (E) proteins, membrane (M) proteins, and nucleocapsid (N) proteins are transcribed by their own ORFs through the RTC in a discontinuous manner (Hussain et al. 2005). While the structural proteins increase in number, so do the transcription and translation of the non-structural proteins and accessory proteins. These non-structural and accessory proteins play a crucial role in the viral pathogenesis (summarized in Table 8.1).

As described in Table 8.1, various proteins interact with the host factors to suppress innate immunity, promote inflammation and cell death. Proteins involved in viral cap formation (nsp10, nsp14, nsp16) are important for the viral mRNAs to remain undetectable from innate immune responses. Viral cap formation along with the inhibitory effects from translation inhibitor nsp1, lead to increased efficiency of viral translation

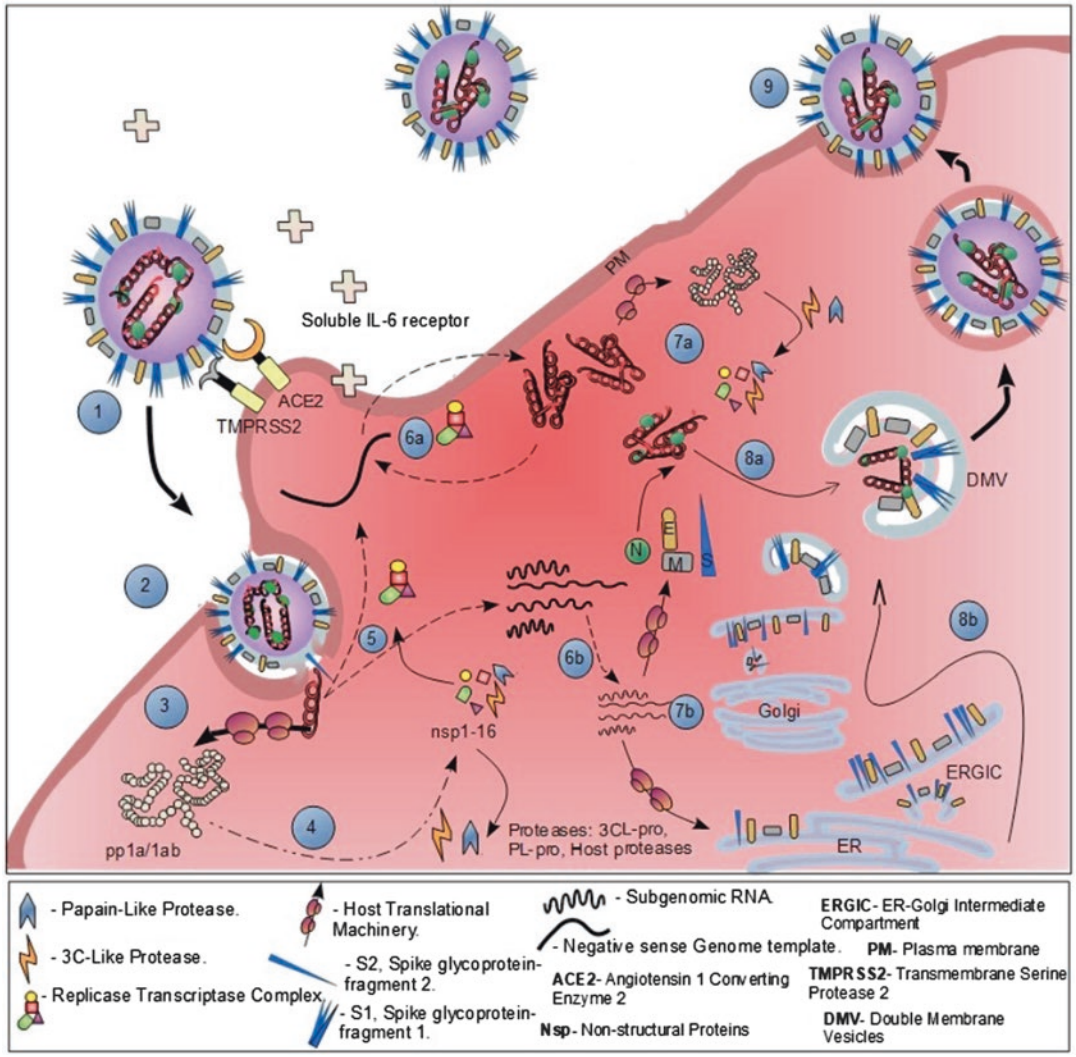

Fig. 8.2 Life cycle of SARS-CoV-2

1. Viral attachment and tropism: SARS CoV-2 attaches to host ACE2 receptor. Fusion (S) glycoprotein is cleaved by host TMPRSS2 into S1 and S2. **2. Endosome formation and membrane fusion:** Viral S2 protein mediates host-endosome membrane fusion. SARS CoV-2 enters host cell. **3. Translation of positive strand RNA genome:** RNA genome enters the cytosol and is translated by host translation machinery to viral polyproteins. **4. Proteolytic processing of the viral polyprotein:** The polyprotein is cleaved by proteases such as 3CL-pro, PL-pro or host proteases to give 16 nsps (non-structural proteins). The nsps include 3CL-pro and Pl-pro, as well as proteins that form the RTC (replicase/transcriptase complex) which is indispensable in subsequent steps. **5. Formation of negative sense RNA templates**: The genome is used to transcribe negative sense templates of the genomic RNA (gRNA), as well as sub genomic template RNA (sgRNA) of the other 10 ORFs using the RTC. **6a. Replication of genomic RNA**: Negative sense RNA template is used to transcribe the positive sense genome, which can be repeated to generate many copies. **6b. Transcription of positive sense ORFs**: The neagtive sense sgRNA are used to transcribe positive sense RNAs for translation. **7a. Production of nsps**: The replicated gRNA can be used for the production of nsps through host translation machinery. Polyprotein is cleaved by proteases to give rise to nsps. Some of them are crucial for DMV (double membrane vesicle) formation. **7b. Translation of structural proteins**: Positive sense ORFs are used to translate structural proteins. Nucleocapsid (N) protein binds to gRNA. Other structural proteins- Spike (S), Envelope (E), Membrane (M) travel to the ER, and undergo modification. **8a. The gRNA is packaged** by the (N) protein and translocate into the forming DMV. **8b. The S, E, and M proteins travel from the ER into the Golgi** bodies through ERGIC. Golgi forms exocytic vesicles, and the DMV forms through the trans-Golgi system**. 9. Viral Exit:** The SARS-CoV-2 virus exits the cell and infects new host cells

Table 8.1 A comprehensive list of all proteins encoded by SARS-CoV-2 genome and its possible functions based on Homology studies, and functional characterization of related viruses such as SARS-CoV-1)

Name of protein/ ORF	Function of different proteins encoded by genome of SARS-CoV-2
Nsp1, ORF1a	Host translation inhibitor. Promotes host mRNA degradation, provides exclusive translation machinery to viral mRNAs (Lokugamage et al. 2012).
Nsp2, ORF1a	May play a role in interacting with PHB1 and PHB2 mitochondrial proteins (Cornillez-Ty et al., 2009).
Nsp3, ORF1a	Papain like protease. Involved in proteolytic cleavage of the polyprotein towards the N-terminal end. Also possesses deubiquitylation and de-ISGylating activity. It is involved in double membrane vesicle (DMV) formation (viral replication) and cytokine dysregulation through suppression of IRF3 activity. Suppresses NF-kB signaling (Angelini et al. 2013; Chen et al., 2014; Frieman et al., 2009; Lindner, 2007).
Nsp4, ORF1a	Assembly of viral replication vesicles. (DMV) (Angelini et al. 2013).
Nsp5, ORF1a	3-C like-protease. Involved in polyprotein processing towards the C terminal of the polyprotein. (At 11 sites). ADP-ribosyl phosphate binding activity (Lin et al., 2005).
Nsp6, ORF1a	Forms (DMV) with nsp3 and nsp4. Autophagosome dynamics. Putative determinant of virulence (Angelini et al. 2013; Cottam et al. 2014).
Nsp7, Nsp8-ORF1a	Putative primase of RNA-dependent RNA polymerase (RdRp) (Peng et al. 2020; te Velthuis et al., 2012).
Nsp9, ORF1a	Essential for efficient viral growth, putative single-strand binding protein (Miknis et al., 2009).
Nsp10, ORF1a	Essential role in viral mRNA cap methylation. Stimulates viral exoribonuclease activity and O-methyl transferase activity (Bouvet et al., 2012).
Nsp11, ORF1ab	Same as nsp12, translated by a-1 frameshift, caused by the slippage of RNA during translation due to putative RNA pseudoknot structure formation.
Nsp12, ORF1ab	Viral RdRp (Ahn et al., 2012; Peng et al. 2020; Wang et al., 2020)
Nsp13, ORF1ab	Helicase activity (Tanner et al., 2003).
Nsp14, ORF1ab	Exoribonuclease activity, N7-guanine methyltransferase activity. Has proofreading activity (Agostini et al., 2018; Bouvet et al., 2012; Minskaia et al., 2006).
Nsp15, ORF1ab	Uridylate specific endonuclease
Nsp16, ORF1ab	O-methyl transferase activity, viral mRNA cap formation (Decroly et al., 2008).
Protein 3a, ORF 3a,	Upregulates expression of fibrinogen subunits FGA, FGB, and FGG in host lung epithelial cells (Tan, 2005; Tan et al., 2005). Induces apoptosis in cell culture (Law et al. 2005). Downregulates the type 1 interferon receptor by inducing serine phosphorylation within the IFN alpha-receptor subunit 1 (IFNAR1) degradation motif and increasing IFNAR1 ubiquitination (Minakshi et al., 2009).

and suppression of translation of the host proteins (most importantly cytokines) (Lokugamage et al. 2012). The formation of double membraned vesicles (DMV) is considered to be a determinant of the viral virulence, which has previously been targeted in Nidoviruses (Rappe et al. 2018) suggesting many non-structural proteins responsible for DMV formation (nsp3, nsp4 and nsp6) (Angelini et al. 2013) and can be considered potential drug targets for SARS-CoV-2 specific antivirals. Recently, the Cryo-EM structure of core polymerase (RdRp (RNA-dependent RNA polymerase)) of SARS-CoV-2 formed by nps12 (catalytic subunit)-nps7-nsp8 (accessory subunits) was solved. RdRp being the most critical polymerase for the viral genome replication, is an attractive target for potential antivirals (Peng et al. 2020). Lastly proteins such as PLpro and protein 3a can give an intuitive idea about the nature of the activity of these proteins that may affect the pathological outcome of the viral infection. PLpro possesses deubiquitination and de-ISGylation activity which suggests its association with protection of the viral proteins from degradation and interference through ISG (Interferon stimulated gene) (Lindner et al. 2005; Lindner et al. 2007). Additionally, the cytokine storm is attributed to cause the inflammation and atypical pneumonia associated with ARDS, which has been recently shown to be due to de-mono-ADP-ribosylation (de-MARylation) of STAT1 by nsp3 of SARS-CoV-2 (Claverie 2020). Protein 3a

forms homo-tetrameric potassium sensitive ion channels and behaves like a porin. This protein is said to be involved in the release of viral particles.

Other than the proteins already described, there are several other proteins whose functions are still unknown (protein 6, ORF 9b, ORF 10, and uncharacterized protein 14) due to insufficient similarity in sequence/motif with the existing proteins, averting homology-based structure-function prediction. These proteins possibly hold cryptic information regarding their involvement in other cellular processes of the host cells. The expression and activity of these viral proteins post-infection by SARS-CoV-2 lead to a plethora of pathological outcomes that are together termed as Covid-19. Covid-19 is an acute disease, which shows variable pathologies in the infected people. SARS-CoV-2 infection does not always present itself as Covid-19 disease and will most probably depend on various factors such as viral load, viral genotype, host factors, and effective intervention in the early stages of the disease. Preliminary phylogenetic analysis of the SARS-CoV-2 genome suggested its 70–90% sequence homology with SARS-CoV-1, and ~ 90% sequence homology to SARSr-CoV (RaTG13), a bat coronavirus, suggesting the closest relative from which SARS-CoV-2 has possibly diverged (Zhou et al. 2020). It has between 50–60% sequence homology with MERS-CoV (Coronaviridae Study Group of the International Committee on Taxonomy of, 2020). Since SARS-CoV-1, MERS-CoV, and Bat-CoV (RaTG13) all have bats as a reservoir or host, bats have been considered as the possible primary reservoir of SARS-CoV-2.

8.3 Currently Applied Therapeutic Strategies Against COVID-19

Due to the sudden outbreak of Covid-19 and inadequate understanding of the mechanism of pathogenesis, there are no such specific preventive or therapeutic agents that are available to contain the disease currently. Thus, only non-specific and symptomatic treatments are being administered to patients. Due to the urgency of the discovery of effective therapeutic agents, most clinical trials are aiming to repurpose existing drugs against Covid-19. Broad spectrum antivirals such as Lopinavir/Ritonavir, Remdesivir, and anti-malarials like chloroquine or hydroxychloroquine have been used in clinical trials to check the efficacy against Covid-19. Antivirals like Lopinavir and Ritonavir (antiviral used against HIV) which are known protease inhibitors have shown promising inhibitory activity against SARS-CoV-2 (Fig. 8.3) (Hung et al. 2020). The efficacy of these protease inhibitors against SARS-CoV-2 is possibly due to their ability to inhibit the 3CL- protease that cleaves the viral polyprotein into non-structural proteins. Remdesivir, another antiviral which works as an inhibitor of viral RdRp, was first developed by Gilead Sciences Inc. as an antiviral for the treatment of Ebola Virus infections and it is also being administered against Covid-19 (Pardo et al. 2020). This drug was found to be an effective antiviral against previous significantly pathogenic coronaviruses like SARS-CoV-1 and MERS-CoV (Fig. 8.3) (Pardo et al. 2020). The compound is an adenine nucleoside-analog prodrug, which possesses appreciable affinity to the SARS-CoV-2 replicase/transcriptase complex, making it a potential antiviral for Covid-19 (Beigel et al. 2020; Wang et al. 2020b). However, the efficacy of Remdesivir as an effective antiviral against SARS-CoV-2 is still controversial due to contradictory experimental results from different groups. A double-blind, randomized controlled trial involving 60 different trial centers across the globe was conducted recently and the study showed a significant improvement of Covid-19 patients with Remdesevir in comparison to the placebo control group in terms of faster recovery time and less lower respiratory tract infection. The authors showed that the median recovery time improved to 11 days with Remdesivir administration as compared to 15 days in the placebo control group. This study also showed improvement in Kaplan-Mier estimates of mortality with Remdesivir (7.1%) compared to the control group (11.9%) (Beigel et al. 2020). On the contrary, a study by a group in China has shown the

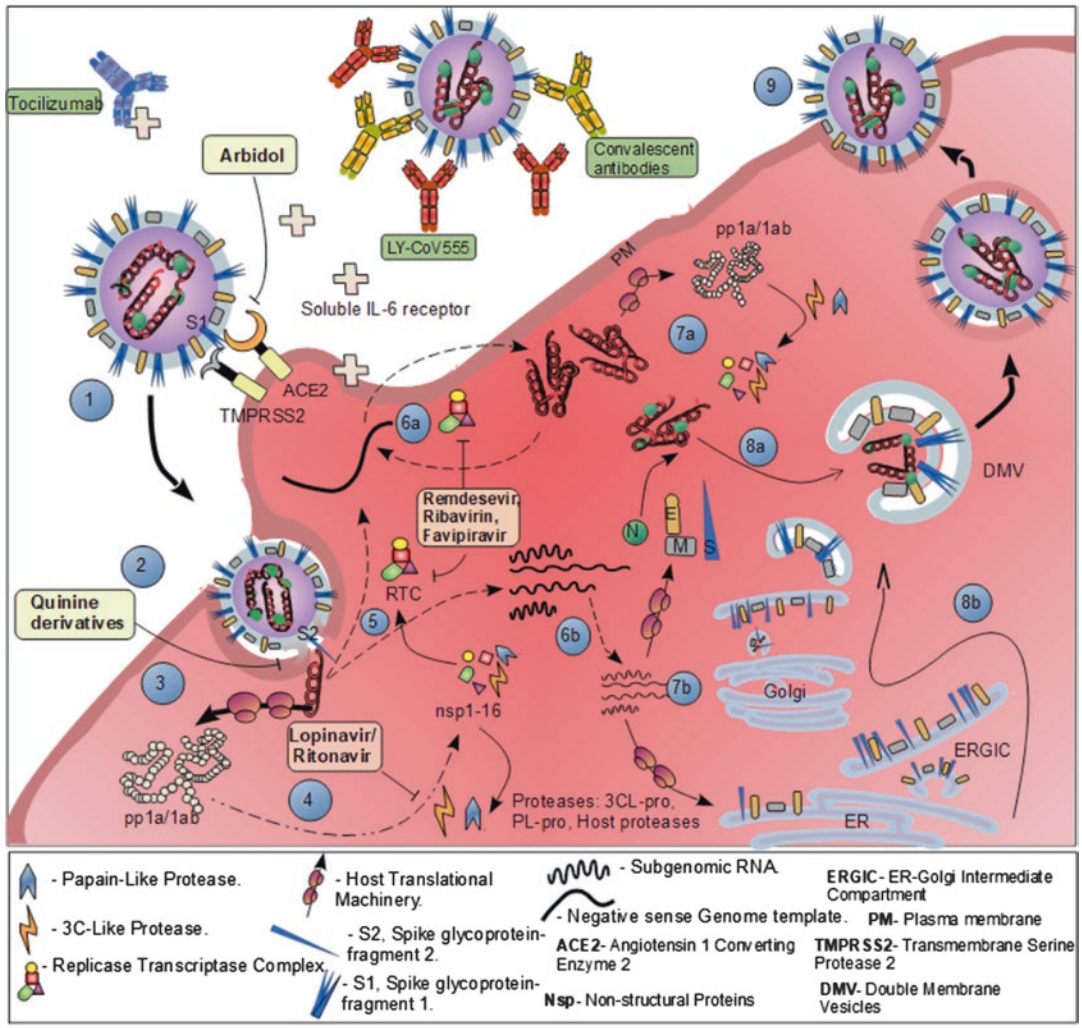

Fig. 8.3 Currently used therapeutic agents and its molecular targets in SARS-CoV-2 life cycle
Boxes labelled in peach represent xenobiotic drugs which are used to contain SARS-CoV-2 infection. Arbidol is used with anti-SARS activity as a putative Spike-ACE2 binding inhibitor. Arbidol also possesses other antiviral activities described in text. Quinine derivatives inhibit viral entry through interference with host protein glycosylation, it also exerts other activities such as alteration of endosomal pH, autophagosome development, cytokine production, and protein processing. Remdesevir, Favipiravir, and Ribavirin inhibit or reduce the activity of the RTC (Replicase-Transcriptase Complex). Lopinavir and Ritonavir in combination inhibit 3CL-pro (3C-Like Protease), a protease essential for polyprotein cleavage. Boxes labelled in green represent interventions by biologics that are used to contain SARS-CoV-2 infection. Tocilizumab binds to soluble IL-6 Receptor (sIL-6R), inhibiting its proinflammatory activity. LYCoV555 is a neutralizing monoclonal antibody against the RBD (Receptor-Binding Domain) of the Spike glycoprotein. Convalescent Plasma taken from individuals who have recovered from SARS-CoV-2 infection, usually contains high titers of anti-SARS-CoV-2 polyclonal antibodies. It inhibits viral growth by promoting opsonization, complement activation and improving proinflammatory responses early in the course of the disease

absence of statistically significant improvement with Remdesivir therapy in comparison to the placebo control group (Wang et al. 2020b). However, the study by Chinese group faces some criticism of much smaller study group size and the study was terminated prematurely as many patients enrolled in the trial suffered from adverse effects of Remdesivir and subsequently there were not enough numbers of eligible patients who could be enrolled due to decrease

in number of incidences of Covid-19 cases in China (Wang et al. 2020a). Apart from these antivirals, various other known drugs are being repurposed for Covid-19 treatment currently. For example, chloroquine and hydroxychloroquine are well known antimalarial drugs although these drugs possess non-specific antiviral activity due to their endosomal pathway—modifying properties. Chloroquine and its derivatives interfere with viral entry by inhibiting host receptor glycosylation, protein processing and by altering endosomal pH (alkalization) (Fig. 8.3) (Bonam et al. 2020; Chen et al. 2011; Mauthe et al. 2018). Inhibition of viral entry through this mechanism has been demonstrated in vitro for other RNA viruses like Dengue Virus, Chikungunya Virus and has been discussed in later sections of these chapter in detail (Yang et al. 2004). Additionally, chloroquine and hydroxychloroquine have immune-modulatory effects such as inhibition of cytokine production, and inhibition of autophagosome-lysosome fusion in the host cells, which may further aid their antiviral activity through regulation of the "cytokine storm" and viral maturation (Bonam et al. 2020). The drugs described so far are at the frontline of our current intervention strategies against Covid-19, and many other drugs such as Ribavirin, Arbidol, and Favipiravir have been used in combination therapy. While Ribavirin and Favipiravir are well known RdRp inhibitors like Remdesivir, Arbidol, however, is a repurposed drug which was used as an antiviral against influenza. In a recent study, the efficacy of various anti-influenza drugs were tested against SARS-CoV-2, systematically (Wang et al. 2020a). In this study Arbidol, Laninamivir, Baloxavir, Oseltamivir, Peramivir, and Zanamivir were studied for their efficacy against SARS-CoV-2. Amongst all, only Arbidol showed promising result as an antiviral against SARS-CoV-2 by significantly reducing the cytopathic effect of the virus as well as viral protein expression in an in vitro system (Wang et al. 2020a). This study also showed that Arbidol can reduce the ACE2 receptor binding efficiency of Spike protein (S) of the virus by 67% in SARS-CoV-2 infected Vero E6 cells in vitro (Wang et al. 2020b).

Apart from xenobiotic drug candidates, several biologics have also been used for the treatment of Covid-19. Due to the absence of a specific treatment regime, passive immunization through plasma donation has been considered as a viable treatment for this disease. Previously, convalescent plasma therapy was shown to be effective against SARS-CoV-1. A study from 2005 on treatment of SARS-CoV-1 reported significant improvement of hospital discharge rates of patients after receiving convalescent plasma from recovered patients of SARS (Cheng et al. 2005). A very recent study has reported significant improvement of clinical symptoms and decrease in the number of mortality of critically ill patients of Covid-19 with convalescent plasma therapy (Xia et al. 2020). Apart from convalescent plasma, the search for specific antibodies are also going on. LY-CoV555 is a monoclonal antibody that has been developed by Eli Lilly and AbCellera for the treatment of SARS-CoV-2 infection. It is a neutralizing IgG antibody that binds to the spike protein of the virus. It is currently under phase 1 clinical trials. Apart from plasma therapy or therapeutic antibodies, interferon therapy or the use of recombinant interferons have been demonstrated to have antiviral activity in almost all viral diseases. Use of interferons such as Interferon B-1b in combination with drug therapy for Covid-19 cases have also been reported (Hung et al. 2020). Tocilizumab, a drug used for rheumatoid arthritis, has been repurposed for the treatment of Covid-19. Tocilizumab is a monoclonal antibody that works as IL-6 receptor antagonist and IL-6 is one of the key players in ARDS as a cytotoxic proinflammatory molecule. Thus, Tocilizumab can block proinflammatory pathways, and consequently improve the respiratory health of the patients. A preliminary small-scale study conducted in China demonstrated that use of Tocilizumab significantly alleviated signs and symptoms of severe disease such as fever, lymphocytopenia, increased C-reactive protein, and ground-glass opacity of lungs associated with Covid-19 (Xu et al. 2020). It improved the clinical outcome on a small scale and is proposed as an effective therapy for critically ill Covid-19 patients (Xu et al. 2020).

While broad spectrum antivirals play an invaluable role in the treatment of viral infections, there are some drawbacks like the development of resistance as well as the chance of development of viral quasi-species. Due to the high rate of genomic mutation of viruses especially in RNA viruses and due to their endemic prevalence in many developing countries, antiviral resistance has a higher likelihood of occurrence (Lipsitch et al. 2007). Like other viral diseases, SARS-CoV-2 needs broad-spectrum as well as narrow-spectrum antivirals to combat it effectively. The search for new drugs or novel therapeutic interventions are the need of the hour as prophylaxis by vaccines are projected to be complicated. There are growing concerns about the safety of vaccination, due to recent reports of antibody dependent enhancement (ADE) of Covid-19 due to low levels of non-neutralizing antibodies (Iwasaki and Yang 2020). ADE causes increased pro-inflammatory cytokines and reduced anti-inflammatory cytokines which may lead to exacerbation of the pathologies. As previously stated, there are currently no specific and very few effective drugs available for treatment of this complex disease. It is extremely important now to broaden our search for new antivirals or combination therapy of antivirals with small molecules that can tweak the cellular pathways utilized by the virus for its pathogenesis. In the following sections of this chapter, we aim to provide evidences from relevant literature and discuss the possibilities of the prospective use of alteration of cellular proteostasis pathways as an effective therapeutic intervention strategy against SARS-CoV-2 in different stages of its lifecycle.

8.4 Host Cell Proteostasis Status Modulates the Rate of Evolution of Viral Pathogens

Viruses, especially RNA viruses, mutate and evolve quickly. Research from RNA viruses like influenza virus has shown that the mutational landscape attainable by the virus, largely depends on the host cell proteostasis status (Phillips et al. 2018a, b; Phillips et al. 2017). During evolution, viral pathogens need to maintain a fine balance between the rate of mutation and fitness cost of its mutated proteins. Recent research with influenza virus shows that one of the mutations of its Nucleoprotein (NP), at Proline 283 (P283 variants), which conferred resistance to host defense protein Myxovirus resistance protein A (MxA) restriction factor to the virus, is a compromised folder but is efficiently chaperoned by the host proteostasis machineries (Phillips et al. 2018b). The variants of P283-NP were found to have compromised thermal stability in chaperone-depleted conditions of the host cells, especially during elevated temperature of the host cell mimicking febrile conditions. The chaperone depletion was achieved by chemical genetic inhibition of Heat Shock Factor1 (HSF1) of host cells (Phillips et al. 2018b). The downstream chaperones of the HSF1 pathway, mainly Hsp70 and Hsp40 are known to assist the influenza virus pathogenesis by interacting with its NP protein and helping in its nuclear export (Hirayama et al. 2004) and import (Batra et al. 2016), respectively, during viral replication. During chaperone depletion condition, reduced or lack of NP-chaperone complex formation might result in misfolding and aggregation of the P283-NP variants which remains protected by these chaperones at their physiological concentrations in the host cells. These results indicate that the chaperone molecules or their master regulators (like HSF1) can be potential drug targets for designing effective antivirals. Genome sequence analysis of SARS-CoV-2 demonstrated specific new mutations in its NP protein which might have similar chaperoning effect from host proteostasis machinery that in turn might have helped the virus to evolve. The mutational landscape of SARS-CoV-2 needs to be analyzed rigorously, followed by systematic study of the enriched mutations and its relation to host cell proteostasis machineries. Molecular chaperones like Hsp70, Hsp40, Hsp90, if found to assist the virus in its evolution by expanding the mutational landscape, can be potential drug targets to combat the virus replication and its subsequent evolution within the host system.

8.5 Ubiquitin Proteasome System, a Double-Edged Sword During Coronavirus Infections

The Ubiquitin Proteasome System (UPS) is one of the major protein quality control pathways involved in cellular protein homeostasis (also known as proteostasis) and maintains critical level of cellular proteins to regulate diverse cellular activities, involving antigen processing, signal transduction, transcriptional regulations, cell cycle maintenance, and apoptosis (Glickman and Ciechanover 2002). During the first step of UPS-mediated proteolysis, substrate proteins are tagged with ubiquitin (a small protein molecule composed of 76 amino acids, having molecular weight of ~8.6 kDa) by the process of ubiquitination or ubiquitylation. The whole process of ubiquitination is a multistep process completed by a cascade of three different groups of enzymes: Ubiquitin activating enzymes or E1 enzymes, ubiquitin conjugating enzymes or E2 enzymes, and ubiquitin ligases or E3 enzymes (Weissman 2001). Following a series of ubiquitination, a poly-ubiquitin chain is attached to the substrate proteins, which is further recognized as signal by 26S proteasome that govern the degradation of the target protein (Glickman and Ciechanover 2002). During degradation process, tagged ubiquitin gets released from target protein with the involvement of deubiquitinating enzymes or DUBs (Glickman and Ciechanover 2002; Weissman 2001).

Evidence from previous studies on the mechanism of pathogenesis by different RNA viruses show the interactions of viral proteins with host cell UPS and the important role of UPS at multiple steps of infection (Gao and Luo 2006; Isaacson and Ploegh 2009). Viruses take over the host UPS and utilize this machinery for its uptake and subsequent propagation within the host cells by either directly modifying certain characteristics of host cell UPS or by encoding proteins having DUB or E3 like activities. The host cell UPS acts as a double-edged sword for most of the viral pathogenesis process due to its pro and antiviral activities. UPS can facilitate the viral pathogenesis by post-translational modifications of viral proteins or by maintaining viral proteins in its stable and functional conformations. On the other hand, UPS facilitates degradation of viral proteins and thus contribute to antiviral defense mechanisms of the host (Gao and Luo 2006; Isaacson and Ploegh 2009). As stated earlier, many viruses encode proteins possessing E3 enzyme or DUB like activities. It was shown that E3 enzymes ubiquitinate cellular p53 and facilitates its degradation via UPS. P53 acts as a key negative regulator of viral pathogenesis by suppressing viral replication by triggering apoptosis of infected cells (Fig. 8.4) (Scheffner et al. 1990). Similarly, all coronaviruses, including SARS-CoV-1, encodes few papain-like proteases (PLpro), which has shown DUB like activities both in vivo and in vitro (Barretto et al. 2005; Lindner et al. 2005). PLpro possesses DUB-like activity via its N-terminal UB-like domain, which facilitates the interaction with host proteasome assembly (Barretto et al. 2005; Lindner et al. 2005). These unique properties of the viral proteins suggest that coronaviruses can exploit host ubiquitin-proteasome system to facilitate its replication and in turn can evade the host immune response. In the studies with previous pathogenic coronavirus (SARS-CoV-1 and MERS-CoV), UPS was found to assist in the cellular entry process of coronavirus as well as in its replication (Raaben et al. 2010). Small molecule inhibitors of UPS thus can be combined as one of the effective approaches to combat SARS-CoV-2 infection and subsequent pathologies.

Studies based on murine hepatitis virus (MHV1) belonging to the same virus family, which have many characteristics in common with SARS pathophysiology, provided evidence of virus release from endosome to the lysosome in a UPS-dependent manner (Ma et al. 2010). MHV1 replication cycle was blocked by the treatment with UPS inhibitors like MG132 and lactacystin, supporting the fact that UPS is involved in the replication cycle of the virus. However, virus entry was not affected in the presence of proteasome inhibitors. Moreover, after MG132 treatment, viral particles stayed protected from the RNAse digestion because it remained entrapped inside the lysosome or endosome vesicles. Taken together, it was shown that inhibiting UPS does

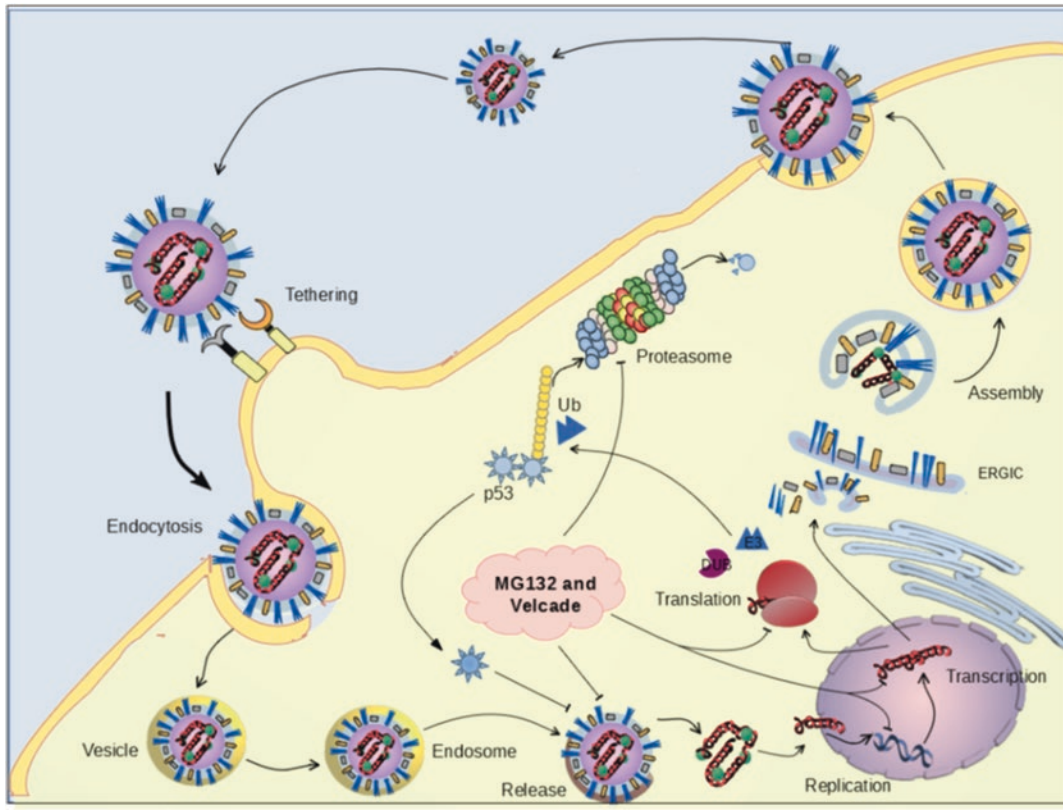

Fig. 8.4 Role of Ubiquitin Proteasome System (UPS) inhibitors in coronavirus life cycle and pathogenesis Internalization of coronavirus into host cells occur via endocytosis followed by its release in the cytosol from endosome. MG132 and other UPS inhibitors block the release of virus from endosome, however it does not affect the viral entry process by endocytosis. The positive sense viral RNA released in the cytosol is translated to viral protein or is transcribed to negative sense RNA strand which serves as template for new positive sense RNA genome. UPS inhibitors inhibit most of these processes suggesting important role of proteasome machinery in development of new virions in the host cells. Simultaneously, viral RNA encodes proteins with DUB (Deubiquitinating enzyme activity) and E3-Ub-ligase activity which targets and ubiquitinates host cell p53 protein to facilitate its degradation via UPS. P53 inhibits the release of virus form endosome and acts as a key negative regulator of viral pathogenesis by suppressing viral replication by triggering apoptosis of infected cells

not affect the virus internalization, but it successfully hinders virus release from endosomal vesicle to the cytosol (Fig. 8.4) (Yu and Lai 2005). SARS-CoV-1 viral RNA is first released into the cytosol after internalization of virus through endocytosis facilitated by Angiotensin-converting enzyme 2 (ACE2) (Inoue et al. 2007). After release in the cytosol, the coronavirus genome encodes two large polypeptide chains, which give rise to all viral proteins including non-structural proteins and replicase via their autocatalytic activity (Tan et al. 2006). These viral proteins assemble into mature virions inside the ER-Golgi intermediate complex (ERGIC), followed by the release of virions by exocytosis (Stertz et al. 2007). Chemical inhibitors like Velcade, Bortezomib, MG132, and Epoxomicin block transcription and translation of different coronaviruses like MHV1, SARS and feline infectious peritonitis virus (Fig. 8.4) (Raaben et al. 2010). It was also shown experimentally that in absence of functional UPS system, viral transcription and translation was strongly diminished, but proteasomal inhibitors facilitated the virus entry into the host cell (Raaben et al. 2010).

Apart from directly modifying the viral life cycle, the UPS also modulates other cellular responses that in turn dictate the severity of the viral pathogenesis. It was shown that in Dendritic cells (DC) infected with SARS-CoV-1, there was low expression of antiviral cytokines; in contrast, pro-inflammatory chemokines were significantly upregulated in the infected cells. This inefficient antiviral response associated with chemokine storm was thought to be one of the key mechanisms of SARS-CoV-1 mediated immune evasion and pathogenesis (Law et al. 2005). In a subsequent study, it was shown that this inflammatory response was drastically reduced after blockage of the UPS. Moutzouris et al. demonstrated that chemical inhibitors of UPS lowered the inflammatory response in airway smooth muscle (ASM) cells (Moutzouris et al. 2010). They further showed that MG132 treatment is effective in downregulating the level of IL-6, IP-10, esICAM-1, MCP-1, RANTES, and MIF cytokines and upregulating the level of MKP-1, which is an effective inhibitor of protein serine/threonine kinases, MAPK (Moutzouris et al. 2010). MAPK participates in a broad range of cellular events like cell migration, proliferation, synthesis of cytokines, and other inflammatory proteins (Chi et al. 2006). MG132 treatment or inhibition of UPS showed a dual inhibitory effect on pro-inflammatory cytokine storm, thus it can be applied as a promising therapeutic agent to control the inflammatory responses due to coronavirus infections. However, Schneider et al. explained MG132 effectively blocks the viral replication, but other UPS inhibitors are not similarly effective. Therefore, they concluded the impact of MG132 on virus replication is not correlated well with the exclusive involvement of UPS. However, authors demonstrated that the inhibitory activity of MG132 on m-calpain, a cellular caspase, eventually leads to inhibition of virus replication (Schneider et al. 2012). Recently, in an in-silico docking study, followed by MD (Molecular Dynamics) simulation with several drugs for repurposing against Covid-19, a known anticancer drug that acts as a proteasome inhibitor, Carfilzomib, was found to be a promising candidate. Carfilzomib showed the best binding free energy with SARS-CoV-2 main protease and can be checked experimentally for its efficacy as an antiviral as performed before for other proteasome inhibitors (Wang 2020).

From these experimental evidences, it is clear that MG132 as well as other UPS inhibitors can prevent coronavirus replication, virus release by exocytosis, and pro-inflammatory cytokine storm (Raaben et al. 2010). From these findings, we can speculate that UPS inhibitors like MG132, lactacystine, bortezomib, carfilzomib, etc. can be combined with other antivirals as effective therapeutic interventions against Covid-19 and other coronavirus mediated pathologies.

8.6 The Implication of Autophagy in Coronavirus Infections

8.6.1 A General Overview About Autophagy

Autophagy is a cellular degradative pathway that involves the lysosome, a terminal degradative organelle of the cell. The lysosome utilizes autophagic and endocytic pathways to degrade macromolecules (Fader and Colombo 2009; Huotari and Helenius 2011). Both the degradative pathways are orchestrated in several steps and a plethora of proteins are involved in the whole process. Cellular autophagy is immensely important in maintaining cellular proteostasis and dysfunctional autophagy is found to be associated with numerous pathological conditions, including several neurodegenerative diseases and cancers (Guo et al. 2018). Targeting of cellular components by autophagy to the lysosome occurs through different mechanisms like (i) macroautophagy, (ii) microautophagy, and (iii) chaperone-mediated autophagy (CMA) (Garcia-Arencibia et al. 2010). Microautophagy occurs via invagination of lysosomal membrane to take up cellular contents (Sahu et al. 2011). In CMA, unfolded proteins are directly targeted and are (Claverie 2020) sent into lysosome via lysosomal-associated membrane protein 2A (LAMP2A) (Orenstein and Cuervo 2010). Macroautophagy

involves sequestration of insoluble aggregates of the misfolded proteins and damaged/aged organelles in double membraned structures (called isolation membranes/phagophores) and their delivery to late endosomes and lysosomes resulting in degradation. The isolation membrane primarily originates from mitochondria-associated endoplasmic reticulum (ER) membrane (Hamasaki et al. 2013; Lamb et al. 2013); however, other cellular components such as plasma membrane, Golgi bodies, and recycling endosomes are also found to contribute to the formation of autoophagosomes (Lamb et al. 2013). Macroautophagy is the best-characterized autophagic pathway and we will refer to it as autophagy hereafter.

The core components of autophagy consist of more than 30 autophagy proteins (ATGs). Macroautophagy occurs in three steps: 1) Initiation, 2) Elongation, and 3) Maturation. Nucleation of an isolation membrane or phagophore marks the initiation step of autophagy. The phagophore then matures into a double membrane autophagosome via the elongation step. The elongation of phagophore depends on ATG12 and LC3 conjugation system. The closure of the phagophore generates double-membraned autophagosomes (Fig. 8.5). Autophagosomes then fuse with late endocytic vesicles or lysosomes to degrade its contents. Apart from ATGs, the various fusion process in autophagy involves several Rab, SNARE, and ESCRT proteins (Garcia-Arencibia et al. 2010).

Autophagy is involved in various cellular processes such as starvation, cell differentiation and development, degradation of misfolded proteins, and damaged organelles and thereby play crucial role in maintaining cellular homeostasis (Mizushima and Levine 2010). Autophagy is also a part of the host innate immune system and protects against pathogens such as viral infections by inhibiting its replication or by eliminating the viruses by delivering them to lysosome for degradation (Deretic et al. 2013). Interestingly, some viruses hijack autophagic machinery and utilize it for its replication (de Haan and Reggiori 2008). In this section, the diverse strategies taken by various coronaviruses to exploit multiple steps of the autophagic pathway to evade host immune response and to facilitate viral replication, will be discussed.

8.6.2 Coronavirus Replication and Autophagy

Autophagy has been implicated in the replication of various positive strand RNA viruses, e.g., dengue virus, and coronaviruses such as mouse hepatitis virus (MHV), SARS-CoV-1 (Lee et al. 2008; Prentice et al. 2004; Shi et al. 2019). However, the general role of autophagy in coronavirus replication is yet to be established in the face of several conflicting reports.

As discussed in previous sections, double membrane vesicles (DMV) in cells infected with positive strand RNA viruses are the site of RNA synthesis and replication. Electron tomography image of MHV and SARS-CoV-1 infected cells showed that DMVs are part of reticular network of modified ER and it contains dsRNA (Gosert et al. 2002; Knoops et al. 2008). The presence of N- glycosylated viral nonstructural proteins (nsp3 and nsp4) in replication-transcription complex also supports the ER origin of DMVs (Kanjanahaluethai et al. 2007; Oostra et al. 2007). Reports citing the favorable role of autophagy in MHV replication in host cells are found to be contradictory (Prentice et al. 2004; Reggiori et al. 2010). Studies in embryonic stem cell line showed autophagy facilitates MVH replication. The replication complexes of MVH (MHV strain A59) are found to co-localize with autophagy markers LC3-II and Apg12 (ATG12). Furthermore, ATG5 knockout embryonic stem cell lines showed significantly impaired (~1000 fold lesser) MHV replication compared to Wild type cells which could be restored by expression of ATG5 in the same genetic background (Prentice et al. 2004). Additionally, co-localization of replication proteins (nsp2, nsp3, nsp8) with endogenous LC3, suggests a connection between virus replication and autophagosome formation (Prentice et al. 2004). Furthermore, it was shown that MHV utilizes the pathway of EDEMosome formation to generate DMV by inhibiting degradation of the EDEM1 and ER chaperone OS-9 (Reggiori et al. 2010).

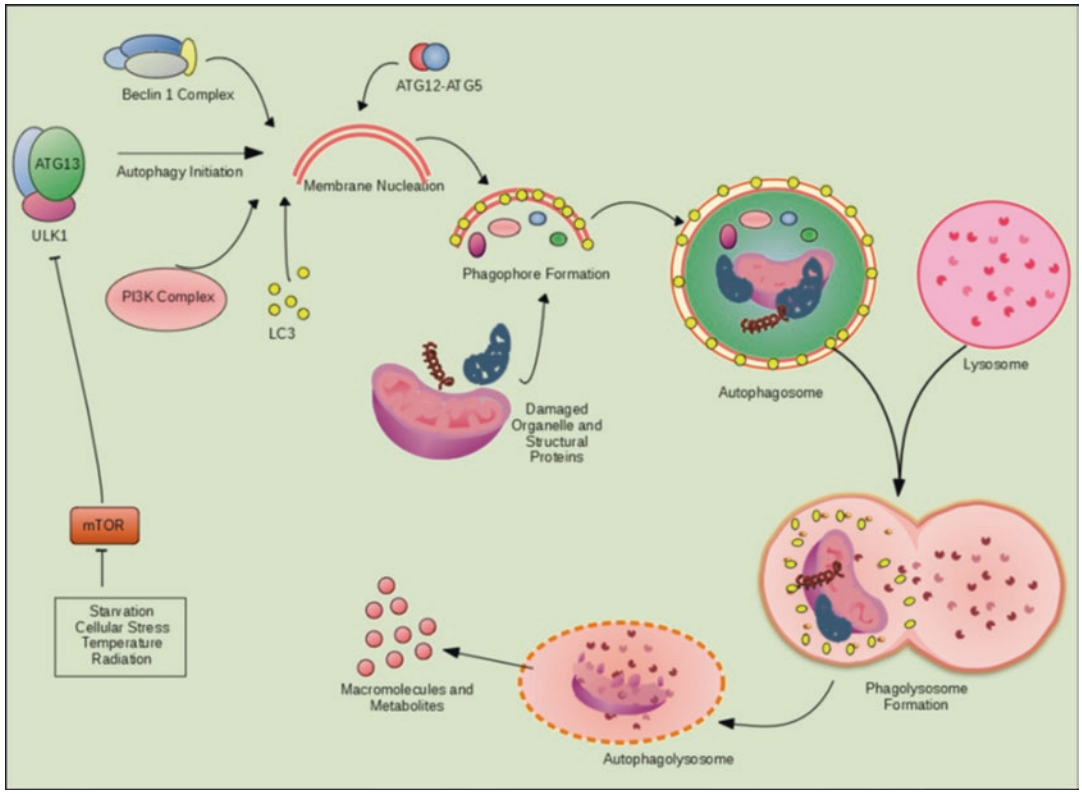

Fig. 8.5 Components and steps of Macroautophagy Physical or chemical stressors induces autophagy by inhibiting mTOR, which is the key negative regulator of autophagy. The core components of autophagy consist of several autophagy proteins (ATGs). Macroautophagy occurs in three steps 1) Initiation, 2) Elongation and 3) Maturation. The process initiates with nucleation of an isolation membrane or phogophore formation. The elongation of phagophore depends on ATG12 and LC3 conjugation system. The closure of phagophore generate double membrane autophagosomes. Damaged organelles like mitochondria, ER, peroxisomes and aggregated proteins are encapsulated within autophagosome, with the help of several protein complexes (ULP-1, Beclin-1, ATG12-ATG5 and LC3-p62 complex). Autophagosome eventually fuse with the lysosome to give rise autophagolysosome where degradation of encapsulated cargos takes place

However, another study indicates that ATG5 is not needed for MHV-A59 replication in primary cells, e.g., primary BMM (bone marrow derived macrophage) and primary low passage MEFs (mouse embryonic fibroblast) (Zhao et al. 2007). This emphasizes that the requirement of autophagic machinery for viral replication depends on host cell type and properties. This observation was further validated by the report showing the presence of non-lipidated LC3-I coats on MVH-induced DMVs which are essential for replication (Reggiori et al. 2010).

Evidence of involvement of autophagy in SARS-CoV-1 infection has been also investigated. SARS-CoV-1 ORF8b (SARS-CoV open reading frame 8b) aggregates in the infected cells, induces lysosomal stress, activates Transcription factor EB (TFEB), and causes eventual cell death. TFEB is the master transcriptional regulator of autophagic and lysosomal machinery (Nabar and Kehrl 2017) and activation of TFEB increases both lysosomal biogenesis and autophagic flux (Settembre et al. 2011). ORF8b triggers calcineurin dependent activation of TFEB and TFEB-target genes, which leads to an increase in autophagic flux (Shi et al. 2019).

Porcine Epidemic Diarrhea Virus (PEDV) is another enveloped, single-stranded positive-sense RNA virus of the Coronaviridae family. PEDV infection leads to the upregulation of autophagy in infected Vero cells as indicated by an increase in LC3-1B level (Sun et al. 2015)

downregulation of mTOR (the mammalian Target of Rapamycin) pathway (Guo et al. 2016). The trigger of autophagy promotes replication of PEDV and inhibition of autophagy by silencing of endogenous Beclin1 or ATG5 gene reduces the PEDV titer (Guo et al. 2017).

8.6.3 Autophagy as Protective Mechanism against Coronavirus Infection

Autophagy has a multifaceted role in viral pathogenesis. Protective response by autophagy has been reported from various coronavirus infection, including SARS-CoV-1, MERS-CoV, and PHEV (Porcine hemagglutinating encephalomyelitis virus). Autophagy can be induced by nsp6 of MHV, SARS-CoV-1 and IBV (Infectious Bronchitis Virus) through an ATG5 and PIK3C3 dependent pathway. Nsp6 protein limits the expansion of autophagosomes induced by starvation, thereby preventing the formation of autophagosomes (Cottam et al. 2014). Analysis of database of SARS-CoV-2 genomes, GISAID (www.gisaid.org) showed presence of leucine to phenylalanine mutation in position 3691 of nsp6 protein. This mutation lowers the stability of the protein structure and favors the affinity of nsp6 protein to ER membrane, which in turn facilitates coronavirus infection by compromising the ability of autophagosomes to deliver the viral components to lysosomes for degradation (Benvenuto et al. 2020).

In MERS-CoV, replication of virus leads to reduction in Beclin 1, a key regulator of autophagy and thus block fusion of autophagosomes with lysosomes. In a recent study, S-phase kinase-associated protein 2 (SKP2) was found to act as E3- ubiquitin ligase for Beclin 1 and its subsequent proteasomal degradation (Gassen et al. 2019). Pharmacological inhibition of SKP2 resulted in decreased Beclin 1 degradation and increased autophagic flux, which in turn was also effective in reducing the replication of MERS-CoV up to 28,000-fold (Gassen et al. 2019). Bioinformatic analysis of kinome data suggests MERS-CoV infection in tissue culture modulates ERK/MAPK and PI3K/AKT/mTOR signaling responses and chemical inhibitors targeting ERK/MAPK and PI3K/AKT/mTOR pathway can inhibit MERS infection (Kindrachuk et al. 2015). Pharmacological or genetic manipulation of autophagy indicates that replication of coronavirus porcine hemagglutinating encephalomyelitis virus (PHEV) in Neuro-2a cells induces incomplete autophagy where autophagosome-lysosome fusion is inhibited. This atypical autophagy is necessary for its replication (Ding et al. 2017).

The use of antimalarial drug chloroquine and hydroxychloroquine for treatment of SARS-CoV-2 has taken huge attention and controversy in recent time (Wang et al., 2020). Chloroquine blocks autophagic degradation in lysosome by altering lysosmal pH (Chen et al. 2011) and by blocking fusion of autophagosome and lysosomes (Mauthe et al. 2018). Elevated pH in endosomes leads to blockage in SARS-CoV-2 entry (Bonam et al. 2020; Hoffmann et al. 2020). Moreover, chloroquine reduces terminal glycosylation of the metallopeptidase ACE2 (Angiotensin-Converting Enzyme 2) and limits the entry of the SARS-CoV-1 and SARS-CoV-2 in host cells (Zhao et al. 2015). However, despite positive reports in the controlled lab culture system, whether chloroquine could be successfully used as a therapeutic agent in fighting SARS-CoV-2 infection has yet not been established beyond doubt.

8.7 ER-Unfolded Protein Response, a Frequently Hijacked Cellular Adaptive Response Pathway by Coronaviruses

8.7.1 A General Overview of ER-Unfolded Protein Response (ER-UPR)

Endoplasmic reticulum (ER) is the hub of post-translational modification and folding of secretory and membrane proteins of any eukaryotic cell. ER possesses a stringent and highly evolved stress response pathway known as ER-Unfolded

Protein Response (UPR) to maintain the quality of the ER-resident and secreted proteome in close collaboration with specialized degradation pathways like ERAD (ER-Associated Degradation). A multitude of viruses employ the ER-Golgi pathway of host cells to produce new viral proteins and subsequent packaging of virions which often leads to overwhelmed ER lumen eliciting ER-UPR. There are three designated sensory pathways for ER-UPR in higher eukaryotes (metazoans), where UPR is elicited upon activation of either one or more of the UPR sensors: 1. Ire1 (Inositol-requiring enzyme 1) -Xbp1 (or Hac1 in unicellular eukaryote yeast); 2. PERK (PKR-like ER kinase); and 3. ATF-6 (Activating Transcription Factor 6) (Korennykh and Walter 2012). The most well conserved ER-UPR pathway that is present from unicellular yeasts to mammals is orchestrated through Ire1-Xbp1 (or Hac1). Ire belongs to cellular kinases and it is an ER-membrane protein having a transmembrane domain and two soluble domains exposed to ER lumen and cytosol, possessing the UPR-sensory and Kinase activity in the ER-luminal domain and RNAse activity in the cytosolic domain, respectively (Korennykh and Walter 2012). Upon sensing stress within ER-lumen, Ire1 oligomerizes and auto-phosphorylates, which in turn activates its RNAse domain, which subsequently splices the Xbp1 (or Hac1) mRNA by a non-canonical splicing process. The spliced Xbp1 (or Hac1) mRNA is translated to the bZIP transcription factor Xbp1 (or Hac1) which moves to the nucleus and then facilitates the expression of ER-UPR target genes. In metazoans, additional pathways for UPR-sensing and activation have evolved. PERK is an ER-membrane resident kinase that possesses possible similar UPR sensing mechanism to Ire1-luminal domain and its kinase domain contains significant similarity to cytosolic kinase PKR. PKR is implicated in providing antiviral innate immunity. PERK and PKR share common targets, and both phosphorylate the translation initiation factor eIF2α which results in attenuation of cellular translation. This attenuated protein translation relieves the ER-UPR by decreasing the incoming protein flux to the ER. During translational shutdown due to eIF2α phosphorylation by PERK, a handful of mRNAs are preferentially translated, among which ATF4 is formed which helps in expression of UPR responsive genes. ATF6 is a transcription factor having an ER-luminal domain with UPR-sensory activity, a transmembrane domain, and a cytosolic domain. Upon sensing the ER-UPR by its ER-luminal domain, ATF6 is exported through membrane vesicles from ER and gets processed by subsequent protease activities of S1P and S2P proteases in the Golgi bodies where the cytosolic domain of ATF6 is cleaved off from its other domains and moves to nucleus to act as a transcription factor for UPR target genes (Korennykh and Walter 2012).

Apart from various evidences of specific viral protein inducing UPRs, studies on SARS-CoV-1 have shown that infection by this pathogenic coronavirus can mount a substantial ER-UPR (Chan et al. 2006; Jiang et al. 2005; Versteeg et al. 2007; Yeung et al. 2008). Even though we lack substantial data from the latest coronavirus, SARS-CoV-2, there is ample evidence in literature regarding ER-UPR induction upon various coronavirus infections. Thus, targeting ER-UPR pathways can be an effective strategy to combat coronavirus infections and subsequent pathologies like Covid-19.

As previously discussed, coronaviruses produce DMVs after infecting the host cells and these DMVs originate from the ER (Angelini et al. 2013; Gosert et al. 2002; Prentice et al. 2004; Snijder et al. 2006). ER being the cellular hub of protein maturation, folding, and secretion, it frequently gets overburdened with excessive flux of viral protein into ER post-infection with viruses like coronaviruses (Delmas and Laude 1990; Nal et al. 2005). Coronaviruses propagate within the ERGIC (ER Golgi Intermediate Center) which is an extended ER structure and, when new virions are exocytosed from ERGIC, it induces ER stress.

The three sensory pathways of ER-UPR as described, are modulated in unique ways by various viral pathogens, including coronaviruses. Here we briefly discuss the evidence of alteration of ER-UPR associated with coronavirus infections from literature and the small molecule-

mediated intervention strategies that can be employed as possible therapeutic agents against pathologies like Covid-19.

8.7.2 Modulation of PERK Branch of ER-UPR by Coronavirus Infections

Various studies using model coronaviruses have reported that the PERK arm of the ER-UPR pathways gets activated due to systemic infections with the viruses (Ye et al. 2007). The increased levels of activated PKR, PERK, and phosphorylated eIF2α signify activation of translation-attenuation pathway (Krahling et al. 2009). The PKR molecule can deactivate eIF2α and activate innate immune responses, although various viruses, including coronaviruses, have evolved strategies to successfully evade the PKR response (Roth-Cross et al. 2008; Zhao et al. 2011, 2012; Zust et al. 2011). The activation of the PERK or PKR pathway eventually leads to eIF2α phosphorylation-mediated global translation attenuation. It was shown that translation attenuation by PERK/PKR can be relieved with the help of ATF4-mediated GADD153 and CHOP/GADD34 activation suggesting the pathway's pro-apoptotic roles, though there are some reports that certain coronaviruses can interfere with this pathway (Liao et al. 2013; Wang et al. 2009). PERK and PKR-mediated GADD153 induction was shown to play a key role in apoptosis induction in cells infected with coronavirus like infectious bronchitis virus (IBV). However, SARS-CoV-1 selectively activates PERK to attenuate cap-dependent translation by phosphorylating eIF2α. This does not culminate in apoptosis but is required for efficient virus replication (Krahling et al. 2009). Small molecule inhibitors of PKR kinase activity like 2 amino purine (2-AP) inhibited GADD153 activation and resulting apoptosis of the IBV infected cells (Liao et al. 2013). Before this study, another report showed that in IBV infected cells, phosphorylation of eIF-2α is severely repressed. The authors showed multiple pathways by which the virus successfully propagates within the host cells; nsp2 of IBV works as PKR antagonist which results in decreased eIF-2α phosphorylation and allows viral protein translation and viral infection mediated induction of GADD34, a component of protein phosphatase 1 (PP1) complex that dephosphorylates eIF-2α to restore cellular translation. Together these studies showed coronaviruses can modulate the host cellular response by modifying the PKR/PERK pathway by multipronged approach, by expressing its nps proteins possessing PKR antagonist activity and inducing GADD34 to recycle phosphorylated eIF2α to ultimately enhance its replication. To intervene in such viral mechanisms of ER-UPR alteration, small molecule modulators of PKR/PERK pathway can be effective and indeed GADD34 inhibitors like okadaic acid was shown to be efficient in controlling the virus replication (Wang et al. 2009). Thus, by modification of specific arms of ER-UPR pathways using specific inhibitor or activators, the fate of virus infected cells can be effectively altered.

8.7.3 Modulation of Ire1 and ATF6 Branches of ER-UPR by Coronaviruses

In a study with murine coronavirus (murine hepatitis virus or MHV), it was shown that Ire1-ATF6 branch of ER-UPR is activated, although the induction of UPR target genes were weak and the viral infection resulted in sustained phosphorylation of eIF2α. Therefore, the host cell protein translation was severely jeopardized, although translation of viral mRNAs proceeded unhindered during MHV infection. Subsequently, it was shown that although ATF4 is produced after infection, its downstream targets, CHOP and GADD34, are not expressed. As discussed in the previous section, GADD34 plays key role in dephosphorylating and recycling of eIF2α to re-initiate cellular protein translation. This study proposed unique mechanisms of MHV by sustained repression of cellular protein synthesis and favoring viral protein synthesis (Bechill et al. 2008). Surprisingly, similar observations were not recapitulated in case of SARS-CoV-1 experi-

ments (DeDiego et al. 2011; Versteeg et al. 2007). In a recent report, it was observed that infections with SARS-CoV deleted of envelop protein (E protein) produce intense ER-UPR and the Xbp1 splicing and amplitude of UPR induction is substantially over and above the SARS-CoV-1 with the E protein (DeDiego et al. 2011). This indicated that the E protein has regulatory role in controlling the amplitude of ER-UPR induction possibly to escape the host cell antiviral response through ER-UPR induction. The SARS-CoV accessory protein 8ab can induce ATF6 (Sung et al. 2009), and physical interaction between ATF6 and the SARS-CoV accessory protein 8ab have been established experimentally (Sung et al. 2009). Like small molecule modulators targeting the PERK/PKR pathway, Ire1, ATF6 pathways can be also modulated and the outcome of viral infections can be altered.

8.8 Conclusion

In this chapter, we have discussed the role of various cellular proteostasis pathways like Hsp70-Hsp40 chaperone system, Ubiquitin proteasome (UPS), autophagy, and ER-UPR pathways during viral pathogenesis as well as host defense mechanism during viral infections, especially during coronavirus infections. We have discussed experimental evidence from previous literatures to demonstrate how different coronaviruses evade the host immune response by manipulating the different arms of cellular proteostasis network. The host defense mechanisms through UPS, autophagy, or ER-UPR in response to viral pathogens have also been discussed in parallel. We have shown manipulation of these pathways by pharmacological inhibitors or activators can be beneficial for us by successfully inhibiting the viral replication and disease progression. In the background of current pandemic caused by SARS-CoV-2, we think that systematic search for modulators of proteostasis have immense potential as therapeutic agents as monotherapy or as combination therapy in the treatment of SARS-CoV-2 pathogenesis or Covid-19.

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Ethical Approval for Studies Involving Animals This chapter does not contain any studies with animals performed by any of the authors.

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9 The Potential Impact of Statins in the Treatment of Patients with COVID-19 Infection

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Abstract

Introduction: Statins are cholesterol-lowering drugs that also have anti-inflammatory/ immunomodulatory properties, and have been suggested as an adjunct therapy for COVID-19.

Methods: To investigate the clinical impact of statins as a potential therapeutic approach in the treatment of cases infected with COVID-19, a systematic search was performed using PubMed and Google Scholar databases. To extend the search results, a set of keywords were used as follows: ("corona virus" OR "Covid-19" OR "SARS-Cov-2" OR "Severe Acute Respiratory Syndrome Coronavirus 2" OR coronavirus) AND (Statins), alongside a manual search in Google Scholar search engine.

Results: It has also been suggested that statins could influence the entry of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) into cells by altering the expression of the angiotensin-converting enzyme 2 (ACE2) and CD143 receptors. Statins may be beneficial for COVID-19 patients according to its pleiotropic effects, although, from the clinical aspect, these pleiotropic effects of statins may not be as strong as in preclinical phase on COVID-19. A retrospective study showed favorable effects for statins in SARS-CoV-2 infection.

Conclusion: Patients with SARS-CoV-2 infection have a high risk of cardiovascular and thrombotic complications and pleiotropic effects of statins may help manage the COVID-19. There is growing evidence that

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supports the need for trials of statin treatment in COVID-19 infection.

Keywords

Statins · Anti-inflammatory activity · SARS-CoV-2 · COVID-19

9.1 Introduction

Statins are HMG-CoA reductase inhibitors best known for their cholesterol-lowering properties. HMG-CoA is the rate-limiting enzyme in the cholesterol synthetic pathway. Statins have been shown to reduce serum cholesterol, triglyceride, low-density lipoproteins, and Apo-lipoprotein (apo) B, while increasing the high-density lipoprotein (HDL) levels. As shown in Fig. 9.1, statins reduce hepatocyte cholesterol concentration and increase the expression of LDL receptors that then leads to an increased LDL clearance. Moreover, HMG-CoA reductase inhibitors also downregulate the production of LDL apoB-100 (Brousseau and Schaefer 2002). The lipid-lowering effects of statins considered their main potential effects and many cardiovascular diseases take benefit of this anti-hyperlipidemic potential. However, it has been argued that the beneficial effects of statins go beyond the lipid-lowering effects (Liang et al. 2017). Statins have also been reported to be involved in the regulation of other biological processes, including inflammatory responses, antioxidant effects, and anti-tumoral activities that could be beneficial in other diseases, except for cardiovascular diseases.

9.2 SARS-CoV-2 and Its Pathogenesis in Humans

SARS-CoV-2 is an enveloped, single-stranded RNA virus. Together with Middle East respiratory syndrome coronavirus (MERS-CoV), SARS-CoV, SARS-CoV-2 is classified as a B coronavirus that predominantly affects mammals. SARS-CoV-2 infection begins with the attachment of the virus to host receptors and entering the cells via membrane fusion or endocytosis (Yuki et al. 2020). The angiotensin-converting enzyme 2 (ACE2) receptor is one of the cellular receptors for SARS-CoV (Lange et al. 2020). The ACE2 receptor is expressed by many mammalian cells, including lung epithelial cells (Lange et al. 2020). After attachment to the cellular receptors, the viral genome then enters the cell's nucleus and replicates (Devaux et al. 2020). Four different components of the coronavirus include nucleocapsid, envelope, membrane, and spike protein (Yuki et al. 2020). After the cellular invasion and the replication of new viruses, the clinical symptoms become evident, ranging from minimal symptoms to severe respiratory symptoms that may lead to death (Yuki et al. 2020). The lung injury usually starts in the distal airways.

Three immune cells are involved in the early responses to coronavirus infection in the lung: dendritic cells, macrophages, and lung epithelial cells (Yuki et al. 2020). These immune cells attempt to control the infection before the activation of the adaptive immune system (Yuki et al. 2020). Dendritic cells and macrophages activate T cells. Both CD8+ and CD4+ T cells play an important role in killing the infected cells and activating B cell against the SARS-CoV-2 (Yuki et al. 2020). Patients with COVID-19 infection may go on to develop various laboratory abnormalities and dysregulated inflammatory responses that may exacerbate the clinical prognosis of patients by causing damage to the lungs and other tissues (Mason 2020). Severe cases show decreased peripheral T cell levels as well as increased circulating pro-inflammatory cytokines, including IL-8, IL-10, IL-6, TNF-a, MCP1, and macrophage inflammatory protein (MIP)1a. The T cells of COVID-infected patients produce granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF may act as a double-edged sword augmenting T cell function, but also causing tissue damage when present in excess (Yuki et al. 2020). Similarly, an increased number of neutrophils may also lead to lung damage. Production of increased numbers of pathologic cytotoxic T cells is also reported in

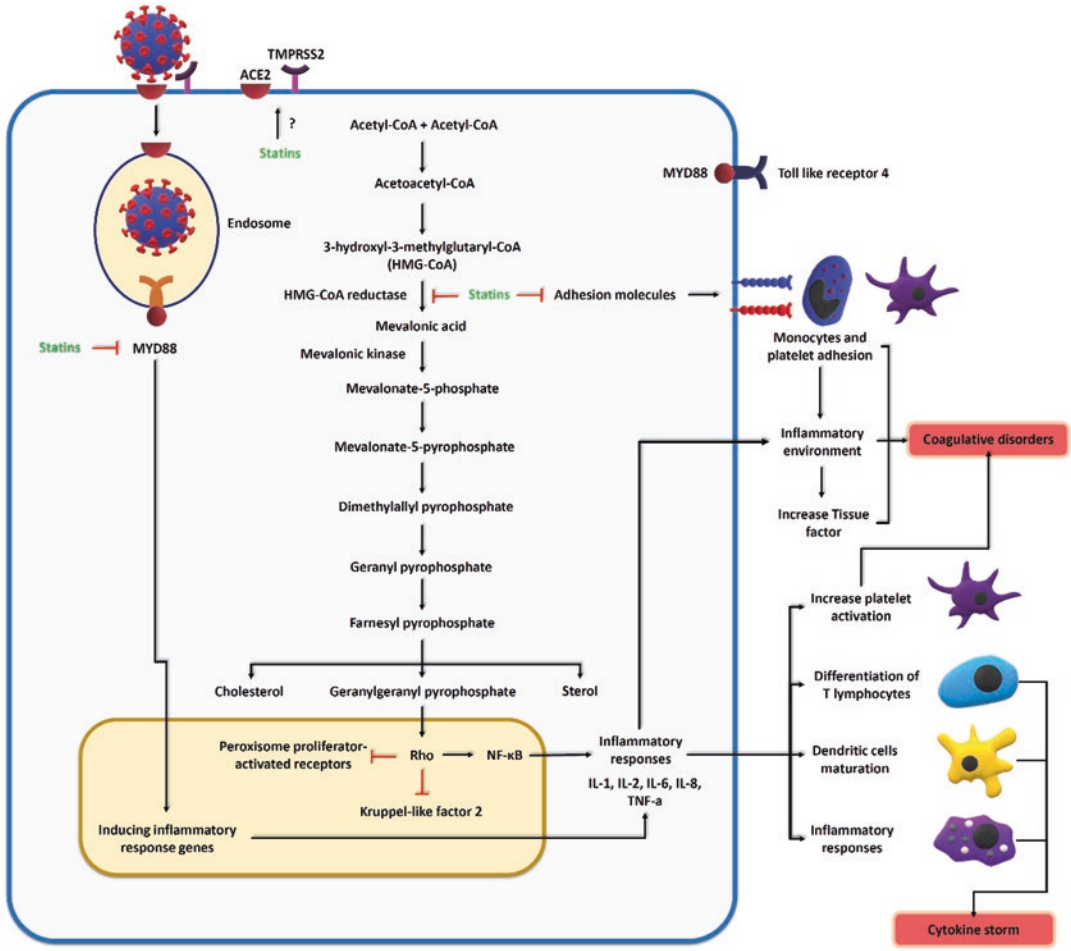

Fig. 9.1 Statins inhibit 3-hydroxy-3-methyl glutaryl (HMG)-CoA and reduce synthesis of mevalonate. Therefore, cholesterol synthesis and isoprenoid production is reduced. Statins also inhibit farnesyl pyrophosphate and geranylgeranyl pyrophosphate synthesis. Reduced isoprenoid production results in increased production of nuclear factor-κB (NF-κB) expression, thereby increasing expression of Kruppel-like factor 2 (KLF2) and inhibiting pro-inflammatory responses. NF-κB induce expression of many inflammatory markers that are crucial in setting up a pro-inflammatory environment, resulting in disrupted inflammatory response and coagulating disorders. Statins also decrease expression of adhesion molecules that reduce coagulation as well as tissue damage induced by monocytes infiltration. Statins also regulate the NF-κB pathway by their effect on toll-like receptors. The effect of statins on angiotensin converting enzyme 2 (ACE2) is still controversial and needs further studies

COVID-19 patients and this can also damage lung tissue (Yuki et al. 2020). These dysregulated immune responses following SARS-CoV-2 infection have been referred to as a "cytokine storm." During COVIID-19, two clinically similar cytokine storm syndromes have been described: secondary Hemophagocytic Lymphohistiocytosis (sHLH) and Macrophage Activation Syndrome (MAS) occur in severe cases (Canna and Behrens 2012). HLH is due to an inadequate anti-inflammatory response (IL-10, TGF-B, and regulatory T cells) in the settings of overproduction of pro-inflammatory components (IL-2, IL-6, TNF-a, GCSF and INF-Y) (Mehta et al. 2020). During a cytokine storm, many other laboratory abnormalities, including leukopenia, increased C reactive protein (CRP), erythrocyte sedimentation rate (ESR), and ferritin, also occur (Canna and Behrens 2012; Mehta et al. 2020). Alongside the cytokine storm,

abnormalities of coagulation is another complication that may be seen in COVID-19 patients. It has been demonstrated that COVID-19 patients are at greater risk of developing thrombotic events and coagulation disorder including disseminated intravascular coagulation (DIC) and pulmonary embolism (Klok et al. 2020). Similar to some other viral infections, coagulopathy may be the result of inflammatory responses during the SARS-CoV-2 infection (Connors and Levy 2020). However, the exact mechanism of accounting for coagulative disorders is not clearly understood.

9.3 Antihyperlipidemic and Non-antihyperlipidemic Effects of Statins

The cholesterol-lowering effect of statins is well established, and many clinical trials have shown the beneficial effects of these drugs on improving outcomes in cardiovascular diseases. However, the effect of statins on infectious diseases is not established. Statins inhibit the mevalonate pathway that leads to a reduction in cholesterol synthesis (shown in Fig. 9.1). However, mevalonate is the precursor of other non-steroidal isoprenoids that are also affected by HMG-CoA reductase inhibitors. Post-translational modifications by isoprenoids located downstream of the mevalonate pathway may lead to some of the "pleiotropic effects" of statins.

HMG-CoA reductase inhibitors also affect small guanine triphosphate (GTP) binding proteins. Statins modulate intracellular signaling, including the pro-inflammatory pathway involving these small molecules, activated by isoprenylation. Xu et al. evaluated the effects of statins on GTP binding proteins (Xu et al. 2006), and demonstrated that the administration of simvastatin modulates proinflammatory cytokines in rheumatoid arthritis. According to their results, simvastatin prevents the activity of RhoA that is one of the GTP binding proteins (Xu et al. 2006). Rho and Rho-like proteins regulate inflammatory pathways including p38 MAP kinase and JNK (Braga 2002). Decreased RhoA activity modulates NF-kB activation induced by TNF-a and decreases the expression of pro-inflammatory cytokines, including IL-6 and IL-1B (Xu et al. 2006). Monocyte chemotactic protein 1 (MCP-1) is another molecule affected by statins. MCP-1 is a chemoattractant for lymphocytes and monocytes (Pasceri et al. 2001). Inflammatory cytokines modulators, including CRP and IL-1B stimulate MCP-1 production and statins inhibit MCP-1 production by epithelial cells (Romano et al. 2000). While Rosuvastatin decreases MCP-1 in animal models, other statins, including pravastatin and atorvastatin, decrease MCP-1 expression (Abeles and Pillinger 2006). Simvastatin and atorvastatin decrease IL-1B production by mononuclear cells (Wæhre et al. 2004). It has been demonstrated that these drugs reduce serum IL-6, IL-1B, and TNF-a in patients with hyperlipidemia (Abeles and Pillinger 2006). HMG-CoA reductase inhibitors also have immune regulatory effects as well, including inhibitory effects on MHC-II expression in contrast to MHC-I expression in epithelial cells (Kwak et al. 2000). Statins inhibit ERK activation modulated by the Ras-dependent mechanism (Kwak et al. 2000), and Rho dependent mechanisms decrease T cell P38 activation (Ghittoni et al. 2005). Another immune modulatory effect of statins is related to autophagy, and they induce autophagy by increasing the expression of LC3-II and Beclin-1 (Gao et al. 2016). LC3-II is a protein that is crucial in the formation of autophagosomes and Beclin-1 is a marker of autophagy (Gao et al. 2016). While the evidence regarding the effect of statins on authophagy capacity of immune system is from in vitro studies on tumor tissues other than respiratory system tumors, Yang et al. is among the few studies that reported the effectiveness of statins in adenocarcinoma of lung cell lines and demonstrated that statins increase LC3-II levels (Yang et al. 2017). Therefore, the effects of statins on the autophagy capacity of immune system needs further studies.

Statins may also interfere with the expression of the ACE2. SARS-CoV-2 downregulate the expression of ACE2. Dong et al. demonstrated that in their animal model study, statins

increased expression of ACE2 in some organs including kidneys and heart (Dong et al. 2008). Other studies on mouse models also demonstrated that ACE2 upregulation is seen in those models receiving statins (Li et al. 2013; Min et al. 2018). Despite the effect of statins on ACE2 expression in animal models, it should be highlighted that these studies are conducted on diseased animals and compared with the healthy subjects. Therefore, the effect of statins on healthy animal models should be addressed in further researches in order to evaluate the protective role of statins on ACE in healthy subjects. A similar issue also relates to the other putative receptor for SARS-CoV-2, the CD147 (EMMPRIN). The SARS-CoV-2 spike protein binds to CD147 and viral invasion occurs (Ulrich and Pillat 2020). Similar to the effect of statins on ACE2, CD147 take effect from this drug. Statins induce impaired translocation of CD147 on cell surface (Sasidhar et al. 2017). As is the case for ACE2 receptors in animal models, animal models for determination of the effects of statins on CD147 was conducted on diseased models (Liang et al. 2017) and clinical trials on humans are lacking in this field.

9.4 Clinical Evidences of Non-antihyperlipidemic Effects of Statins

Although there have been many in vitro and animal studies on the non-antihyperlipidemic effects of statins; the clinical evidence regarding the non-antihyperlipidemic effects of statins, including the immune and inflammatory regulatory effects in man, are still very limited. Primarily results of clinical studies established a relationship between receiving statins and pro-inflammatory markers. Okopien et al.'s study evaluated the inflammatory effects of statins and fibrates on patients with dyslipidemia (Okopień et al. 2004). They randomized their study population to four groups, including Fluvastatin (40 mg/d), Simvastatin (20 mg/d), micronized ciprofibrate (100 mg/d), and micronized fenofibrate (200 mg/d) (Okopień et al. 2004). They showed that in comparison with healthy subjects, both of the groups receiving statins reduced INF-Y and IL-2 secretion by T lymphocytes (Okopień et al. 2004). Rezaei-Majd et al. evaluated the effect of Simvastatin on 107 hypercholesterolemia patients and demonstrated that after six months of treatment, MCP-1 and interleukins including IL-6 and IL-8 reduced significantly (Rezaie-Majd et al. 2002). Cerivastatin, atorvastatin, and simvastatin downregulate cytokine production in a dose- and time-dependent manner (Rezaie-Majd et al. 2002). Kowalski et al. evaluated 54 patients treated with atorvastatin, simvastatin, or fenofibrate for four weeks. These drugs could significantly; reduce serum MCP-1 and therefore the authors concluded that these drugs inhibit monocyte activity directly (Kowalski et al. 2003).

The anti-inflammatory effects of statins have also been demonstrated in some larger clinical studies. One of the earliest clinical trials demonstrating the effect of statins on inflammatory markers was the Pravastatin Inflammation/CRP Evaluation (PRINCE) study enrolling more than 1600 individuals (Albert et al. 2001). This randomized double-blind prevention trial evaluated the effect of 40 mg/d of pravastatin for six months. The primary results indicated an anti-inflammatory effect of pravastatin in terms of reducing serum CRP levels within the first three months of study (Albert et al. 2001). The anti-inflammatory effect of pravastatin was unrelated to the effects of anti-hyperlipidemic effects in terms of reducing the LDL-C levels (Albert et al. 2001). Subsequent to the PRINCE trial, a larger clinical trial on 15,548 participants evaluated the effect of 20 mg/d Rosuvastatin on cardiovascular diseases during five years' follow up (Ridker et al. 2009). The JUPITER trial demonstrated that despite the beneficial effect of Rosuvastatin on the reduction of vascular events, statins might have beneficial effects on reducing the hsCRP levels (Ridker et al. 2009). This trial also showed that serum hsCRP levels <2 mg/L and LDL levels <1.8 mmol/L were associated with improved event-free survival in contrast to decreased LDL cholesterol alone (Ridker et al. 2009).

9.5 Statins and its Emerging Effects in Viral Disease

The regulatory effects of statins have been shown in many inflammatory and immune disorders, including rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis (Zeiser 2018). A systematic review and meta-analysis on the relationship between statins and the development of bacterial infections demonstrated some interesting findings (Björkhem-Bergman et al. 2010). Despite possible publication bias, according to the results of this meta-analysis, there may be an association between using statins and reduced mortality from bacterial infections. Furthermore, using statins may have a prophylactic effect on the prevention of pneumonia and sepsis (Björkhem-Bergman et al. 2010). The possible beneficial role of statins in viral infection has been proposed in the literature. Elahi et al.'s study demonstrated that statins may inhibit HIV replication by increasing cyclin-dependent kinase inhibitor p21 expression and CC-chemokine receptor 5 (CCR5) (Elahi et al. 2016). Moreover, increasing the type I interferon responses decreased both murine cytomegalovirus (MCMV) and a mouse model of gammaherpesvirus infections (Lange et al. 2016; Blanc et al. 2011). This evidence suggests a possible beneficial role of statins in many other viral diseases, including in the novel coronavirus infection. As mentioned earlier, SARS-CoV-2 infection mainly causes its harmful effects through the dysregulation of the immune response and activation of cytokine storm syndrome. Therefore, the beneficial effects of statins in the inflammatory responses and immune modulatory effects may help overcome some of the serious effects of SARS-CoV-2 infection. The other possible beneficial effects of statins, including their effect on the expression of ACE2 and CD134 as well as their beneficial effects on the autophagy, need further clinical evidences.

Activation of NF-kB is an important pathogenic mechanism in viral infections, including coronavirus infections. A recent study by DeDiego et al. demonstrated that SARS-CoV infection induces an inflammatory response by the activation of the NF-kB pathway (DeDiego et al. 2014). In mouse models infected with recombinant SARS-CoV, the administration of NF-kB inhibitors increases mouse survival and reduces pro-inflammatory cytokines in the lungs (DeDiego et al. 2014). As demonstrated earlier in this chapter, statins decrease the activity of RhoA activity and modulate NF-kB activation while decreasing the secretion of pro-inflammatory cytokines including IL-6 and IL-1B (Devaux et al. 2020).

Alongside the regulation of RhoA activity, Toll-like receptors (TLRs) are another important target for statins. TLRs play an important role in the recognition of viral pathogens and the initiation of the innate immune response (Totura et al. 2015). Some TLRs agonists showed protective potentials against SARS-CoV infection in animal models (Totura et al. 2015). Totura et al. evaluated the susceptibility of TLR-3 and TLR4 mice to SARS-CoV infection (Totura et al. 2015). They demonstrated that TLR3 (−/−) and TLR4 (−/−) mice are more susceptible to develop SARS-CoV respiratory infection (Totura et al. 2015). However, TLR3/TLR4 adaptor TIR-domain-containing adapter-inducing interferon-β (TRIF) defecate mice showed greater susceptibility to SARS-CoV infection and developed severe symptoms (Totura et al. 2015). TRIF (−/−) mice developed excessive neutrophil infiltration and other inflammatory cells (Totura et al. 2015). Alongside TLR-3 and TLR-4, myeloid differentiation primary response 88 (MYD88) that is downstream of the TLRs, showed a significant association with SARS-CoV infection (Sheahan et al. 2008). An imbalanced immune response, through TLR MYD88, is crucial in the development of SARS-CoV infections. Even excessive or reduced expression of MYD88 showed increased mortality following MERS-CoV infection (Totura et al. 2015; Sheahan et al. 2008). Activation of NF-kB that can be activated via the TLR-MYD88 is one of the SARS-CoV infection pathogenesis (DeDiego et al. 2014). Sheahan et al. reported that MYD88-deficient mice are more susceptible to SARS-CoV infection and the genetic absence of MYD88 results in greater mortality within the

first week of infection (Sheahan et al. 2008). It is hypothesized that statins, as MYD88 antagonists, may lead to beneficial effects during coronavirus infections. Youan et al. showed that in adult male rats under intermittent hypoxia, administration of atorvastatin before the hypoxia inhibits TLR4 and MYD88 expression and radical oxygen species in the myocardium (Yuan et al. 2014). Galleli et al. evaluated the effect of statins in lung adenocarcinoma cell lines and showed that the administration of simvastatin inhibited IL-8 production, NF-kB activation, and decreased MYD88 (Gallelli et al. 2014). Moreover, the inflammatory response may be controlled by the effect of statins on leukocytes. Statins may bind directly to the L-site located on the integrin lymphocyte function-associated antigen 1 (LFA-1) of leukocytes (Weitz-Schmidt et al. 2001). LFA-1 is important in the development of inflammatory diseases and inhibition of LFA-1 results in amelioration of established diseases (Weitz-Schmidt et al. 2001). Among different statins, Pravastatin does not interact with the L-site and newer drugs have been developed to interact with LFA-1 (Weitz-Schmidt et al. 2001).

In contrast to the inflammatory and immune regulating effects of statins, the exact mechanism behind the thrombotic complications during SARS-CoV-2 infection is not clearly understood. However, disrupted inflammatory responses seem to be a reason for the increase in coagulating disorders in COVID-19 patients. The inhibition of GTPs by statins inhibits the coagulation cascades (Eto et al. 2002). Statins can also reduce blood clot formation by thrombomodulin augmentation (Lin et al. 2007). A meta-analysis by Sahebkar et al. demonstrated that the administration of statins may also reduce the D-dimer level within three months and suggested their possible beneficial role in coagulation disorders (Sahebkar et al. 2015).

Regarding the potential beneficial effects of statins on inflammatory, coagulative, and immune responses against viral infections, Yuan et al. suggested that the administration of statins in the early stages of MERS-CoV infection might be beneficial and reduce the mortality rate. They hypothesized that high doses of statins will attenuate NF-kB activation within 24 h and should be continued up to two days for achieving significant results (Yuan 2015). According to the recent study by Zhang et al., it may be concluded that using statins in SARS-CoV infection, may be beneficial in the clinical settings. The most recent large-scale retrospective cohort study on COVID-19 patients evaluating the effect of using statins on the clinical symptoms has recently been published online. Zhang et al. evaluated statin treatment in 13,981 hospitalized COVID-19 patients and revealed that statin users had a lower risk of all-cause mortality (Zhang et al. 2020). Although the study of Zhang et al. provided some valuable indications, it Is well demonstrated that different statins may not provide the same effect in every disease, including viral infections such as SARS-CoV infection, which is not clearly stated in Zhang et al.'s study. A recent study on the Ebola virus demonstrated that statins may reduce viral infectivity by disrupting the protease activity of the virus (Reiner et al. 2020). The main protease (Mpro) in coronavirus is crucial for its proteolytic maturation. The Mpro/chymotrypsin-like protease of SARS-CoV-2 successfully crystallized and considered as a target for coronavirus replication (Jin et al. 2020). Reiner et al.'s study conducted a molecular ducking study and reported that statins can be efficient inhibitors of SARS-C0V-2 Mpro. Among different statins, pitavastatin showed promising inhibitory effects in contrast to other statins (Reiner et al. 2020). The study of Reiner et al. is not the only in-silico study suggesting the possible efficacy of statins in treating COVID-19. Withanone and Withaferin-A are two structurally similar isolates of Ashwagandha or Withania somnifera that is used in traditional medicine of Asian countries. A recent study by Kumar et al. demonstrated that Withanone and Withaferin-A can inhibit transmembrane protease serine 2 (TMPRSS2). The TMPRSS2 is another surface receptor that is used for cell invasions in viral infections, including SARS-CoV (Kumar et al. 2020). Interestingly, simvastatin has drug similarity with withaferin. However, none of these results regarding the

drug similarity and in-silico predictions have been evaluated in clinical studies.

9.6 Conclusion

Many people who become infected by SARS-CoV-2 have underlying co-morbidities, including cardiovascular disease. These people are vulnerable to severe complications following infection, and usually take various medications that could have potentially harmful or beneficial effects on the COVID-19. However, the effects of taking various medication on the COVID-19 susceptibility and severity is not yet addressed for every drug. Among these drugs, statins are inexpensive and readily available drugs in many countries and are widely used by patients with cardiovascular disease. Moreover, many patients without cardiovascular disease but with cardiovascular risk factors are taking statins as a prophylactic regimen (Elnaem et al. 2017). These drugs have few side effects and are well tolerated by most people. From the molecular aspect and according to the available evidence regarding the usefulness of this drug in COVID-19 disease, we can conclude that statins may be beneficial for COVID-19 patients according to its pleiotropic effects. Statins have pleiotropic anti-inflammatory and immune-regulatory effects that can affect immune dysregulation and aberrant inflammatory responses during the disease course. However, from the clinical aspect, these pleiotropic effects of statins may not be as strong as in vitro or animal studies on COVID-19. Although many large clinical trials demonstrated the efficacy of using statins in different clinical settings varying from healthy subjects to even patients with viral or bacterial infections; however, the clinical efficacy of statins on SARS-CoV-2 infection is limited and only one retrospective study suggested favorable effects for using statins in SARS-CoV-2 infection. Therefore, in our point of view, some important issues should be addressed in future researches on using statins in COVID-19 patients. First, the most effective statin from the wide range of available HMG-COA reductases with the lowest adverse effects and lowest drug interaction should be addressed. Second, the dose and duration for greatest efficacy needs to be evaluated and may not be the same as for the effects on serum cholesterol. It is still unclear that statins can be used as a treatment or as a prophylactic treatment strategy for SARS-CoV-2. The fourth issue is the target population for statins. It is unclear that the patients with severe symptoms admitted in the intensive care unit can benefit or the mildly symptomatic patients that are not receiving any medication. The fifth issue is about patients who are already receiving statins. While it has been suggested that patients who are receiving statins could continue their statin and ACEI/ARB drug regimens (Lee et al. 2020), increasing the statin dose in such patients could be considered as most of these patients are at greater risk of developing COVID-19. The last and most important issue is the side effects and drug interaction of statins. Almost all of the statins have hepatic clearance except Pravastatin and Rosuvastatin that have both renal and hepatic clearance (Ward et al. 2019). It is recommended to discontinue or change statins when it is intolerable for the patient and the side effects, mostly including headache, muscle symptoms, dyspepsia, rash, arthritis, and sleep disorders, overcome the cardiovascular risks (Ward et al. 2019). Moreover, possible drug interactions between statins and antiviral drugs should also be considered. Previously, the combined use of antiviral drugs, including lopinavir and ritonavir in combination with rosuvastatin in HIV-infected patients, has been studied and the safety and efficacy of using such drug combinations are still not well understood (van der Lee et al. 2007). Therefore, researchers should decide to choose statins in COVID-19 patients wisely and consider these important issues in their clinical studies.

Conflict of Interest The authors have no conflict of interest to disclose.

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10

Anticoronavirus Activity of Water-Soluble Pristine C_{60} Fullerenes: *In Vitro* and *In Silico* Screenings

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Abstract

Introduction: The emergence of a new member of the *Coronaviridae* family, which caused the 2020 pandemic, requires detailed research on the evolution of coronaviruses, their structure and properties, and interaction with cells. Modern nanobiotechnologies can address the many clinical challenges posed by the COVID-19 pandemic. In particular, they offer new therapeutic approaches using biocompatible nanostructures with "specific" antiviral activity. Therefore, the nanosized spherical-like molecule (0.72 nm in diameter) composed of 60 carbon atoms, C_{60} fullerene, is of interest in terms of fighting coronaviruses due to its high biological activity. In here, we aim to evaluate the effectiveness of anticoronavirus action of water-soluble pristine C_{60} fullerene in the model and *in vitro* systems. As a model, apathogenic for human coronavirus, we used transmissible gastroenteritis virus of swine (TGEV), which we adapted to the BHK-21 cell culture (kidney cells of a newborn Syrian hamster).

Methods: The shape and size of the particles present in C_{60} fullerene aqueous colloidal solution (C_{60}FAS) of given concentration, as well as C_{60}FAS stability (value of zeta potential) were studied using microscopic (STM, scanning tunneling microscopy, and AFM, atomic force microscopy) and spectroscopic (DLS, dynamic light scattering) methods. The cytopathic effect of TGEV was determined with the help of a Leica DM 750 microscope and the degree of monolayer changes in cells was assessed. The microscopy of the viral suspension was performed using a high resolution transmission electron microscope (HRTEM; JEM-1230, Japan). Finally, the search for and design of optimal possible complexes between C_{60} fullerene and target proteins in the structure of SARS-CoV-2 coro-

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navirus, evaluation of their stability in the simulated cellular environment were performed using molecular dynamics and docking methods.

Results: It was found that the maximum allowable cytotoxic concentration of C_{60} fullerene is 37.5 ± 3.0 μg/ml. The investigated C_{60}FAS reduces the titer of coronavirus infectious activity by the value of 2.00 ± 0.08 $TCID_{50}$/ml. It was shown that C_{60} fullerene interacts directly with SARS-CoV-2 proteins, such as RdRp (RNA-dependent RNA polymerase) and 3CLpro (3-chymotrypsin-like protease), which is critical for the life cycle of the coronavirus and, thus, inhibits its functional activity. In both cases, C_{60} fullerene fills the binding pocket and gets stuck there through stacking and steric interactions.

Conclusion: Pioneer *in vitro* study to identify the anticoronavirus activity of water-soluble pristine C_{60} fullerenes indicates that they are highly promising for further preclinical studies, since a significant inhibition of the infectious activity of swine coronavirus of transmissible gastroenteritis in BHK-21 cell culture was found. According to molecular modeling results, it was shown that C_{60} fullerene can create the stable complexes with 3CLpro and RdRp proteins of SARS-CoV-2 coronavirus and, thus, suppress its functional activity.

Keywords

Water-soluble pristine C_{60} fullerene · Coronaviruses · Anticoronavirus activity · *in vitro* and *in silico* screening

Abbreviations

BHK-21	Kidney cells of a newborn Syrian hamster (Baby hamster kidney cells)
C_{60}FAS	C_{60} fullerene aqueous colloidal solution
3CLpro	3-chymotrypsin-like protease
RdRp	RNA-dependent RNA polymerase
SARS-CoV-2	Severe acute respiratory syndrome-related coronavirus 2
TGEV	Transmissible gastroenteritis virus of swine

10.1 Introduction

The COVID-19 outbreak and the need for nanostructure testing. Outbreaks of infectious diseases are a threat that humanity is facing more and more in the circumstances of globalization. Due to the high mobility of the population and well-developed transport connections, especially dangerous infectious diseases spread quite quickly. At present, viral infections are the predominant part of infectious pathology in humans and animals. Every year they lead to significant losses in almost all age and social groups and impact the livestock industry. The current COVID-19 pandemic is caused by the new coronavirus SARS-CoV-2 which belongs to the same group as the well-known SARS and MERS coronaviruses—the causative agents of atypical pneumonia and Middle Eastern fever.

The development of the infection process, including coronavirus infections, is the result of a long interaction between two biological systems—the host and the parasite (virus). As it is known, this interaction largely depends on the type, strain, structure, state of the virus, cells, and the organism as a whole, which leads to a variety of relationships between the host organism (cell) and the virus and affects the clinical manifestations and pathogenesis of the disease. Pathogenesis of viral diseases begins at the cellular level. The cell is a dynamic system, which is characterized by a continuous and consistent course of intracellular processes. The intensity of these processes under the influence of pathogens is rapidly changing, which leads to various consequences. If we consider the pathogenesis of coronavirus infections, we should bear in mind that the Coronaviridae family contains similar, but not identical viruses. They are complex in their antigenic structure. Antigenic heterogeneity

determines the high frequency of reinfections by other coronaviridae serological variants. Prior to the SARS-CoV outbreak, coronaviruses were considered to be only minor pathogens causing respiratory infections in mild human beings. Coronaviruses affecting humans belong to the following groups: α-coronaviruses (HCoV-229E; HCoV-OC43; and human coronavirus NL63) and β-coronaviruses (HCoV-NL63 (respiratory tract) HCoV-HKU1 (respiratory tract, gastroenteritis possible); SARS, MERS, SARS-CoV-2 (NovCo-19).

Coronaviruses are the largest group of viruses belonging to the order *Nidovirales*. They are divided into four groups: α, β, γ and Δ. A characteristic feature of coronaviruses is their wide range of natural hosts in combination with a pronounced species-specific pathogenicity. By their ability to infect various organs, coronaviruses can be classified as pantropic viruses that infect the epithelium of the intestinal mucosa, respiratory tract, and nerve cells, causing liver, enterotropic, pneumotropic, and neurotropic diseases of varying severity. When comparing the coronaviruses of animals belonging to different groups (transmissible gastroenteritis virus of swine (TGEV), bovine virus – BCoV, and virus of chicken infectious bronchitis – IBV), it was found that α- and β-coronaviruses (TGEV and BCoV) form clusters of particles with a double membrane sometimes due to the tortuous shape of the membrane and is quite conservative among coronaviruses. At the same time, γ-coronavirus IBV induces wide paired membranes and smaller 60–80 nm spheres in addition to double membrane. With the exception of the phylogeny of the coronaviruses studied, the only known characteristic that distinguishes swine (TGEV) and bovine (BCoV) viruses is their unique nonstructural protein 1 (nsp1), which differs in size and sequence as well as the absence of nsp1 in IBV. In addition, homologies of the BCoV genome, compared to IBV and TGEV, indicate only 48.3% and 53% similarity, respectively. Therefore, research on the development of new antiviral drugs should be based on models of coronaviruses belonging to different groups. It is known that due to the unique mechanism of viral replication, coronaviruses have a high tendency to recombine and possess elevated mutation rates that potentially increase the possibility of their adaptation to new hosts and ecological niches and it must be taken into account when modeling the experiment.

Outbreaks of SARS, MERS, and Covid-19 stimulated the study of their pathogens and revealed a large number of relevant antiviral targets, including viral proteases, polymerases, and internal proteins of coronaviruses. It is believed that the ciliated and goblet epithelial cells that cover the nasal cavity are the main targets of the SARS-CoV-2 virus in humans. These cells contain high levels of ACE2 and TMPRSS2 proteins that allow viral attachment by specific envelope proteins. Upon infection, the protein spike on the surface of the SARS-CoV-2 virion also attaches to the receptor protein of human lung cells, in particular ACE2, and penetrates into the cells of the tissue. SARS-CoV viruses (2002–2003) and SARS-CoV-2 (2020) are able to penetrate macrophages and dendritic cells, but in this case, only abortive infection is observed. Patients with COVID-19 show an abnormal decrease in the number of immunocompetent cells and suppression of their functional activity. Therefore, the problem of neutralizing the activity of the coronavirus requires a detailed study of its structural organization—especially components involved in the interaction with target cells of various human organs (binding and penetration) and mechanisms of virus replication in the target cell. With such important information, it is possible to develop effective preventive and therapeutic drugs which will prevent coronavirus entry into target cells or its reproduction in them. In addition, it is possible to identify structural elements of the coronavirus which best elicit an immune response forming the basis for the development of effective antiviral vaccines.

One of the urgent problems of modern veterinary biotechnology is to solve the complex task that lies at the intersection of chemistry, physics, materials science, biology, veterinary medicine is focused design, synthesis, and study of the functional properties of nanomaterials which is characterized by high bioavailability and biocompatibility, low toxicity, and high specific

biological activity. Modern nanobiotechnologies can address the many clinical and social health challenges posed by the COVID-19 pandemic. So, they actively promote the development of antiviral nanocoatings to limit the action of coronaviruses through air filters and protective masks (based on nanofibers) that prevent inhalation of viruses, as well as fundamentally new diagnostic test systems and targeted nanoplatforms for the delivery of antiviral drugs. Finally, they offer new therapeutic approaches using biocompatible nanostructures with "specific" antiviral activity.

About the nanostructure. Therefore, the C_{60} fullerene is an attractive nanosized molecule (Kraevaya et al. 2020; Klimova et al. 2020). Due to the presence of double chemical electron-deficient bonds in the structure, C_{60} fullerene easily attaches to free radicals, and thus is a powerful antioxidant (Gharbi et al. 2005; Ferreira et al. 2018). In addition, this typical nanostructure of carbon atoms in sp^2-hybridization exhibits prooxidant properties by photoexcitation with UV-Vis light, i.e., it is able to produce reactive oxygen species (Grebinyk et al. 2018). With special treatment, C_{60} fullerene forms stable colloidal solutions (Mchedlov-Petrossyan, 2013), that expands its use for biomedical purposes. Due to its hydrophobicity, C_{60} fullerene easily penetrates the biological membrane by passive diffusion or endocytosis (Franskevych et al. 2017), is nontoxic in *in vitro* and *in vivo* systems, at least in the low concentration and dose ranges (Prylutska et al. 2019; Singla et al. 2019). It is established that C_{60} fullerenes and their derivatives show high biological activity in *in vitro* and *in vivo* systems, in particular, anti-inflammatory, antibacterial, antitumor, neuro- and radioprotective effects. They can also serve as enzyme inhibitors, drug delivery vectors, contrast agents for MRT and photodynamic therapy, and most importantly, C_{60} fullerenes show antiviral and immunomodulatory effects (Goodarzi et al. 2017; Moussa, 2018). Earlier we have first shown that water-soluble pristine C_{60} fullerenes at low concentrations (10 μg/ml) under the action of visible light for 30 min interact with virions of mosquito iridovirus in larvae of the large wax moth *Galleria mellonella* and reduce its infectious titer by 4.5 lg ID_{50}/ml (Rud et al. 2012).

Therefore, the goal of this work was to conduct the model (*in silico* screening) and experimental (*in vitro* screening) studies of water-soluble pristine C_{60} fullerenes as potential therapeutic agents against the most common coronaviruses. These biocompatible carbon nanoparticles can be used as promising mono-agents for effective anti-coronavirus therapy, and as components of highly effective pharmaceuticals used for the prevention and treatment of infectious diseases.

10.1.1 Nanomaterial

C_{60} fullerene aqueous colloidal solution (C_{60}FAS) with a maximum concentration of 150 μg/ml was prepared using the original ultrasonic technology (Ritter et al. 2015). It is known that the size of water-soluble particles of C_{60} fullerenes directly correlates with the manifestation of their specific biological activity (Goodarzi et al. 2017; Moussa, 2018). Therefore, the shape and size of the particles present in C_{60}FAS of given concentration, as well as C_{60}FAS stability (value of zeta potential) were monitored using microscopic (STM, scanning tunneling microscopy, and AFM, atomic force microscopy) and spectroscopic (DLS, dynamic light scattering) methods.

The study of C_{60} fullerene films deposited from C_{60}FAS revealed a high degree of molecules dispersion in solution. It turned out that used C_{60}FAS contains both single C_{60} fullerene and its labile spherical-like nanoaggregates with size of 1.3–100 nm. The majority of C_{60} molecules were located chaotically and separately along the surface (see the dotted objects with a height of ~0.7 nm in Fig. 10.1), or in the form of bulk clusters consisting of several tens C_{60} molecules (Prilutski et al. 1998) (objects with a height of 1.3–2 nm in Fig. 10.1). Such arrangement of C_{60} molecules formed because of electrostatic repulsion between them: the zeta potential value was −25.7 mV at room temperature, indicating a high solute stabilization.

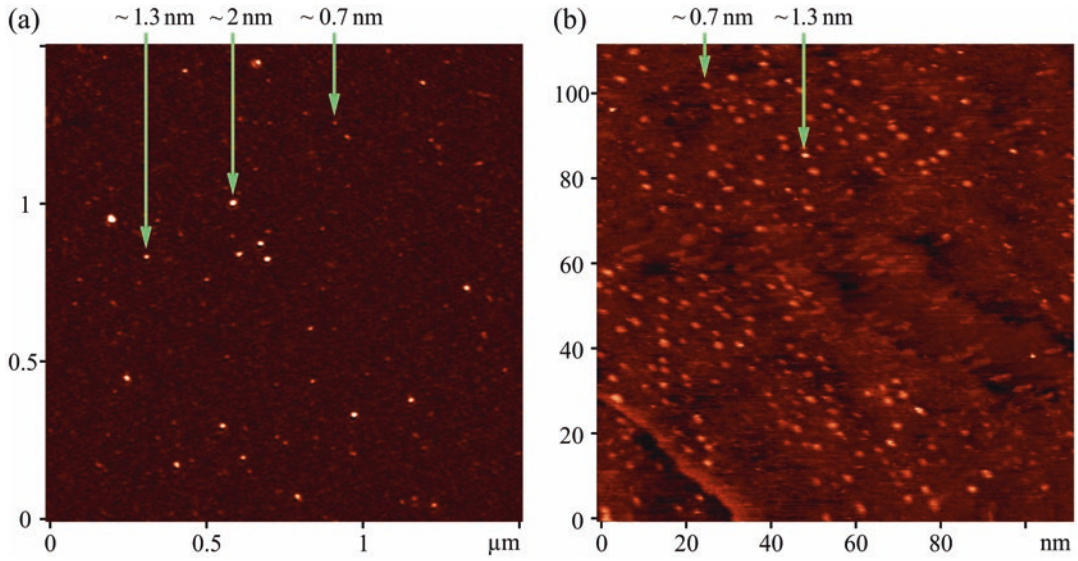

Fig. 10.1 AFM (**a**) and STM (**b**) images of the C_{60} fullerene layer. Numbers with arrows show the height of nano-objects (a concentration of C_{60} fullerenes in C_{60}FAS corresponds to 37.5 μg/ml)

10.1.2 *In Vitro* Experiment

As a model, apathogenic for human coronavirus, we used TGEV, namely, epizootic isolate "Chuguev-02-55", which later we adapted to the BHK-21 cell culture (kidney cells of a newborn Syrian hamster). The virus was stored in a special container in a freezing chamber at −70 °C.

Recall that infectious swine gastroenteritis is viral gastroenteritis, transmissible gastroenteritis (TGE), Doyle and Hutchings disease (Doyle and Hutchings 1946). TGE is an acute high-contagious disease that occurs mainly in 2-week-old piglets and is characterized by catarrhal hemorrhagic gastroenteritis (Wood 1979). TGEV is antigenically related to hemagglutinous coronavirus, which causes encephalomyelitis in piglets, as well as dog coronavirus and coronavirus, which causes infectious peritonitis in cats. It agglutinates the red blood cells of cattle, guinea pigs, and chickens. TGE agent is epitheliotropic, multiplying and accumulating in epithelial cells of the small intestine, alveolar macrophages of the lungs and tonsils (Laude et al. 1984). TGEV adapts and multiplies easily in the cytoplasm of the primary and continuous cells of pig organs. Its incubation period, depending on the age of the piglets, lasts from 12 to 36 h. As animals age, the incubation period increases and the mortality rate decreases. In pigs, the TGEV enters mainly through the fecal-oral route, passing through the stomach, into the intestines (Mayer et al. 1984). After a few hours, a large amount of the virus accumulates in the lumen of the intestine, from where it enters the blood and all internal organs. In the epithelium of the lungs, there is a secondary cycle of virus reproduction, which leads to significant damage to alveolar macrophages and the epithelium of the lungs. Disease begins with a decrease in appetite, oppression, and sleepiness. Patients of piglets have increased thirst, vomiting, and body temperature is 41–41.5 °C. The clinical period of the disease is characterized by occupational diarrhea with the release of gases. The mortality rate among piglets of 2–3 weeks of age can reach 100% (Ditchfield and Pearce 1967).

All manipulations related to BHK-21 cell culture were performed in the Bio II Advance laminar box (Telstar, Spain). Cell incubation was carried out in Memmert CO_2 incubator (Germany) at 37 °C, 5% carbon dioxide, and 88% air humidity. DMEM nutrient media with sodium pyruvate, L-glutamine and high glucose content (PAA and

HyClone), embryonic calf serum (PAA and HyClone), Hanks solution, 0.25% trypsin solution, and 0.02% Versen solution ("Bio-Test Laboratory") were used. Monolayer cells were grown in plastic culture vials with ventilation lids of 50 and 250 cm^3 (Sarstedt, Nest, and Orange) and in 96-well culture flat-bottomed plates (Sarstedt and Nest). When crossing, the cell culture was washed several times with Versen solution, a mixture of trypsin and Versen solutions (1:3) was added and left for six min in a thermostat at 37 °C. In the process of contact with reagents, the cells began to round off, lose contact with other cells and the substrate to which they were attached, and were separated. After that, a nutrient medium was added to the cell and solution suspension, which contained 10% of cattle embryonic serum heated in advance at 56 °C for one hour. Then the cell suspension was intensively resuspended, the number of cells was counted and poured into vials or plates at a sowing concentration of 200,000 cells/cm^3.

TGEV was passaged in BHK-21 cell culture during 24–48 h at 37 °C. Its cytopathic effect was determined with the help of a Leica DM 750 microscope (Switzerland) and the degree of monolayer changes in cells was assessed, in particular, rounding, destruction, and separation of cells from culture dishes were observed. After detecting the cytopathic action of coronavirus, the obtained material was frozen/thawed and centrifuged three times at 3000 rpm for 15 min.

TGEV titration was performed by the generally accepted method of final dilutions in BHK-21 cell culture in 96-well flat-bottomed plates (final concentration 200,000 cells/cm^3), its cytopathic effect was observed and 50% tissue cytopathic dose (TCD_{50}/cm^3) was determined. To determine this value, we prepared a series of 10-fold dilutions of the virus on the nutrient medium for cell cultures. Each dilution of 150 µl virus infected cells in four wells and incubated in a CO_2 incubator at 37 °C for 4 days. The results of cytopathic action were taken into account every day and the TGEV titer was calculated using the Reed and Mench method (Reed and Muench 1938).

For electronic microscopy, the virus-containing suspension was preliminarily concentrated using PEG-6000 (polyethylene glycol). For cell detritus deposition the virus-containing suspension was centrifuged 7 min at 100 g. To the supernatant suspension 8% PEG with pH 7.4 was added and left for 16 h at +4 °C. Then the mixture was centrifuged for 20 min at 16,000 g, after which the sludge was dissolved in a phosphate buffer 1/100 of the initial volume of the viral suspension. Electron microscopy of the viral suspension was performed at the M.G. Kholodny Institute of Botany of NAS of Ukraine on a transmission electron microscope (TEM; JEM-1230, Japan) with a resolution of 0.2 nm and a magnification range of 50–600,000. TEM was conducted to confirm the presence of the TGEV. Fixation of the material was carried out with 3% glutaraldehyde at 0.1 M of sodium-cacodylate buffer at pH 7.2, left for 3 h, after vacuum infiltration was washed with sodium-cacodylate buffer two times for 15 min. Additionally we fixed (postfixation) with four osmium oxide which simultaneously stained cell structures. It was left for 1 h at room temperature, and then—for 14 h at 4 °C. After that it was washed with a buffer and dehydrated with ethyl alcohol in increasing concentration (30° – 50° – 70° – 80° – 96°), adding drops of uranium nitrate. Then dehydrated with 100% ethyl alcohol, 100% acetone, and 100% alcohol in a ratio of 1:1. The dehydrated material was poured with synthetic epoxy resins: 5 drops of DDSA, 3 drops of Epon-812, 2 drops of Araldite®, 10 drops of DMP. Cuts of the investigated material were carried out on a special device—an ultratome—using glass knives. The thickness of the obtained ultra-thin cuts was 30–50 nm. Painting (contrasting) slices were performed with heavy metal salts (lead, osmium, uranium, etc.), which are differently associated with individual structural components, giving them different electronic density. Painted ultrafine slices were placed on a metal substrate and studied with TEM.

After introduction of the virus-containing material to pigs (in biological probe), they got sick with all basic clinical signs of TGE. In 20–24 h after infection, one of the first symptoms

of the disease in animals was vomiting, then developed profiled diarrhea with release of yellow-green feces, clots of coagulated milk. There was also a strong thirst and lack of appetite in animals. Their skin acquired a dark grey color. Body temperature at the beginning of the disease was 38.5–39 °C, and before death it dropped to 36–37 °C. The piglets were knocked down and died 48–60 h after infection. The main pathological and anatomical changes were observed in the stomach and small intestine: there were hemorrhages in the stomach, fibrinous inflammation; the intestine was dilated, overflowing with liquid and semi-liquid content of yellow, its wall was soft, flabby; mucous membrane of the small intestine was swollen, hyperemic, covered with cloudy mucus, its folds were dotted with spot hemorrhages. Animals that were in contact were also sick with signs of TGE, but 18–20 h later than the studied pigs. Control animals were left without signs of disease.

TGEV was isolated from the pathological material of animals obtained in the bioassay (small intestine) and further used for consecutive passages both in sensitive animals and in adaptation to the culture of BHK-21 cells. This allowed to study in detail the morphological structure of the virus, its morphogenesis in an infected cell, and to search for candidates for antiviral drugs, in particular, to test the action of water-soluble pristine C_{60} fullerene nanoparticles.

Coronavirus, the causative agent of TGE, was typified in the neutralization reaction and studied by TEM (Figs. 10.2 and 10.3).

It was found that at 55 passages in the cell culture of BHK-21 (Fig. 10.4), the titer of infectious activity of TGEV was 7.50 ± 0.22 lg TCD_{50}/cm^3 ($p < 0.05$). Cell lysis was observed under the influence of coronavirus; mass yield of viral particles into intercellular space was accompanied by maximum infectious activity (Fig. 10.5).

Since, together with the broad perspective of using C_{60} fullerene for the prevention and treatment of various diseases (Kepley 2012; Zhang et al. 2019) there are potential risks of their toxic impact on biological objects (Singla et al. 2019), in the beginning we investigated the cytotoxic effect of water-soluble pristine

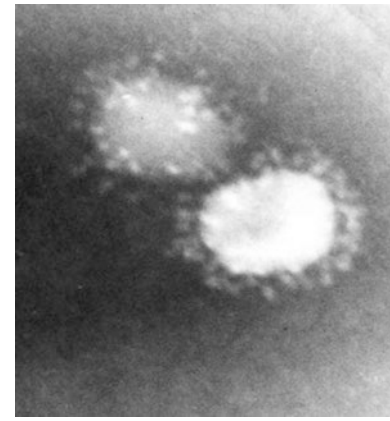

Fig. 10.2 Two coronavirus virions of TGE (2% treatment with aqueous solution of molybdenum ammonium acid with pH 6.55; magnification ×540,000)

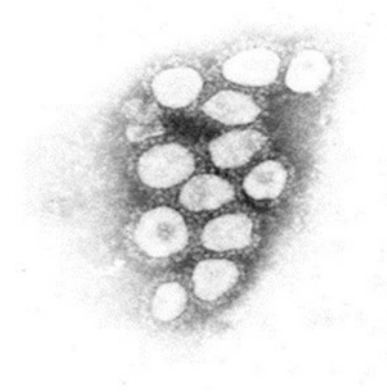

Fig. 10.3 TGEV, field isolate (treatment with 4% aqueous solution of phosphorus tungsten acid with pH 6.55; magnification ×180,000)

C_{60} fullerenes in BHK-21 cell culture. In experiments, we used at least ten repetitions (in tablets with cell culture) for each concentration of nanocompound (150; 75; 37.5; 19; 9; 5 μg/ml). Tablets with cell culture were incubated for 96 h at +37 °C in a CO_2 incubator with 5% CO_2 content. The maximum allowable cytotoxic concentration of C_{60} fullerene was determined, which was 37.5 ± 3.0 μg/ml ($p < 0.05$).

It is important to note that C_{60}FAS is a relatively safe substance: *in vitro* and *in vivo* experiments proved the absence of toxic effect of C_{60} fullerene in low concentrations (3.6–144 μg/ml)

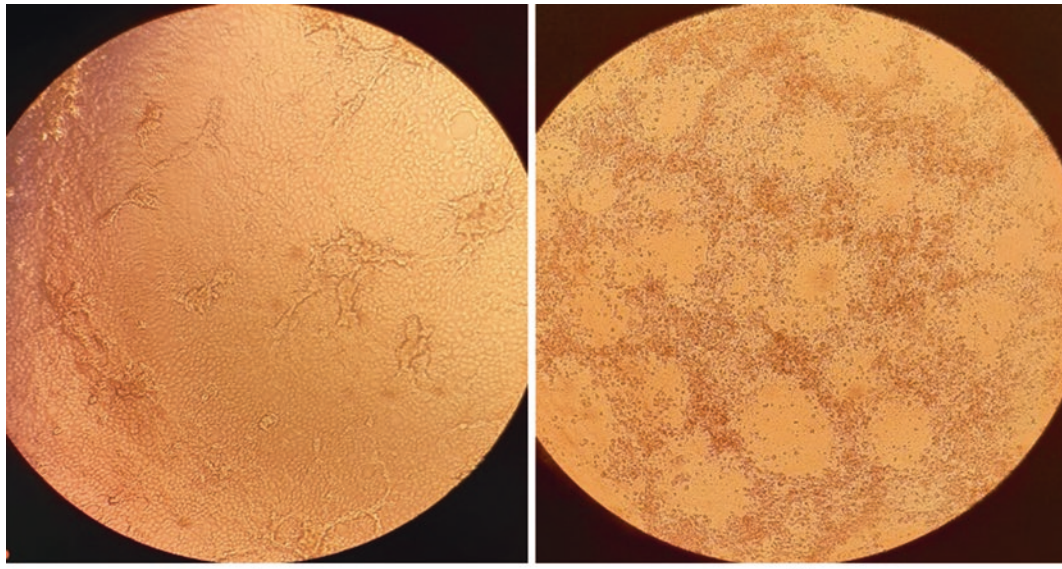

Fig. 10.4 BHK-21 cells: (**a**) control; (**b**) cytopathic action of TGEV

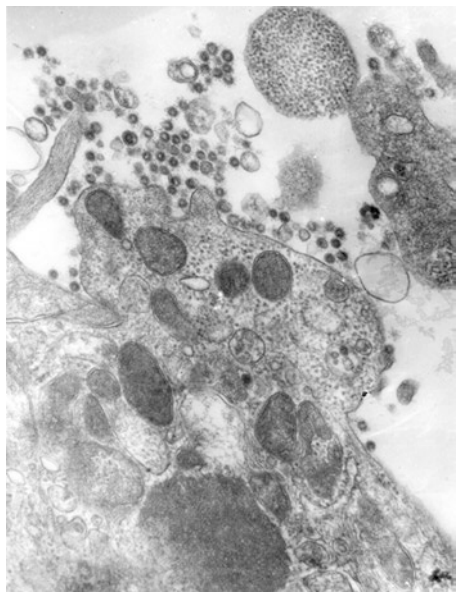

Fig. 10.5 TGEV in extracellular space after release from cell (enterocyte) (magnification ×165,000)

and doses (75–150 mg/kg), respectively, using it in the form of an aqueous colloidal solution and found that IC_{50} and LD_{50} values are 383.4 μg/ml for human embryonic kidney cells of the HEK293 line and 721 mg/kg of mouse weight, respectively (Prylutska et al. 2019).

The next stage of the *in vitro* study was to detect the antiviral activity of C_{60} fullerene using TGEV as a biological model of the coronavirus in BHK-21 cell culture. It turned out that the investigated nanosubstance reduces the titer of coronavirus infectious activity by the value of 2.00 ± 0.08 $TCID_{50}$/ml ($n = 5$; $p < 0.05$). Therefore, the obtained result allows recommending water-soluble pristine C_{60} fullerenes for further preclinical studies in the field of anticoronavirus therapy.

It is important to emphasize that all of the above biostudies were conducted in a laboratory with a biosafety level of BSL-2.

10.1.3 Model Experiment

One can assume that the inhibiting the functional activity of the studied SARS-CoV-2 coronavirus can be realized through the following molecular mechanisms of action of water-soluble C_{60} fullerene: (1) C_{60} fullerene accumulates in the coronavirus membrane, forms pores in its shell, thus violating the integrity of the structure, that leads to the violation of the membrane-dependent stages of the cycle of virus replication; (2) C_{60}

fullerene interacts directly with coronavirus proteins, such as RdRp (RNA-dependent RNA polymerase; plays a key role in viral RNA synthesis) or 3CLpro (3-chymotrypsin-like protease is actively involved in causing mutations Nsps (non-structural proteins), that is critical for the life cycle of the coronavirus) (Wua et al. 2020) and thus inhibits its functional activity; (3) C_{60} fullerene blocks the penetration of the coronavirus into the cell by directly interacting with its structural components, in particular S-crown glycoproteins, or the surface protein of host cells, such as ACE2 or TMPRSS2. The potential implementation of at least one of these types of blockages will prevent the formation of, for example, S-glycoprotein-ACE2 complex and, thus, prevent the penetration of coronavirus into the cell (Wang et al. 2020). Note that since water-soluble C_{60} fullerene is able to penetrate inside the cell (Grebinyk et al. 2018; Prylutska et al. 2019), mechanisms (1) and (2) can be implemented in both extracellular and intracellular spaces. Thus, based on the above assumptions, the search for and design of optimal possible complexes between C_{60} fullerene and target proteins in the structure of SARS-CoV-2 coronavirus, evaluation of their stability in the simulated cellular environment were performed using molecular dynamics (MD) and docking methods (Melnyk et al. 2019).

Here, based on available structure data (https://www.rcsb.org; Yin et al. 2020) as targets for molecular docking and MD simulations of SARS-CoV-2, RdRp (PDB ID 7BV2) and 3CLpro (PDB ID 6M2N) structures were selected. Before molecular docking simulation, to determine possible binding pockets for C_{60} fullerene, literature and structure analysis of investigated proteins were performed. As a result, three and one pockets were detected for RdRp and 3CLpro, respectively (Figs. 10.6, 10.7 and 10.8). First binding pocket for RdRp locates in RNA synthesis channel (Wua et al. 2020), and according to the fact that without the assistance of Nsp7 and Nsp8 as co-factors RdRp can't function (Kirchdoerfer and Ward 2019), two other pockets are in the RdRp-Nsp8 binding interface (Fig. 10.6). In a case of 3CLpro, the active pocket is located in the space between two domains (Kirchdoerfer and Ward 2019; Qamar et al. 2020). This space contains a catalytic dyad (Cys145 and His41) (Wua et al. 2020), which must be inhibited to prevent 3CLpro correct functionality. Molecular docking simulation was carried out by utilizing a flexible C_{60} fullerene structure and rigid protein molecule. An algorithm of systematic docking (SDOCK+) built-in QXP docking software was used (Warren et al. 2006). The number of SDOCK+ steps was set to 300, and the one best complex to each explored model according to the integrated QXP scoring function (the number of hydrogen bonds, the RdRp/3CLpro-C_{60} fullerene contact surface area, and the distance between the ligand and the key points of the corresponding pharmacophore model) (McMartin and Bohacek 1997) were selected for further analysis.

As a result of molecular docking, it has found that C_{60} fullerene can inhibit catalytic dyad. In our binding model, C_{60} fullerene tightly stuck near the catalytic dyad and shielded it. Furthermore, a few more important interactions in this potential complex were identified, namely, the stacking interactions with His 41, Cys 145, Met 49, Met 165, and some steric interactions with Met 49, Gln 189, and Asn 142 (Fig. 10.7).

As in a previous case, C_{60} fullerene filled out the whole selected potential binding pockets of RdRp and fixed there by different stacking and steric interactions (Fig. 10.8a, c, e). In pocket 1 (Figs. 10.6 and 10.8a), by interaction with C_{60} fullerene, it is possible to prevent RNA placement in RNA synthesis channel. Here, C_{60} fullerene creates π-caton interactions with Arg 570 and Lys 578, T-stacking with Tyr 690 and steric interactions with Asn 497 and Leu 577. On the other hand, C_{60} fullerene interacting with pocket 2 or 3, as it was mentioned above, makes impossible the complex formation between RdRp and Nsp8 (Figs. 10.6 and 10.8c, e). As a result, RdRp is not able to carry out its initial functions. In pocket 2, C_{60} fullerene clamped between Trp 510 and Phe 369 by stacking interactions, and between Leu 372 and Leu 515 by steric interactions. Tyr 516 and Phe 507 are located at the bottom of the binding pocket (Fig. 10.8c). Those two amino acids

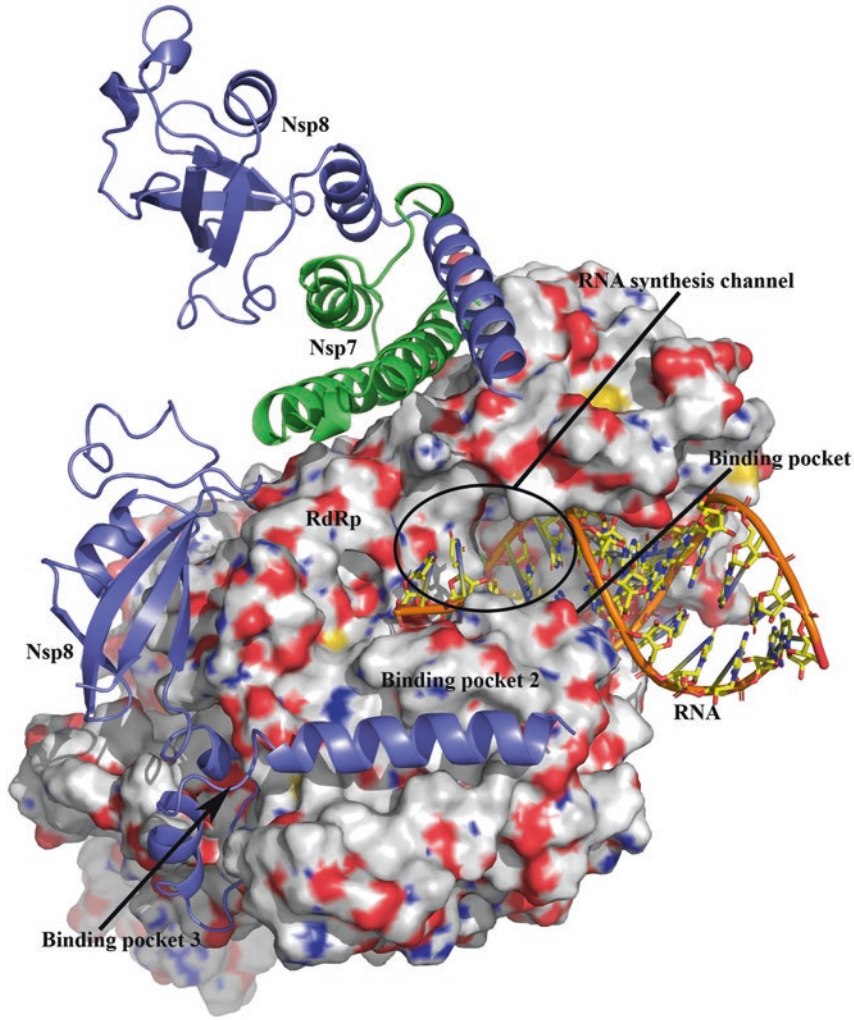

Fig. 10.6 Structure of SARS-CoV 2 RdRp (surface presentation) in complex with Nsp7 (in green) and Nsp8 (in purple) co-factors, and RNA molecule (stick model). In one case, RdRp directly interacts with Nsp8; in another, it binds to heterodimer of Nsp7 and Nsp8. Pocket 1 locates in RNA synthesis channel, pockets 2 and 3 locate in direct binding interface between RdRp and Nsp8

are able to hold C_{60} fullerene in the current position during MD simulation by stacking interactions. Pocket 3 is located deep inside RdRp structure (Figs. 10.6 and 10.8e). Despite the fact that in this pocket almost not presents any aromatic amino acid (to create stacking interactions with C_{60} fullerene), we think that pocket is promising because of its depth. Here, C_{60} fullerene locates among Ala 384, Val 331, Val 399, Thr 325, and Leu 271. Although it should be noted that binding pocket comprises Phe 397 and Tyr 274 and there is a possibility of stacking interaction with those amino acids.

Next, MD simulation was used to evaluate the stability of obtained complexes. The calculations were done by applying Gromacs 2020 software tool (http://www.gromacs.org) in force field Charmm36 (Huang and MacKerell 2013). Protein structures were protonated in accordance to build-in function in Gromacs 2020. C_{60} fullerene topology was generated by SwissParam (Zoete et al. 2011). Each investigated system was set in

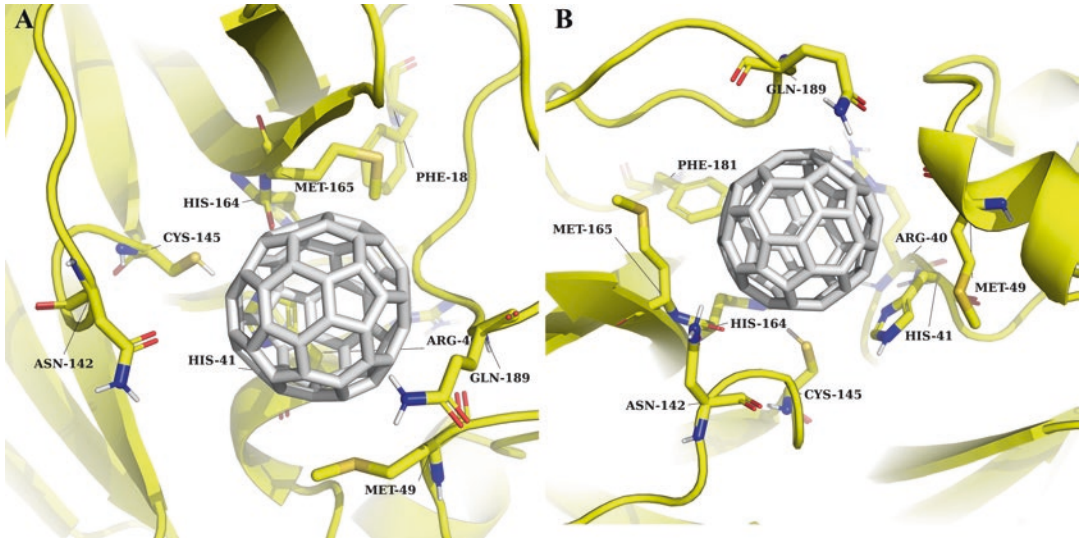

Fig. 10.7 Catalytic binding pocket of 3CLpro (3CLpro highlighted in yellow, and C_{60} fullerene in grey): molecular docking result (**a**); MD simulation result (**b**)

the center of the periodic cubic box. The minimum distance between the complex and the edge of the box was 0.9 nm, so that complex is able to freely rotate and immerse within the environment. Then box with investigated complex was filled with TIP3P water molecules and Na^+ and Cl^- ions were added to bring the ionic concentration to 150 mM (mimicking the cellular environment). In the system, solvent molecules were randomly replaced with monoatomic ions. Then, energy minimization (to relieve any steric clashes) and two steps of equilibration (NVT, 100 ps; NPT, 1 ns) were performed. And finally, MD simulation within 50 ns was launched. All calculations were done at a temperature of 300 K and constant atmospheric pressure.

The MD results showed that the complex of C_{60} fullerene with each explored target is stable. The Rmsd (Root mean square deviation) movement during MD simulation of each complex is in a range 2–3 Å. Furthermore, in some instances, C_{60} fullerene forms new, in our opinion, more profitable interactions.

In the catalytic pocket of 3CLpro, the C_{60} fullerene shifts by 3.2 Å. Asn 142 moved for 4.8 Å toward to C_{60} fullerene. But on the opposite side of pocket, C_{60} fullerene forced out Gln 189 for 1.8 Å. In the bottom part of the pocket, C_{60} fullerene stayed closer to Arg 40, and therefore there is a possibility of π-cation interaction between those two structures. For the interactions between C_{60} fullerene and Met 156, Phe 181, and His 164, no fundamental changes were detected. The most interesting is that C_{60} fullerene forced out both His 41 and Cys 145 (catalytic dyad) from their initial positions (Fig. 10.7b). Thus, the integrity of catalytic dyad is violated and without any doubts, it has negative impact on 3CLpro functionality.

During MD simulation for the C_{60} fullerene bind with pockets 1 and 2, it was detected that C_{60} fullerene slightly forces out from both pockets (4.1 Å and 3.5 Å, respectively) (Fig. 10.8b, e). The opposite effect was discovered for pocket 3: here C_{60} fullerene pushed inside the binding pocket by 4.0 Å. However, the key interactions between C_{60} fullerene and RdRp in whole models remain (Fig. 10.8b, e, f). For pocket 1 (Fig. 10.8b) almost no changes were determined. So, amino acids Ile 590, Tyr 690, Leu 577, and Gln 574 moderately changed the initial position (less than 0.75 Å). But despite that mentioned amino acids have strong steric interaction with C_{60} fullerene. On the other hand, Lys 578 and Arg 570 in comparison to other amino acids are characterized by large displacement (1.5 Å and 1.6 Å, respec-

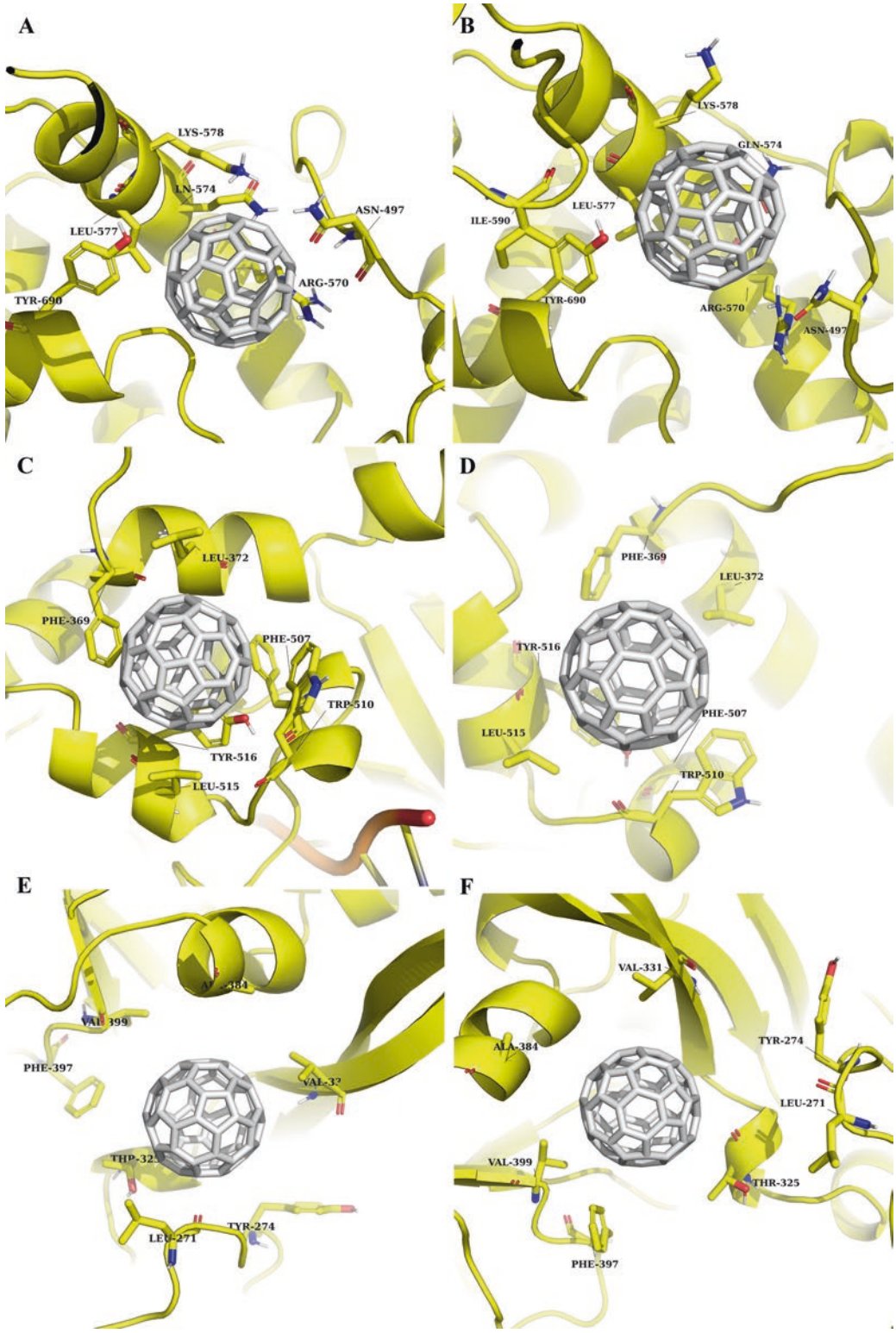

Fig. 10.8 Molecular docking (**a**, **c** and **e**) and MD simulation (**b**, **d** and **f**) results of complex between C_{60} fullerene (in grey) and investigated pockets of RdRp (in yellow): (**a** and **b**) – pocket 1; (**c** and **d**) – pocket 2; (**e** and **f**) – pocket 3

tively). Also, it should be noted that the π-cation bond with Lys 578 is not stable in contrast with Arg 570. Pocket 2 (Fig. 10.8e) is characterized by a stable position of whole key amino acids during MD simulation (the displacement is about 1 Å). In spite of C_{60} fullerene displacement, it sticks between Leu 372, Leu 515 (steric interactions), and Phe 369, Trp 510 (stacking interactions). Finally, a reversed situation compared to the above was observed in pocket 3 (Fig. 10.8f): C_{60} fullerene shifted inside the binding pocket and tightly stuck among the surrounding hydrophobic amino acids. C_{60} fullerene creates stacking with Phe 397 and steric interactions with Val 399, Ala 384, Val 331, and Thr 325. Such interactions become possible due to huge shift of Phe 397 (4.9 Å), Val 399 (3.5 Å), and Ala 384 (3.2 Å); other amino acids, Val 331 and Thr 235, located in the pocket 3, were almost not shifted (about 0.5 Å).

10.2 Conclusions

Pioneer *in vitro* studies to identify the anticoronavirus activity of water-soluble pristine C_{60} fullerenes with selected maximum allowable cytotoxic concentration in the kidney cell culture of a newborn Syrian hamster (BHK-21) 37.5 μg/ml indicate that they are safe and highly promising for further preclinical studies, since a significant inhibition of the infectious activity of swine coronavirus of transmissible gastroenteritis in BHK-21 cell culture was found, namely, by the value of 2.0 $TCID_{50}$/ml. According to molecular modeling results, it was shown that C_{60} fullerene can create the stable complexes with 3CLpro and RdRp proteins of SARS-CoV-2 coronavirus and, thus, suppress its functional activity. In both cases, C_{60} fullerene fills the binding pocket and gets stuck there through stacking and steric interactions.

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Conflict of Interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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11 Application of Nanoscale Materials and Nanotechnology Against Viral Infection: A Special Focus on Coronaviruses

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Abstract

Introduction: In recent years, viral infections and associated diseases have become a big challenge for humanity due to high morbidity rates globally. However, timely, accurate, and rapid detection of viral infection may lead to the control of morbidity as well as provide enough time for vaccine preparation and early antiviral therapy. Existing virus detection methods based on immunological and molecular diagnosis found drawbacks, such as its time-consuming and costly one. Recently, the introduction of nanomaterials having multiple unique properties with a series of smart and innovative nano-based technologies have been under investigation for rapid viral detection. This chapter aims to critically review recent literature to illustrate the encompassing applications of nano-engineered materials and further highlighting the role of their active surface in improving the virus detection with high sensitivity and detection range, and in a short time.

Methods: The authors review the research findings related to emerging nanotechnology-based virus detection systems and their applicability for diagnostics of infectious viruses.

Results: Recent advances in nanotechnology allow for the development of robust, rapid, and sensitive detection of infectious virus to overcome deficiencies of conventional detection technologies. Nanoparticles have several distinctive physical and chemical characteristics such as unique optical, electronic, and magnetic properties compared to their bulk form enabling them the detection of biological agents like viruses. Further, high surface area to volume ratios of nanoparticles also provides a platform for multi-functionalization with various organic or biological ligands for the selective binding and detection of biological targets like viruses. For instance, colloidal gold nanoparticle-based lateral-flow (AuNP-LF) provides rapid diagnosis and on-site diagnosis of SARS-CoV-2 virus via the IgM detection using the indirect immune-chromatography method.

Conclusion: The distinct properties of nanomaterials such as plasmon resonance absorption, conductivity, redox behavior, etc. along with surface functionalization might be used in the development of the nano-sensing system with high accuracy and rapid detection of infectious viral diagnosis at the point of care application.

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Keywords

Viral infection · Rapid sensing · Point of care · Nanoparticles

11.1 Introduction

According to WHO, nearly 17 million people die due to infectious diseases every year (WHO 2015). Such diseases are a leading cause of death worldwide, particularly in low-income countries, especially premature death in young children (Holmes et al. 2017). Infectious diseases are caused by microscopic agents or organisms, such as bacteria, viruses, fungi, parasites, that invade the host body, grow within, finally cause harm to the host's tissues, and get transmitted to other individuals directly or indirectly, from one person to another. Infectious diseases majorly cause five kinds of contagious agents, namely, bacteria, viruses, fungi, protozoa, and helminths. Among these, viral and bacterial infections are more predominant. However, the significant difference between viral and bacterial infections is that it is easier to control bacterial infection using antibiotic drugs, while viruses cannot be controlled by antibiotics. Viral infections can only be prevented with the availability of safe, effective, and affordable vaccines (Drexler 2014).

Moreover, viral-based diseases demonstrate increase in infection rate and death due to their extremely contagious nature and the lack of cheap and highly efficient control systems which is indeed an important reason for causing latent health impacts (Draz and Shafiee 2018). For instance, the Spanish flu pandemic from 1920 to the present COVID-19 have become a significant threat to the health care system. Spanish flu caused by H1N1 influenza resulted in infecting more than a quarter of the world's population, with nearly 30 million to 100 million deaths (Johnson and Mueller 2002). According to reports, contagious viral diseases like HIV, hepatitis, and influenza, causes almost 8 million human deaths every year. Besides, there is a resurgence of many new virulent types of viruses at an increasing pace due to the ability of viruses to change the genetic makeup rapidly through point mutation and recombination and threaten health care issues (Malik et al. 2017). Boniotti et al. (2016) have reported the detection of new porcine coronavirus detected during the porcine diarrhea virus outbreak that resulted due to genetic recombination of transmissible gastroenteritis virus and porcine epidemic diarrhea virus (Boniotti et al. 2016).

Viruses are holoparasitic microorganisms that require a host body for their proliferation as well as utilize host cell machinery to replicate their genetic material. Further, viruses have a complex-intrinsic defense mechanism against the host immune response that continuously changes very rapidly and helps the viruses adapt themselves for multiplying by modification of the host immune response. This ability of a complex-intrinsic mechanism of viruses to adapt quickly to the host defense system leads to the emergence of new-novel viruses capable of destabilizing the host immune system, thereby causing infections and death. Notably, the Ebola virus epidemic in 2014 and influenza A H1N1 subtype in 2009 are examples of such outbreaks that gained attention (Chan and Gack 2016). Since the discovery of the first enteric virus, Norovirus (Caliciviridae), in 1972 using the electron microscope, different types of viruses, such as Parechovirus, Bocavirus, and Aichivirus (Picornaviridae), Astrovirus (Astroviridae), Rotavirus (Reoviridae), enteric Adenovirus (Adenoviridae), Torovirus (Coronaviridae) Picobirnavirus (Picobirnaviridae), Sapovirus (Calciviridae), corona virus (COVID-19), and several others, have been identified as being related with gastroenteritis infections (Malik et al. 2019).

The respiratory system of the humans is the primary site of entry for several viruses for infection or invasion, including parainfluenza virus, influenza virus, and respiratory syncytial virus, etc. These virulent viruses initially invade the upper respiratory tract and then transmit to the lower respiratory tract leading to death. The lower respiratory tract infection is reported as one of the primary causes of nearly 3 million deaths annually worldwide (WHO 2016). Most of these virulent strains are airborne and transmit

as aerosols/droplets as well as by direct contact, thereby effectively proliferating in the respiratory tract and causing clinical manifestations such as pneumonia, cough, fever, bronchiolitis, and dyspnea (Kutter et al. 2018). Severe/acute respiratory syndrome (SARS), influenza virus, rhinovirus, and respiratory syncytial virus (RSV) have been reported as frequent respiratory viruses causing the majority of respiratory tract infections in the lower respiratory tract (Wong and Yuen 2008).

Furthermore, presently, respiratory viral infection has been the primary reason for the hospitalization of immune compromised patients, older people, infants, as well as children. It has been reported as nearly 40% and 45% of pediatric hospitalizations of children due to acute respiratory illness caused by Respiratory syncytial virus (RSV) (Mazur et al. 2018) and Parainfluenza viral infections (Branche and Falsey 2016), respectively. Besides these, adenovirus infections cause about 3~5% of the lower respiratory tract infection (Lu et al. 2013).

In 2003, Severe Acute Respiratory Syndrome (SARS) became a viral respiratory illness in the acute form of bronchopneumonia caused by a corona virus called SARS-associated corona virus (SARS-CoV). The primary emergence of the SARS outbreak for about eight months was driving a fatal outbreak rate of ~10%, causing around 8000 confirmed cases (Wong and Yuen 2008). Again in 2012, the Middle East respiratory syndrome (MERS) caused by a novel coronavirus (MERS-CoV) that was first identified in Saudi Arabia and became a universal outburst by transmitting to 27 countries (De Wit et al. 2016).

In 2019, there was an emergence of a new public health crisis that threatened the world by the spread of the severe acute respiratory syndrome corona virus 2 (SARS-CoV-2) or 2019 novel coronavirus (2019-nCOV) (Munster et al. 2020). Astoundingly, there is a rising infection of SARS-CoV-2 that has affected specifically the elderly population, as well as people having respiratory diseases, coronary heart disease or cardiovascular heart problems, diabetes, and hypertension. The infected patients with SARS-CoV-2 reported clinical symptoms such as acute respiratory distress syndrome (ARDS), septic shock, cytokine storm, and a metabolic coagulation dysfunction, finally leading to death (da Silveira et al. 2020; Ye et al. 2020). Now the world is facing a severe threat due to transmission of SARS-CoV-2 with more than 30 million positive cases so far, with nearly 1 million deaths, transmitted from China to more than 213 countries. Currently, several proposed antiviral agents and drugs are under clinical trials to test their possible effects against SARS-CoV-2. However, these proposed antiviral agents have critical issues such as side effects, drug availability, and pharmacokinetic properties that ought to be analyzed perceptively (Sportelli et al. 2020). Indeed, under circumstances of unavailability of effective antiviral drugs or particular treatments to contain pandemics like SARS-CoV infections, the only best and possible way to reduce such public health challenges to manage the viral infection is to prevent and control the pathogenicity of viruses. Then the focus should be on the development and improvement of an effective vaccine, which is the most effective way to control viral respiratory diseases.

Thus, the best possible strategy to contain the viral infection before the availability of an effective vaccine is to prevent the spread of infection using prompt diagnosis and isolation. Hence, there is a requirement for timely diagnostics with precision as a fundamental step for preventing the spread of viral illness on a provincial level, thereby further damage or mortality may be avoided. The accurate and rapid detection of such viral infection requires a biosensor having high sensitivity that is capable of detecting the target viral particles in body fluids even at relatively low concentrations in short duration, i.e., rapid processing time for ensuring timely treatment. However, timely diagnosis suffers due to limited availability of resources and essential medical personnel at the point-of-care setting leading to a significant challenge to the health care system for countries like India, which has a considerable population of about 1.3 billion.

Thus, there is an urgent need for simple and inexpensive diagnostic tools with high sensitivity that can be used at the point-of-care setting to

enable timely diagnosis of infectious diseases like SARS-CoV-2 and for containing them (Lien et al. 2011). However, most of the conventional viral assays like enzyme-linked immunosorbent assay (ELISA), plaque assay, real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR), hemagglutination, and endpoint dilution are unable to satisfy all the above requirements. For instance, enzyme-linked immunosorbent assay (ELISA), which is the most commonly used and established technique for viral detection, cannot be used at the point-of-care setting as it requires specific laboratory equipment, as well as sample preparation, takes nearly four hours or more, thereby making ELISA impractical for rapid diagnostics (Lien et al. 2011). Similarly, another vital technique for viral detection is a plague assay where contagious viral (Ammersbach and Bienzle 2011) samples have to be inoculated over a layer of host cells for several weeks observed for unique cytopathic effects. Although this plague assay is very sensitive, it suffers because it requires several weeks, making it unsuitable for timely rapid diagnosis (Ammersbach and Bienzle 2011). Hence, there is a requirement of simple and inexpensive diagnostic tools for point-of-care application to enable timely diagnosis for containing infectious diseases like SARS-CoV-2 to avoid epidemics and the increased spread of disease. These timely diagnosis kits must be capable of short, rapid, and precise detection of viral targets within a small trial volume.

Over the past decades, Nanomaterials have gained enormous attention in the field of biomedicine as disease diagnostics, drug delivery applications, as well as targeted therapeutics (Somu and Paul 2018, 2019a, b; Elahi et al. 2018). The recent advancement in nanotechnology enables the manipulation of nanoscale, its size, shape, and spatial organization at the nanoscale level for tuning optical, electrical, magnetic, as well as biological properties (Chen et al. 2014). The significant advantage of nanoparticles is their high surface area, which allows enhancing interactions with targeted particles as well as its surface can be easily functionalized for the selective or specific recognition required for bio sensing (Elghanian et al. 1997; De et al. 2009). These enable the application of nanomaterials and nanostructures for the design of new biosensors and bioelectronics with enhancing sensitivity for developing high-performance sensing systems for the target pathogen in body fluids such as viruses, genomic material, and complementary antibodies for health monitoring.

11.2 Prevailing Methods of Pathogen Detection and Their Drawbacks

The spread of SARS-CoV-2 is basically through respiratory droplets from people that harbor the infection. Though symptomatic individuals happen to be the origin point of the virus yet, a significant health risk lies in transmission from somewhat mildly ill or asymptomatic people during their incubation period. Quick diagnostic testing for SARS-CoV-2 is essential to recognize these people and carry out protective and preventive measures, such as social distancing, isolation, and segregation, that help avoid the spread of the infection to the surroundings. The advancement in rapid viral diagnostics distinguishes antibodies post-contamination giving an idea about an individual's exposure to condition and amount of viral load, which can be used to monitor the affecter's immunity and chances of re-infection (D'Cruz et al. 2020).

Viral respiratory tract infections (RTIs) have similar clinical symptoms as bacterial infections. Hence, it is essential to differentiate viral infection from bacterial and its other kinds. Sometimes uncertainty in diagnosis results in over-prescription of antibiotics and extra detection tests to rule out bacterial infections, adding to additional costs. Fast identification of viral pathogens could beat these drawbacks. Additionally, rapid viral detection tests prompt quick implementation of disease control measures, early consumption of the antiviral drug, if accessible, and shorter hospital stays, resulting in minimal health care costs (Bruning et al. 2017).

The earlier studies on viral antigen detection and isolation began in the early 1950s with the

development of cell culture methods and the invention of the electron microscope. For quite a long time, these procedures were the principal devices for examining and exploring the biochemical and morphological properties of pathogens and infections that formed the primary basis of all known classification and detection techniques. However, gradually, the use of these methods of infection identification has become debatable because of a few issues, including lab hardware costs, time of detection, and security concerns. In the mid-1980s, the field of diagnostics was supported with two other significant turns of events: (1) the introduction of different immunoassays and (2) the innovation of polymerase chain response (PCR). This was trailed by the advancement of a broad scope of serological and molecular identification methods, which quickly developed to establish the standard methodologies for both lab research and the clinical detection of infections (Draz and Shafiee 2018; Ellis and Zambon 2002).

11.3 Detection by Isolation: Cell Culture

It is one of the comprehensive known strategies for isolating pathogens utilizing cell lines. The cell lines vary depending on the type of infections (for instance, rhesus monkey kidney cells are being used for separation of Influenza A virus). Evidence of infection development is seen through the cytopathic impact (CPE), displaying explicit characteristics and modifications of the cells (Leland and Ginocchio 2007). Then viral pathogen was identified using Immunofluorescence (IF) staining. The limitation of virus detection by cell culture is not ideal if there is an occurrence of infections not manageable to develop in cell lines (norovirus, hepatitis infection) or delivering CPE (Papafragkou et al. 2014). Also, little volumes of the samples may not permit the injection of numerous cell types, and subsequently, affect the outcomes. The time required for detecting infections by cell culture is exceptionally long (weeks), restricting the value of this strategy when a fast diagnosis is needed.

11.4 Serological Methods for Detection

Serology remains the standard method for virus detection. It primarily relies on testing for the presence of specific viral antigens or the corresponding antibody responses of the immune system. The most common types of serological tests include the neutralization assay, complement-fixation test (CFT), immunoprecipitation assay (IPA), hemagglutination-inhibition (HAI) assay, enzyme immunoassay (EIA), radioimmunoassay (RIA), chemiluminescent immunoassay (CIA), particle agglutination, immunostaining, immunofluorescence assay, single radial hemolysis, immunoblotting assay (IBA), and immunochromatographic test (ICT).

11.5 Complement Fixation Test (CFT)

It is probably the oldest established strategy through the existence of clinical virology (Casals and Palacios 1941). The complement system attacks the antigen-antibody complex in a non-specific manner, and so in the presence of the complex, the RBCs remain unattached and hence remain intact. The test is considered to be 'positive' in the fact of intact RBCs (Casals and Palacios 1941). The limitation of CFT is the tedious nature and lack of sensitivity. Further, in-house standardizing through titration of the reactants preparing control is critical to obtain for effective testing (Bommana et al. 2019).

11.6 Hemagglutination-Inhibition Assay (HAI)

It is for viruses that hemagglutinate RBCs of some species, for example, vast numbers of the arthropod-borne infections, flu infections, and parainfluenza infections, HAI have been broadly

utilized. The objective of the test is simple—viruses attach to receptors on RBCs, while antiviral antibodies compete to bind these receptors and prevent hemagglutination (Souf 2016). The significant limitations of HAI are that *c*are ought to be taken in deciphering numerous earlier serostudies while analyzing results of HIA tests, especially for paramyxoviruses as non-specific inhibitors of agglutination for them, delivered some false-positive results in previous investigations (Comin et al. 2013).

11.7 IgM Class-Specific Antibody Assay

The most widely used method is the IgM antibody capture assay, in which the viral antigen is bound on a solid-phase substrate such as a microtiter well. The test serum containing IgM antibodies binds only to the compatible viral antigen and is further detected by labelled anti-IgM antibody matched to the species from which the specimen was obtained. The drawback of this method is that it is not appropriate for use in vaccinated organisms as the IgM reactions to that antigen has just happened in response to the immunization (Storch 2000).

11.8 Enzyme Immunoassay (EIA)—Enzyme-Linked Immunosorbent Assay (ELISA)

EIAs are a type of solid-phase immunoassays in which the antibody against the suspected viral antigen in the sample is attached to the wells of polystyrene or polyvinyl microtiter plates. Virus, as well as dissolvable viral antigens from the sample, are permitted to bind to the bound antibody. After unbound segments are washed away, the "indicator" enzyme-labelled antibody is added; different enzymes can be attached to the indicator; however, horseradish peroxidase and alkaline phosphatase are the most commonly utilized. After a washing step, a suitable substrate for the enzyme is added, and readout is carried out based on the color change that follows (Schuurs and van Weemen 1980).

Enzyme immune assay (EIA) is a comprehensive tool for diagnosing infections or pathogens. Its various applications include Enzyme immunosorbent assay (ELISA), micro-particle immune assay (MEIA), fluorescence polarization immune assay (FPIA), and chemiluminescent (CLIA) (Bramhachari et al. 2019). The limitation with this method is the inactivation of labelling of antibodies could lead to false, as well as labor-intensive and expensive, procedures.

11.9 PCR Based Detection

Real-time PCR is a modification of conventional PCR that uses a closed tube system, subsequently diminishing contamination rates, and do not require post PCR processing. It monitors the PCR products in "real-time" in contrast to end-point PCR, where amplicons are detected toward the end of the reaction. This results in the quantitation of nucleic acids, which is valuable in observing the movement of specific infections, for example, human immunodeficiency infection (HIV) and hepatitis C infection (HCV), and diminishes turn-around time since no post PCR preparation is required. As the reaction proceeds, fluorescence produced by the reporter molecule increases, this forms the basis of detection by real-time PCR (Josko 2010). The major limitation of Real-time PCR-based detection is that high mutation rates may prompt broad changes in viral nucleic acid sequences making devoted PCR primer use immaterial. Moreover, the process is costly and complicated due to thermal cycling and fluorescence detection co-occurring (Smith and Osborn 2009; Lassauniere et al. 2010).

11.10 Loop-Mediated Isothermal Amplification (LAMP) Based Detection

LAMP is an isothermal intensification technique utilized for distinguishing nucleotide sequences. Two sets of primers are being used on both inward and outward sides for flanking the target segment. Bst DNA polymerase (DNA polymerase obtained from *Bacillus stearothermophilus*) with high replication and strand displacement capacity is used for the amplification of the DNA (Bhadra et al. 2015). Thus, the LAMP reaction creates a mix of stem-loop DNAs of various sizes containing different loops. A positive amplification can be effectively observed either by gel electrophoresis or by estimating the amount of magnesium pyrophosphate (Mori et al. 2004). RNAs can likewise be amplified through LAMP by including a reverse transcription step along with the addition of different primers and is called RT-LAMP (Kabir 2018).

11.11 Nucleic Acid Sequence-Based Amplification (NASBA) Based Detection

In this process, the RNA sequence is amplified using RNA dependent DNA polymerase. At first, a DNA primer binds to the T7 promoter, which is further elongated by reverse transcriptase. The RNA is then excised from the RNA/cDNA hybrid by RNase-H, and the DNA is then amplified by reverse transcriptase by another primer. T7 polymerase integrates numerous duplicates of RNA from this DNA template (Ammersbach and Bienzle 2011). The technique was applied for the diagnosis of a few infections, for example, West Nile and St. Louis encephalitis viral infections (Lu et al. 2017). The limitation of both LAMP- and NASBA-based methods is that it is expensive; these tests are usually unaffordable for monumental testing purposes such as in health care departments.

Further, we have tabulated the various techniques used for viral detection as well as limitation in Table 11.1 as follows

11.12 Challenges and Limitations in SARS-CoV-2 Detection

The fast transmission rate of SARS-CoV-2 has made the requirement of a rapid diagnostic kit of utmost importance with easily accessible and minimum assets requirement for testing with acquiring accurate results. As such, the quick and accurate recognition of suspected cases using the rapid diagnostic kit is appropriately required to prevent community transmission (Webb et al. 2004). Consequently, numerous diagnostic tests for COVID-19 have been made accessible until now, with additionally gaining emergency approval every day (Younes et al. 2020).

However, in the above context, cell culture is not recommended for diagnostic purposes for SARS-CoV-2 (Tang et al. 2020). Since SARS-CoV-2 is delegated as a biosafety level-3 pathogen, the biosafety of cell culture and the accessibility of biosafety level-3 facilities raise the main concern. Additionally, the handling of specimens and interpretation of culture results and analysis through electron microscope requires an experienced research center staff, accordingly making them hard to be applied in ordinary clinical practice (Wu et al. 2020).

Concerning RT-PCR-based SARS-CoV-2 detection, the process is complex, costly, and time-consuming. An RT-PCR test unit may cost more than USD 100, while setting up a diagnostic/handling lab requires more than USD 15,000; moreover, the examination time is 4–6 h, and test to-result turnaround time is more than 24 h (Sheridan 2020). Moreover, the molecular diagnostics are not deliverable to end-clients and are expected distinctly for qualified clinical research facility staff and medium-or high-multifaceted nature labs. Many cases also reported false-negative results of RT-PCR diagnostics for COVID-19 (Huang et al. 2020a). Errors in RT-PCR results might be brought about by improper sample collection, stockpiling, move, refinement, and handling as well as the nature of the RNA extricated from the swabs likewise influences the outcomes. Different factors, for example, degradation of purified RNA, the presence of RT-PCR inhibitors, or genomic transfor-

Table 11.1 Methods of detections and their limitations

Method	Description	Detection	Limitations	References
Cell culture	Isolating viruses using cell lines.		Isolation of viruses using cell culture is time-consuming, avoiding its usefulness for rapid diagnosis.	Malik et al. (2017).
Complement fixation test (*CFT*)	The complement reacts with the antigen to form an antigen-antibody complex. This is indicative of the result to be positive.	Chlamydial infections, rabies, Japanese B encephalitis, Eastern equine encephalomyelitis, Western equine encephalomyelitis, St. Louis encephalitis, spontaneous encephalomyelitis of mice (Theiler's disease), and lymphocytic choriomeningitis.	It is labor-intensive and lacks sensitivity.	Boniotti et al. (2016) and Chan and Gack (2016)
HIA	The objective of the test is simple—viruses attach to receptors on RBCs, while antiviral antibodies compete to bind these receptors and prevent hemagglutination.	Arthropod-borne viruses, influenza viruses, and parainfluenza viruses.	Prior sero surveys need to be done before HIA. False-positive results due to non-specific inhibitors.	WHO (2016)
Single Radial hemolysis	Antibodies quantified by determining the area of hemolysis.	Leukemia virus; Vaccinia virus; Influenza virus, and *Bordetella pertussis.*	The results obtained do not distinguish between the antibody responses to different types of antigens.	Haaheim (1978)
IgM Class-Specific Antibody Assay	IgM antibodies in the sample sera bind to viral antigens fixed in the microtiter wells and detected by labelled anti-IgM antibodies.	Epstein-Barr virus (EBV) and parvovirus B19.	Due to cross-reactivity, false-positive IgM results. Immunosuppressed samples were found seronegative.	Miller et al. (2018)
Enzyme-Linked Immunosorbent Assay (ELISA)	The primary antibody is fixed in the microtiter well to which sample antigen is added. To it, either an enzyme-labelled secondary antibody is added and color developed with the substrate or direct Color formed on substrate addition.	Clostridium perfringens, staphylococcal enterotoxins, botulinum toxins, and enterotoxins from *E. coli*.	Labelling of antibodies could lead to their inactivation. A false signal was received due to the cross-reactivity of the secondary antibody involved. The construction of two antibodies makes it a labor-intensive and expensive procedure.	Lu et al. (2013)

(continued)

Table 11.1 (continued)

Method	Description	Detection	Limitations	References
Real-time PCR	The method differs from traditional PCR by the use of dsDNA binding reporter dyes and monitors the reaction products in real-time and not at the end of the process.	Respiratory syncytial virus, influenza viruses A and B, parainfluenza viruses, adenovirus, human corona viruses, human bocavirus, and human metapneumovirus.	Tragically, high mutation rates may prompt broad changes in viral nucleic acid sequences making devoted PCR primer use immaterial. It is costly and complicated due to thermal cycling and fluorescence detection occurring simultaneously.	da Silveira et al. (2020)
LAMP	Its principle lies in the use of four different primers to recognize six separate sequences of the target gene.	*Escherichia coli, Salmonella enteritidis, Salmonella typhimurium, Bacillus cereus,* Middle East respiratory syndrome coronavirus (MERS-CoV).	Designing primers is complicated. Difficult to detect unknown and unsequenced target.	Sportelli et al. (2020)
NASBA	This is primer-dependent process where continuous amplification of nucleic acids is carried out in a single mixture at one temperature.	Detection of enterotoxin related genes from Bacillus sp. in milk. West Nile and St. Louis encephalitis viral infections.	Needs the bacteria to be viable, requires the gene to be expressed, RNA handling might be troublesome.	Ammersbach and Bienzle (2011)

mations may cause false-negative outcomes. False-positive results, in this case, is also common due to cross-contamination of samples during assortment, pipetting, and technical errors. Despite the likelihood of these untoward occurrences, these diagnostics are at present the most exact and the most delicate accessible answers for the earliest and large-scale detection of SARS-CoV-2 (Afzal 2020).

Among the diagnostic kits approved for SARS-CoV-2 detection, antibody capture assay has been reported to be rapid, but have a few constraints due to difficulty in quick detection as a result of slow antibody response to SARS-CoV-2 infection. This makes the virus undetectable until three days from onset of symptoms or else 7–10 days after infection. Moreover, these tests cannot be relied on to detect infected individuals in the beginning phases of COVID-19. For instance, below 40% of diseased people are seropositive (IgM/IgA) in the initial seven days, making it inconsistent for finding out intensely infected individuals (Younes et al. 2020). Significantly, there have been reports that those with mild contamination of coronavirus don't deliver antibodies (Zhao et al. 2020a). Lateral flow assays for both IgM and IgG antibodies might assume a significant job in the COVID-19 outbreak by determining the load of infection, the contribution from asymptomatic infections, the basic proliferation number, and the general mortality rate. However, due to non-specificity and inconsistency in IgM reactions and the time taken by IgG reactions to occur after the initial onset of infections, serological diagnostic methods aren't probably going to assume a major role in active case management (Tang et al. 2020). Hence, there is a need to create fast, antigen-based COVID-19 tests for various reasons, one of them is to reduce or avoid the use of lengthy RT-PCR.

11.13 Nanomaterial-Based Detection System

Over the last few years, nanostructured materials such as quantum dots, metal nanoparticles, and magnetic nanoparticles have been used for sensing the presence of metal ions, small molecules such as protein and nucleic acid biomarkers. The field of nanotechnology capitalizes on the distinctive properties of materials at nanoscale form its bulk form (Wang 2005). The distinctive underlying properties of nanomaterials are critically linked with their size that made the fundamental basis for each nanoscale effect. The physical and chemical of material change with the dimensions and nanomaterials acquires distensive novel properties as well as existing properties also change entirely from that of bulk scale (Wang 2005). These unique attributes of nanomaterials make them promising synthetic scaffolds for the construction of novel chemical and biological sensors for the detection of chemical and biological components like viruses (Niemeyer 2001) as well as disease diagnosis and control (Prylutska et al. 2017; Shapoval et al. 2016). Further, nanoparticle's spectral and chemical properties can be easily modified by changing their size and shape as per the requirement for the specific applications in electronic, chemical, and biological areas like electro catalysis, redox reaction recognition, and electrochemical sensor devices for diagnosis (Wang 2005; Hu and Dong 2008). Besides, nanomaterials possess a large surface-to-volume ratio that favors functionalization with a wide range of small organic ligands and large bio macromolecules as an effective technique of surface modification that allows the design of novel diagnostic systems with significant advantages sensitivity, selectivity, reliability, and practicality as well as drug delivery application (Borowik et al. 2018). Moreover, nanomaterials also reported possessing antiviral activity, for instance, water-soluble C60 fullerenes reported photodynamic inactivate of mosquito iridescent virus (MIV) Aedes flavescens (Rud et al. 2012).

Nanoparticles have been used as the probe in studying the deep internal cellular structure and dynamics of a range of biological systems, including the vesicular secretion of neurotransmitters and characterizing healthy colon cells from adenocarcinoma cells (Zhang et al. 2009). The application of magnetic nanoparticles as Magnetic Resonance Imaging (MRI) contrast agents has led to their purpose in identifying atherosclerotic lesions in cardiovascular tissue (Frías et al. 2008). The advanced growth of nanoparticles led to the study of several other biological systems that have established their functionality and versatility.

11.14 Gold Nanoparticles

Metal nanoparticles have become a crucial group in the development of nanotechnology-based detection for respiratory viruses. Among the metal nanoparticles, gold nanoparticles have gained much of the attention in this field due to their unique chemical and physical properties. AuNPs have a brilliant surface binding property that acts as a soft metal ion to easily bind to soft ligands such as thiols. This specific reactivity endows with a practical approach to readily functionalize particles with appropriate biological molecules (Lippard and Berg 1994). The result of the Surface Plasmon Band (SPB), a broad absorption band in the visible region between 510 and 580 nm, is due to the property of AuNPs that is the deep red color found in the solutions (Daniel and Astruc 2004). Compared to the bulk gold counterpart, the Surface Plasmon Band (SPB) of AuNPs can be smoothly tuned since it experiences wavelength shifts based on the temperature, shape, size, and nature of functionalized ligands. All these properties have made it the subject to focus on in the wide-ranging studies of the optical spectroscopic properties of AuNPs. The alternation of AuNPs refractive index caused by ligand shell functionalization resulting in SPB shift either red or blue which can be effectively utilized for studying the extent of ligand functionalization or target binding to the AgNP surface (Aboelfetoh et al. 2017).

The potential of AuNP to be functionalized with various biological molecules helps in molecular recognition and led to the development of new techniques for respiratory infection detection. Further, a fluorescence quencher ability of AuNPs might be effectively used for detecting infectious viruses (Daniel and Astruc 2004). The efficiency of quenching by the AuNP depends on the distance between the surface of the AuNP and the chromophore, such as antibodies or carbon-based linker molecules. The quenching ability of AuNPs decreases with the increasing distance between the AuNP surface and the chromophore. All these properties of AuNPs provides exciting platforms for the development of diagnostic tools for respiratory infection detection.

11.15 FRET-Based Detection Based Gold Nanoparticles Quenching Ability

For instance, Grant et al. (2006) have developed a FRET immune sensor for the detection of porcine reproductive and respiratory syndrome virus (PRRSV) (Grant et al. 2006). The sensory system consists of two components, firstly, the antibody PRRSV (SDOW 17) is conjugated with donor fluorophore Alexa Fluorophore (AF546), and secondly, protein A has conjugated to AuNPs at a distance of 20 nm. Both these constructs incubated together to form the intact fluorescence bio-probe for viral detection. In the presence of the PRRSV, the binding of the antibody with fluorophore as well as AuNPs to the virus bring in fluorophore and AuNPs is near enough, helping AuNP fluorescence quenched. The AuNP conjugated FRET pairs were considerably more sensitive in the presence of PRRSV than other related structures developed using typical organic accepting molecules. These studies highlight the effectiveness of the sensor towards frequently detected contaminants (Grant et al. 2006). Using the benefits provided by AuNP, a fast, precise, and responsive FRET platform for the detection of PRRSV was created.

11.16 Detection Based on Other Properties of AuNPs

Due to their relatively easy chemical synthesis and the possibility of surface modification with various small molecules or bio-polymers, including DNA, peptides, proteins, and antibodies, the AuNPs ranging from 1 to 250 nm are widely used in various biological applications (Giljohann et al. 2010). A unique phenomenon called surface plasmon resonance (SPR) of AuNPs, which are responsible for its wine red color, which can be effectively used for easy detection of the target as the binding of the target results in AuNP agglomeration as well as the change in color from red to blue. For instance, Driskell et al. (2010) have reported the detection of influenza A virus with a detection limit of 100 $TCID_{50}$/mL, where an antibody labelled AuNP used as a sensing system, and the presence of the target virus of agglomeration of AuNPs detected using dynamic light diffusion (DLS) (Driskell et al. 2011).

Li et al. (2013) have reported immunochromatographic gold Strips with naked eyes to detect AIV where the sensing system consists of monoclonal antibodies functionalized AuNPs and second antibodies embedded in the nitrocellulose membrane in a particular detection region (Li et al. 2013). The test system consists of a sample pad, the conjugate release pad, the analytical membrane (which includes both the control line and test line), and the absorbing pad. The antibodies specific to AIV is immobilized in the test line, where the control line contains immobilized IgG goat antibodies. Further, the AuNPs are attached to the control line as negative samples flow through the analytical membrane, and the test line is not detected, and thus a single band is observed. During testing, a single-band is observed for negative samples and two distinct bands in the strips for the positive samples. Since antibodies in the test line are captured by targets in case of the positive test sample, while the test line remains un-captured for negative test samples. These techniques showed nearly more than 100-fold sensitivity than commercial test strips with the detection limits of 2 pg mL^{-1} (Li et al. 2013).

11.17 Quantum Dots

QDs are nanocrystalline semiconducting fluorophores with a standard diameter of about 2–20 nm, usually composed of elements of group II, III, V, or VI (Kairdolf et al. 2013). QDs have a range of advantages compared to standard fluorescence dyes, e.g., highly enhanced photo bleaching stability, high quantum yield, broad excitation spectrum, extended fluorescence lifetime, and narrow emission (Petryayeva et al. 2013). These particles display size-tunable emissions, indicating that the wavelength of the emission can be predicted based on the QD's size. Multiple QDs of various sizes can be excited simultaneously by a single source of light, substantially improving their use in multiplexing assays. QDs were also used to diagnose the H9 Avian influenza virus (AIV), which relies on an antibody-antigen reaction (Yun et al. 2007). Avian influenza virus (AIV) binding to F_0F_1-ATPase by an antibody/biotin-streptavidin linkage causing changes in this enzyme's function (Yun et al. 2007). As F_0F_1-ATPase is an enzyme that carries protons through a bio-membrane, changes in the behavior caused by AIV binding contribute to a shift in local pH, which can be easily detected using pH-sensitive QD fluorescence (Yun et al. 2007). Compared with the organic dyes, the narrow fluorescence emission spectrum of QDs can allow simultaneous virus detection using various QDs.

Further, the potential of QDs to be functionalized with various biological molecules helps in molecular recognition and led to the development of new techniques for respiratory infection detection. For instance, Levy et al. (2005) have QDs-based sensing system where QDs' surface is conjugated to anti-target aptamers, which is further tagged with Fluorophore molecules as a quencher, as shown in Fig. 11.1a (Levy et al. 2005). The construction of aptamer-QD-quencher oligonucleotide is prepared such that quencher close enough QD surface for quenching the QD fluorescence (Levy et al. 2005). The presence of target viral protein causes the displacement of the quencher molecule due to the structural change of aptamer resulting in the reinstates of the original emission of QD, as shown in Fig. 11.1a. Thus this technique might be used for successful multiplex detection of numerous targets like viruses by tagging different aptamers specific to viruses onto different QDs.

11.18 Magnetic Nanoparticles

Magnetic nanoparticles consist of various forms of iron oxides that possess varying magnetic properties based on the material's stoichiometry. These magnetic properties of magnetic nanoparticles with extensive surface modifications, including DNA, antibodies, or viral proteins, is available to allow functionality with a detection agent as it can be withdrawn from the test assay by an external magnet after binding with the target. The target can then be identified with designated antibodies or with other antigen-specific reagents. This method can minimize processing times substantially and can also boost the recycling of useful analytes. The magnetically labelled diagnosis (MLD) technique using nanoparticles for diagnosis makes possible the identification of the target by analyzing differences in bio-functionalized magnetic nanoparticles' magnetical properties. Antibodies fixed on the surface of magnetic Nanoparticles sense a particular bio-molecule, resulting in the aggregation of particles and the simultaneous change in the magnetic signals (Fig. 11.1b) (Yang et al. 2008). These significantly bigger/aggregated magnetic nanoparticles get stimulated less to external magnetic fields as compared to original magnetic nanoparticles. Besides, the mean diameter of magnetic nanoparticles pre- and post-incubation with samples varies from 50 to 200 nm or more, based on the concentration of the target molecule to be detected as revealed by dynamic light scattering technology. These MLD method can be used for AIV detection with a detection limit as low as 5 pg mL^{-1}.

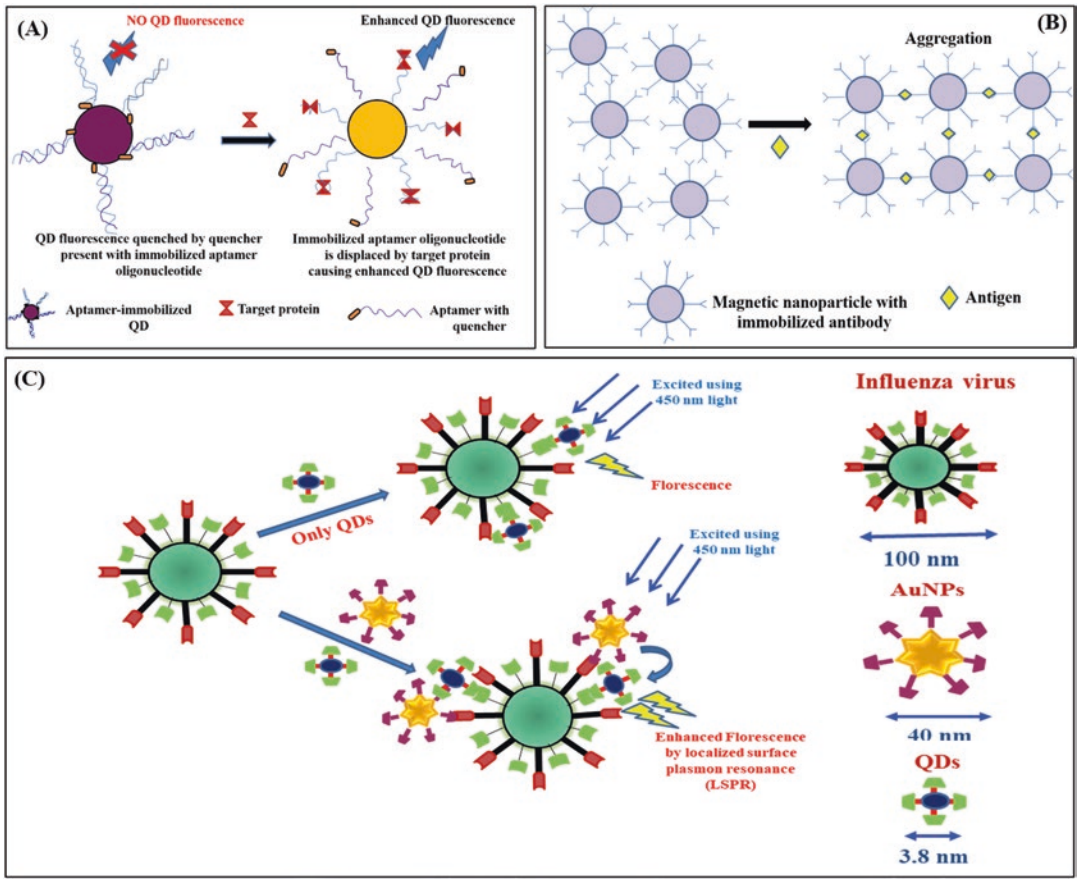

Fig. 11.1 (**a**) Schematic representation showing QDs Fluorescence and Quencher-based detection system where the aptamer tagged quencher is functionalized to QDs' surface such that quencher is close enough to QD surface in order quench the QD fluorescence. The presence of target viral protein causes the displacement of the quencher molecule due to the structural change of aptamer, resulting in the reinstates of the original emission of QD (Levy et al. 2005). (**b**) Schematic representation showing magnetically labelled diagnosis (MLD) technique based on aggregation and DLS detection, here magnetic nanoparticles' bio-functionalized antibodies specific to the virus and the aggregation of particles takes place in the presence of the virus. These were causing the size of the nanoparticle to increase, detected using DLS (Yang et al. 2008). (**c**) Schematic illustration showing the bio sensing for the influenza virus based on localized surface plasmon resonance (LSPR) and Plasmon Resonance Energy Transfer (PRET) of plasmonic materials, AuNPs (Lee et al. 2007). Here, both AuNPs as well as QDs, are functionalized with different antibodies specific to a virus. When both QD and AuNPs nearby, as shown in the virus/P-NM/QD structure, result in the enhanced fluorescence intensity of QD by energy transfer used for viral detection

11.19 Sensing-Based Interactions Between Nanomaterials

The metal nanoparticle, like gold, silver, and platinum, has an enhanced plasmonic effect. The hybrid plasmonic nanomaterials have been developed for viral detection based on optical properties, such as a localized surface plasmon resonance (LSPR) effect, the magneto-optical (MO) effect, plasmonic resonance energy transfer (PRET), and surface-enhanced Raman scattering (SERS) (Lee et al. 2017). For instance, Takemura and colleagues developed LSPR-based sensing systems consisting of plasmonic materials, non-spherical AuNPs, as well as QDs detection of an influenza virus (Takemura et al. 2017).

The fluorescence of quantum dot (QD) is utilized to monitor the viral antigen where the fluorescence intensity of QD increases when non-spherical AuNPs near it due to the LSPR-based PRET effect (Fig. 11.1c) (Lee et al. 2007). When the target virus is present, antibody (Ab) modified non-spherical AuNPs/QD system interact with the virus by key-and-lock interaction and bring the non-spherical AuNPs close to QDs. These result in the fluorescence intensity of QD enhanced due to energy transfer in the virus/P-NM/QD structure. The fluorescent enhancement of QDs depends on the concentration of the viral antigen. These systems reported having the limit of detection (LOD) for the influenza virus as low as 0.03 pg/mL in deionized water and 0.4 pg/mL in a serum matrix. Therefore, hybrid plasmonic nanomaterials-based detection platforms might be developed for the highly accurate biosensing devices for public health.

Similarly, Medhi et al. (2020) have reported a viral detection system based on the principle of photoluminescence of ZrQD- Fe_3O_4@Au core–shell magnetoplasmonic nanohybrid system (Medhi et al. 2020). The nanohybrid was formed by autoclaving Zr NPs in ascorbic acid at 120 °C for one hour, which yielded Zr QDs. These were then conjugated with viral antibodies, followed by the addition of viral antibody-conjugated MNPs. The subsequent addition of viral particles induced immune-linking between the QDs and MPNPs, forming magnetoplasmonic–fluorescent nanohybrid structures. After the nanohybrids, coupled with the viral particles, an external magnet was used to draw them away from the free particles in the solution. The varying concentrations of the virus in the solution showed in the photoluminescence signal fluctuations of the nanohybrid structure. This method was successfully able to detect the infectious bronchitis virus (IBV) and can be expected to perform well in the detection of SARS-CoV-2 (Medhi et al. 2020).

Further, we have also summarized various nanomaterials-based detection systems for the infectious virus in Table 11.2 as follows.

11.20 Applications of Nanomaterials in SARS-CoV-2 Detection

Following the outbreak of COVID-19, nanoparticles assisted RT-PCR for diagnosis of COVID-19 have reported reducing the time of diagnosis as well as efficacy. Somvanshi et al. (2020) have proposed magnetic nanoparticles (MNPs) assisted RT-PCR-based detection where RNA extraction is carried out using MNPs (Somvanshi et al. 2020). The MNP's of zinc ferrite (ZNF) were prepared by the cost-effective sol-gel auto-ignition method, and further, its surface was functionalized with carboxyl-containing polymers (CPoly). Because of the strong interface among nucleic acids and carboxyl groups, the surface-functionalized MNP's encourage fast and efficient adsorption of viral RNAs.

Rajil et al. (2021) reported a methodology focusing on IgM and IgG antibodies against SARS-CoV-2 in the blood. Blood was mixed with fluorescent nanoparticles (FNPs, for example, nitrogen-opening nanodiamonds, upconversion nanoparticles, or semiconductor quantum dabs (QDs), which have been coated with an antigen protein mimicking the surface spike protein of SARS-CoV-2. Now, the QDs, subsequently coated, resembles SARS-CoV-2 infection to the antibodies and get bound to them, which are attached to secondary antibodies in the wall. The following step includes compelling the antibody QD fluid through the cylinder or fiber-optics waveguide, where it binds with the anti-antibodies (against immune response) that are themselves coated properly on the walls. The QDs are presently fixed and, when driven by an outer layer, can comprise a lasing setup. In this manner, when mirrors are incorporated, the system will "lase" when the antibody molecule count increases beyond a particular number (Rajil et al. 2021).

Shan et al. reported a nanomaterial-based sensor for detection of COVID-19 from the exhaled air of an infected person. The sensors are made out of various gold nanoparticles connected to

Table 11.2 Nanomaterials-based detection systems for virus infection diagnosis

Nanoparticle construct	Detection System	Detection target	Pathogen detected	Reference
Au/MNP-CNT (carbon nanotube of Gold (Au) -magnetic nanoparticles (MNP))	Multi-functionalized CNTs-based sensing platform.	DNA	Influenza virus A (H1N1) and norovirus	Lee et al. (2018)
Gold nanoparticles conjugated to organic ligands	Biosensing array	Volatile organic compounds	Coronavirus	Shan et al. (2020)
Carboxyl-modified MNPs	Chemiluminescence combined with RT-LAMP	Hemagglutinin (HA) and neuraminidase (NA) genes of the H7N9 virus	The H7N9 virus and other avian influenza viruses	Wang et al. (2016)
Core-shell Ag@SiO_2 nanoparticles (NPs)	Metal-enhanced fluorescence (MEF) sensor	Recombinant hemagglutinin (rHA) protein	H5N1 influenza virus	Pang et al. (2015)
Monoclonal antibody conjugated gold nanoparticles (mAb–AuNPs)	Colorimetric immunosensor	Hemagglutinin (HA) surface protein	H3N2 Influenza A virus	Liu et al. 2015)
Anti-NA antibody labelled AuNP and anti-HA antibody labeled CdSeTeS QD	Local plasmonic resonance induced immune-fluorescence Nanosensor	HA and NA antigens	Influenza virus H3N2 and Norovirus-like particles (Nov-LPs).	Takemura et al. (2017)
Erythrocyte membrane cloaking on PGLA NPs core-shell structure with SPION core	Isolation of virus by host-pathogen interaction	Viral surface antigen	Influenza virus	Han et al. (2017)
Europium NP conjugated mAB	Fluorescent immunochromatographic strip test (FICT) assay	rHA antigen	Avian Influenza (AI)-H7N1, H7N7, H7N9	Yeo et al. (2017)
Glassy Carbon electrode surface modified by thiol-graphene quantum dots (QGD-SH) and AgNPs	Fabricated electrochemical immune sensing with Riboflavin probe	Core antigen of HCV	Hepatitis C virus (HCV)	Valipour and Roushani (2017)
Silica-coated magnetic nanobeads (MagNBs) conjugated primary antibody, and Au nanozyme (AuNZ) conjugated secondary antibody	Magnetic nanoenzyme linked immunosorbent assay	HA antigen	Influenza virus A (H1N1) and (H3N2)	Oh et al. (2018)

natural ligands, making a different detecting layer that swells or shrinks upon introduction to volatile organic compounds (VOCs), resulting in a varied electrical resistance. In these layers, the inorganic nanomaterials are liable for the electrical conductivity, with the organic film component allowing for adsorption of VOCs. The presence of VOCs in the surrounding results in its diffusion into the detecting layer or falls on the detecting surface and reacts with the organic film elements covering the inorganic nanomaterials. The result of the associations causes a volume change (expanding/shrinkage) in the nanomaterial film. Therefore, the contacts among the inorganic nanomaterial block the change (higher/lower) with an increase/decrease in conductivity.

The rationale behind this methodology is based on reports indicating that viral agents as well as their microenvironment transmit VOCs that can reach out through expired air. The rise of VOCs in exhaled breath could occur in the initial phases of the infection, subsequently serving for immediate identification of COVID-19 (Shan et al. 2020). Further, we have also summarized various nanomaterials-based detection systems for SARS-CoV-2 infection in Table 11.3 as follows.

11.21 Future Prospects and Conclusion

Nanomaterial-based diagnosis systems provide a new frontier toward new applications in clinical diagnostic and biological applications. This current chapter reviewed the different nanomaterial-based biosensing techniques that have reportedly been developed so far based on the unique physico-chemical properties of noble metal nanoparticles as well as QDs for future applications toward point-of-care diagnostics for viral infection.

Noble metal NPs have already proven that they are among the main groups of biosensing materials, as well as for biomedical applications (e.g., viral detection, cancer diagnosis, etc.). Groups of nanomaterials possess a specific and efficient attribute that paves the way for the early identification of disease and contaminants for the establishment of new diagnostic systems for disease markers and pathogens like a virus. The merits and demerits of any approach toward a diagnosis of real specimens depend on the various methods studied to date, for example, colorimetric image, NIR imaging, fluorescence quenching/enhancement, SERS, electrical and electrochemical sensing. Evaluation of such approaches, in most cases, has been documented using managed synthetic bioanalytics and samples. In contrast, validation of such biosensors with a statistically more significant population of

Table 11.3 Nanomaterials based detection systems for SARS-CoV-2 infection diagnosis

Nanoparticle construct	Detection system	Detection target	Reference
Lanthanide-doped polystyrene nanoparticles (LNPs)	Lateral flow immunoassay (LFIA)	Anti-SARS-CoV-2 IgG in human serum	Chen et al. (2020)
Gold nanoparticles (AuNPs) thiol-modified antisense oligonucleotides (ASOs) specific for N-gene (nucleocapsid phosphoprotein) of SARS-CoV-2	Naked-Eye Detection by selective aggregation of AuNPs in the presence of its target RNA sequence of SARS-CoV-2, as well as, colorimetric assay due to change in surface plasmon resonance	mRNA of N-gene (nucleocapsid phosphoprotein) of SARS-CoV-2	Moitra et al. (2020)
Carboxyl groups (PC)-coated magnetic nanoparticles (pcMNPs)	The pcMNPs-based viral RNA extraction method and RT-PCR	ORF lab and N gene of viral RNA	Zhao et al. (2020b)
Colloidal gold nanoparticle-based lateral-flow (AuNP-LF)	Indirect immune chromatography method, where analytical membrane coated with SARS-CoV-2 nucleoprotein (SARS-CoV-2 NP) for sample capture, and antihuman IgM was conjugated with AuNPs to form the detecting reporter	IgM antibody in blood against the SARS-CoV-2 virus	Huang et al. (2020b)
Selenium nanoparticles modified SARS-CoV-2 nucleoprotein	Lateral flow immunoassay kit based helps to read the results by the naked eye wherein 10 min, where selenium nanoparticle-modified SARS-CoV-2 nucleoprotein, which detects anti-SARS-CoV-2 IgM and anti-SARS-CoV-2 IgG in human serum	Anti-SARS-CoV-2 IgM and anti-SARS-CoV-2 IgG in human serum	Wang et al. (2020)
Magnetic nanoparticles	Giant magnetoresistive (GMR) biosensor along with MNPs	Viral ssRNA, and S (spike) – protein of SARS-CoV-2	Islam and Ahsan (2020)

actual specimens needs to be done for demonstrating the possible application of such approaches in clinical and health care diagnosis. Besides, the increased demand for greater sensitivity and selectiveness at minimum expense and the ability to track biosensing in real-time, particularly for point-of-care systems, for whom simplicity should also be taken into account, is a critical issue in biosensing in real-time. In this context, colorimetric and electrochemical approaches are the most promising because of their simplicity, sensitivity, and specificity, and in most cases, need no costly and complex instruments to construct a biosensor platform for sampling. Also, such methods allow biosensing amplification to be implemented in a spectrum from low to high-performance diagnoses.

Specific strategies designed using noble metal nanoparticles (SERS) and fluorescent quenching methods can also be implemented in research laboratories so that existing paradigms and new problems in biology and medicine can be identified. For instance, Shanmukh et al. (2006) have developed a surface-enhanced Raman scattering (SERS) substrate with an array of silver nanorod having excellent SERS enhancement ($\sim 10^8$) factors providing novel and powerful biosensing methods. These help in the rapid detection of trace levels of viruses with a high degree of sensitivity and specificity based only on spectroscopic assay using SERS substrate of silver nanorod (Shanmukh et al. 2006). Indeed, some of the nano-based sensing systems have already been used for sensing bio molecules, such as DNA, RNA, etc., as is the case with Nanosphere, Inc. (Northbrook, IL, USA) Verigene® Method, which examines the scatter ability of gold nanoparticles on a micro-array platform and provides a diagnostic medium for DNA/RNA and protein sensing. The knowledge of the biological mechanisms and advances in clinical practice with their inclusion in future diagnostic platforms would most likely revolutionize the mentioned NP metal biosensors that have currently been developed so far. Presently, some of the noble metal NP-based biosensors are very likely to broaden our understanding of the fundamentals of biological systems and to promote clinical practice by integrating them into future diagnostics platforms.

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Obesity: A Risk Factor for COVID-19

12

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Abstract

Introduction: Emerging data have demonstrated increased mortality of COVID-19 patients suffering from comorbid conditions such as Type II diabetes, hypertension, and cardiovascular diseases. Underlying risk in all these patients is an increase in bodyweight or obesity. The adverse health effects of obesity and how these factors enhance the risk of mortality in COVID-19 patients is still unexplored.

Objective: The enhanced fat deposition might be a risk factor for increased mortality in COVID-19 patients.

Method: We have reviewed and collected the information from online databases: Pubmed, Google scholar, Researchgate, to highlight the systematic link between obesity with associated risks in COVID-19.

Result: We have reported the first study during the pandemic from France and New York, to a currently reported study in Mexico and found individuals with BMI ≥35 kg/m^2 or >40 kg/m^2 have greater risk of developing critical illness due to COVID-19, thereby increasing mortality.

Conclusion: Our study suggests obesity in childhood, adolescence, and adulthood can be considered a profound risk factor for greater susceptibility and severity of COVID-19 and is associated with nutritional, lifestyle, cardiac, respiratory, renal, and immunological alterations, which may potentiate the complications of SARS-CoV-2 infection. Further suggesting to check on BMI during this pandemic situation.

Keywords

Obesity · COVID-19 · Inflammation · Immune dysregulation

Abbreviations

COVID-19	Coronavirus Disease 19
kg/m^2	kilogram per meter square
WHO	World Health Organization
BMI	Body Mass Index
SARS-CoV	Severe Acute Respiratory Syndrome Coronavirus
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
ACE2	Angiotensin-Converting Enzyme 2
MHO	Metabolically Healthy Obese
ER	Endoplasmic Reticulum

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TMPRSS2	Transmembrane Protease Serine 2
HC	Hip circumference
WC	Waist circumference
WSR	Ratio of waist to height/stature
WHR	Ratio of waist to hip
SNS	Sympathetic Nervous System
RAS	Renin-Angiotensin System
RAAS	Renal- Reabsorption-Aldosterone System
ARDS	Acute Respiratory Distress Syndrome
TNF	Tumor Necrosis Factor
IL	Interlukine
NFκB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NE	Norepinephrine Levels
PRA	Plasma Renin Activity
TH1	T helper 1
IFNγ	Interferon gamma
T2D	Type 2 Diabetes
IKK-β	IkappaB kinase beta
IRS1	Insulin receptor substrate 1
FVC	Forced Vital Capacity
FEV1	Forced Expiratory Volume in One Second
IMV	Invasive Mechanical Ventilation
FRC	Functional Residual Capacity
OSAS	Obstructive Sleep Apnea Syndrome
CVD	Cardiovascular Diseases
MCP1	Monocyte Chemoattractant Protein-1
CCL	Chemokine Ligand 2
UPR	Unfolded Protein Response

12.1 Introduction

Coronavirus disease 2019 (COVID-19), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was declared as an outbreak by Public Health Emergency of International Concern on January 30, 2020 and a global pandemic situation on March 11, 2020 by the World Health Organization.

Individuals globally are experiencing a standstill in their day-to-day life due to COVID-19 pandemic. Coronavirus Disease 19 (COVID-19) was first reported in China in late December 2019 and has caused a global pandemic situation with large number of cases worldwide. It is caused by severe acute respiratory syndrome coronavirus 2 (SARS CoV-2) with fever, dry cough, shortness of breath, muscle and body aches as primary symptoms, and infections resulting into life threatening form of the disease.

Recent studies have reported that, globally, more than 1.9 billion adults are overweight and 650 million are obese (WHO, Obesity and Overweight). Emerging data have demonstrated that Coronavirus disease 2019 (COVID-19) and the risk of severe acute respiratory syndrome is higher in people living with pre-existing conditions of cardiovascular diseases, diabetes, hypertension. Persons with obesity around the world are at high risk of severe complications of COVID-19 by virtue of the increased risk of the chronic diseases that obesity drives. Obesity, a condition that often coexists with diabetes and hypertension is now recently linked to higher mortality even in young patients. Low grade inflammation which is associated with obesity may be one of the major factors which is responsible for the adverse effects of these patients suffering from COVID-19. It is well known that chronic inflammation in obese people can lead to an imbalance in cytokine levels and higher activation of nuclear transcription factor kappa B (Lee et al. 2013). However, among the obese, a sub-group called metabolically healthy obese (MHO) exists, which has no inflammation (Karelis et al. 2004; Geetha et al. 2011). The MHO group may have better survival rate in case of hospitalization due to the phenomenon of obesity paradox (Ades and Savage 2010).

12.2 Morphology of Coronavirus

The coronavirus has acquired its name from the crown like morphology. The size of coronavirus ranges from 26–32 kbs in length and 65–125 nm in diameter with nucleic material containing 5′ capped positive single stranded sense RNA (Shereen et al. 2020).

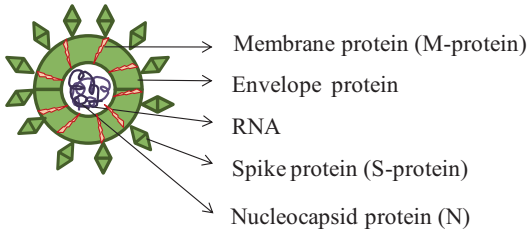


Fig. 12.1 Structure of SARS-CoV-2

As per morphology, the structure of coronavirus consists of a spike protein (S), nucleocapsid protein (N), membrane glycoprotein (M) and a lipid bilayer with an additional membrane glycoprotein (Velavan and Meyer 2020) (Fig. 12.1).

12.3 Key Features and Entry Mechanism of SARS-CoV-2

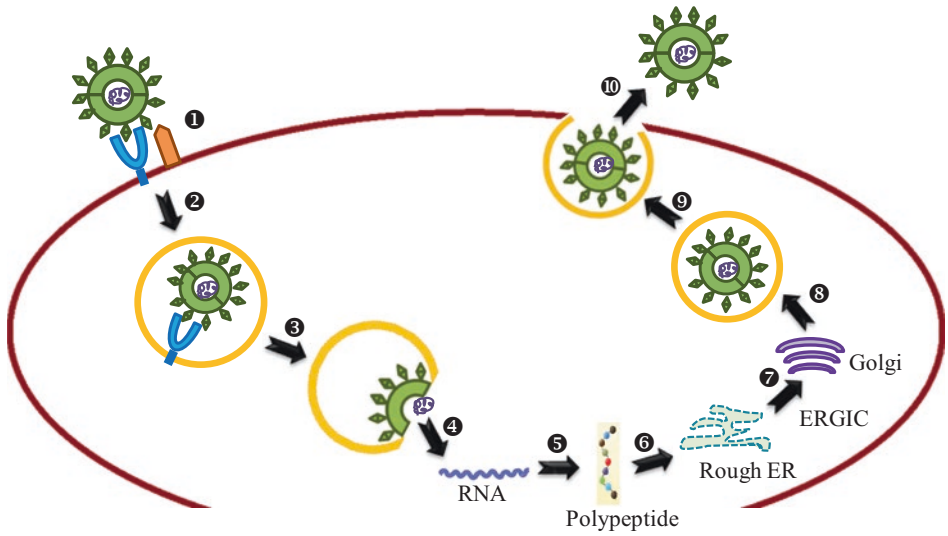
The binding of SARS-CoV-2 glycoprotein spikes (S protein) to the surface receptor, angiotensin-converting enzyme 2 (ACE2) by membrane fusion is responsible for the viral admission to the host cell (Hoffmann et al. 2020) Further, the entry of SARS-CoV-2 depends upon the cellular proteases, transmembrane protease serine 2 (TMPRSS2), which is responsible for splitting the spike S protein and promotes penetration changes (Alanagreh et al. 2020). Followed by endocytosis and release of RNA into the target cells (Shereen et al. 2020). Thereafter, the largest gene, ORF1a/b of SARS-CoV-2 encodes the pp1ab protein and 15nsps; while ORF1a gene encodes pp1a protein and 10nsps (Chen et al. 2020). The synthesized protein is further cleaved into small products via viral proteinases. Subsequently, viral proteins and genomic RNA assemble into rough endoplasmic reticulum (ER) and Golgi. Afterwards, it is transported and released out of cells via exocytosis process (Sheeren et al. 2020) (Fig. 12.2).

12.4 Symptoms

Fever is considered as initial symptom of COVID-19, later accompanied by dry cough, difficulty in breathing, dizziness, body ache, sore throat, chest pain, diarrhea, nausea, and vomit (Yi et al. 2020). Guan et al. (2020) recommends that dyspnea/hypoxemia was also experienced by some patients within one week after inception of CoV. During the initial stages of SARS, some patients are reported to develop acute respiratory distress syndrome (ARDS), accompanied by respiratory failure and other severe complications (Chen et al. 2020). The human to human transmission of this novel virus is reported to be acquired by people either by inhalation of droplets which can travel and spread up to 2 m or touching the contaminated surfaces and further touching your face, eyes, mouth or nose (Kampf et al. 2020). The binding of ACE2 with the virus might result in release of pro-inflammatory cytokines and dysfunction of multiple organs.

12.5 COVID-19 and Inflammation

Currently the attention of worldwide research is on the COVID-19 and cytokine storm. The symptomatic treatment of inflammation caused by cytokine storm, leading to respiratory dysfunction is one of the primary focuses. According to recent literature, the uncontrolled or overproduction of pro-inflammatory cytokines such as tumor necrosis factor [TNF], IL-6, IL-12 and IL-1β is known as "cytokine storm," which leads to an increase in various risk factors, including systemic inflammation, vascular hyper permeability (outflow of fluids from blood vessels), ARDS (Acute respiratory distress syndrome), multiple organ failure, and mortality if cytokine levels are persistently high (Jose and Manuel 2020). The T helper 1 (TH1) cells which are lineage of CD4+ cells and promote cell-mediated responses, secrete these pro-inflammatory cytokines, with similar mechanism observed in SARS-CoV and MERS-CoV56. Additionally, studies suggest monocytes and T lymphocytes are immune cells that are attracted by the secreted cytokine and chemokines into the infected site (Tay et al. 2020). Observations from the case studies also indicate an increase in the level of cytokines (IL1B, IL1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IFN-γ, MCP1, TNFα,) and chemokines (CCL2, CCL3, CCL4, CCL5) in COVID-19-infected



❶Attachment; ❷ Entry; ❸ Fusion; ❹ Uncoating; ❺ Translation, polypeptide synthesis; ❻ & ❼ Assembly and budding; ❽&❾ Exocytosis; ❿ Viral release

: ACE2 receptor : TMPRSS2 : S protein : RNA : SARS-CoV-2

ACE2, angiotensin-converting enzyme-2; TMPRSS2, transmembrane protease serine 2; ER, endoplasmic reticulum; ERGIC, ER–Golgi intermediate compartment

Fig. 12.2 Key features and entry mechanism of SARS-CoV-2

individuals and have higher chances for being admitted to Intensive Care Units as compared to less critical patients (Huang et al. 2020). With the presence of SARS-CoV-2 in the epithelial lung cells, there is a secretion of various cytokines and chemokines from the immune cells such as: macrophages, dendritic cells, and T-lymphocytes, eventually leading to development of acute respiratory distress syndrome (Coperchini et al. 2020). More evidences suggested the association of COVID-19 with cytokine storm that causes multiple organ exhaustion, including lung and kidney dysfunction, risk of cardiovascular diseases that resulted in increased mortality rate (Nile et al. 2020). A few recent case studies reported that comorbidities such as diabetes mellitus (type 2), chronic pulmonary diseases, hypertension, and cardiovascular disease appear to increase the chances of COVID-19 infection (Caussy et al. 2020). Apart from other risks, underlying observation in many of the patients is higher Body mass Index and obesity, which is also emerging as a risk factor for severe COVID-19 infection (Dietz and Santos-Burgoa 2020) (Fig. 12.3).

12.6 Obesity

Obesity is considered as a chronic and metabolic disorder with increasing public health issue which is attributed to multiple factors like diet, hormonal, genetics as well as environment (Lee et al. 2013). Body mass index (BMI) is one of the traditional anthropometric measurements which are generally used to evaluate the degree of obesity (Mikhail et al. 1999). Body Mass Index is defined as the ratio between weight (kilogram) and height (meter square) (Center for Disease Control and Prevention, Body Mass Index). Apart from BMI, hip circumference (HC), waist circumference (WC), ratio of waist to height/stature (WSR), ratio of waist to hip (WHR), sagittal

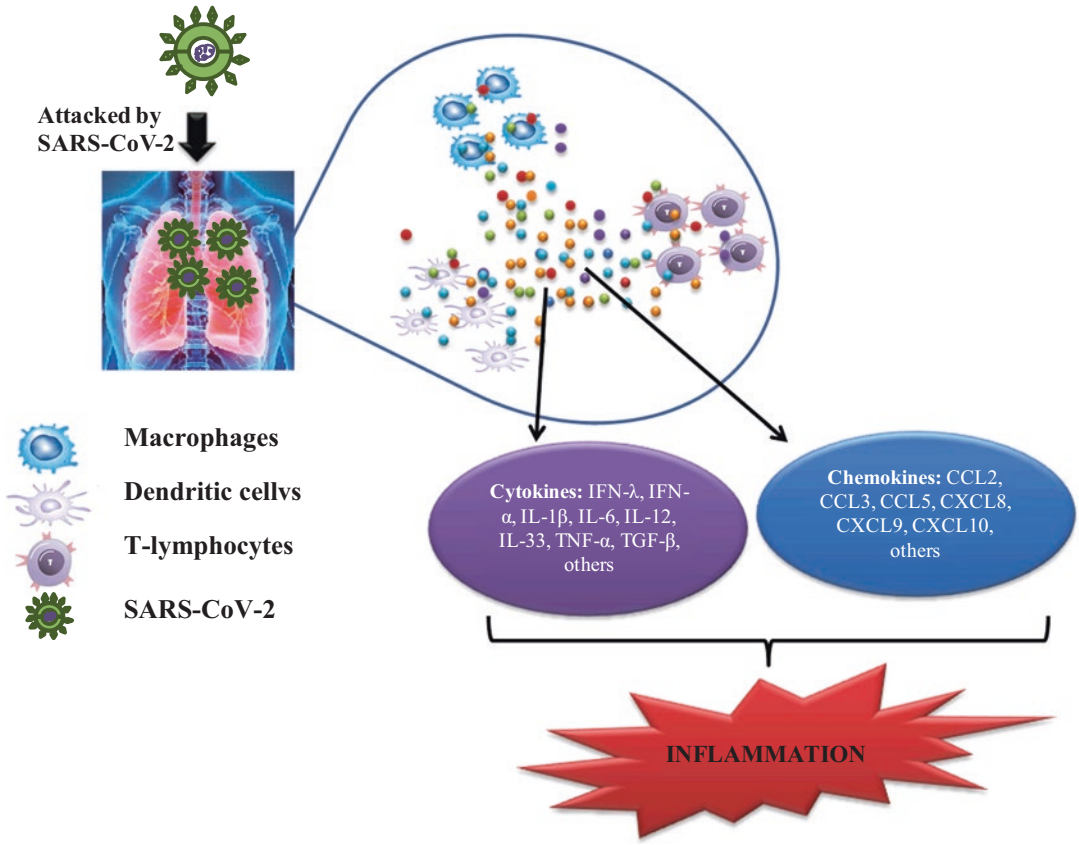


Fig. 12.3 COVID 19 infection and role of inflammation

depth, and body adiposity index are also considered as anthropometric measures, which could help in enhancing the BMI data and measures the risks related to obesity. The current classification of Body Mass Index is shown in Table 12.1.

Overweight and obesity affects multiple organs of the body, mainly heart, liver, kidney, and brain (Hall 2003). There are various life-threatening risks associated with obesity which majorly include hypertension, cardiovascular diseases, diabetes mellitus (type 2), breast cancer, infertility, and other chronic metabolic syndrome (Jiang et al. 2016).

Table 12.1 Body mass index classification of obesity

Sl. no.	Weight status	BMI (kg/m^2)	Asian population BMI (kg/m^2)
1.	Under weight	<18	<18.5
2.	Normal weight	18.5–24.9	18.5–22.9
3.	Over weight	25–29.9	23–24.9
4.	Obese	≥30	≥25
(a)	Class 1 obese	30–34.9	
(b)	Class 2 obese	35–39.9	
(c)	Class 3 obese	40.0–59.9	

WHO, Mean Body mass index

12.7 Obesity and Lifestyle

Obesity among young adults is growing rapidly all over the world due to physical inactivity, high calorie intake, and other unhealthy lifestyle habits. It is well known that obese individuals are especially vulnerable to chronic diseases such as type 2 diabetes (T2D) and cardiovascular dis-

eases. Studies have shown that genetic tendency for weight gain does not lead to obesity. The pattern of dietary intake and physical activity might play an essential role in weight gain (Jiang et al. 2016). Reports suggest young adults and adolescents are more prone to weight gain and obesity as increased calorie intake with sedentary lifestyle accompanied by change in lifestyle might incline them for becoming less active. The prolonged sedentary lifestyle can contribute to various psychological issues such as depression, insomnia, and low self-esteem further resulting into overeating and weight gain (Nestle and Jacobson 2000; Racette et al. 2005). Moreover obesity or overweight are clearly because of increase in calories intake to those utilized by the body, and excess being stored in fat tissues (Jiang et al. 2016).

12.8 Obesity and Type II Diabetes

There is a strong relationship between Body mass index, diabetes, and insulin resistance (Kahn et al. 2006). The release of various pro-inflammatory cytokines, fatty acids, glycerol in an obese individual increases the chances of insulin resistance. Being a chronic and metabolic disorder, diabetes mellitus is caused by the progressive inability to produce insulin by β-Langerhans islet cell in pancreas (Al-Goblan et al. 2014). Diabetes mellitus is categorized into two: Type I and Type II diabetes mellitus. Adipocytes (storage of energy in the form of triglycerides) and adipose tissues play an essential role in the development of insulin resistance with obesity. Studies suggest adipose tissues create adipokines such as leptin, adiponectin, retinol-binding protein-4 (RBP4), monocyte chemoattractant protein-1 (MCP-1), resistin are primarily involved in the development of insulin resistance related to obesity (Kasuga 2006). Due to chronic inflammation, there is activation of macrophage infiltration which leads to secretion of TNF-α, IL-1, IL-6, and MCP-1 and other cytokines and chemokines causing insulin resistance (Xu et al. 2003). The trigger of local inflammatory responses with secretion of various chemokines and cytokines lead to systemic insulin resistance (Kasuga 2006). The increase in the free fatty acids levels is also considered as one of the factors that are dominant in obese individuals resulting in insulin resistance as there is a decrease glucose uptake by muscles (Felber and Golay 2002). Moreover, the storage and conversion of lipid in the form of triglycerides is accumulated in both skeletal muscles as well as liver is also associated with insulin resistance. These triglycerides consist of three long chain of fatty acids and consisting acyl coenzymes A, ceramides, and diacylglycerol as metabolites which majorly contribute to impairment of both hepatic and peripheral insulin action. Hence, the process is stated as lipotoxicity (Soodini and Hamdy 2006), which is also proven to decompensate β islet cells in diabetes type 2 (Shimabukuro et al. 1998). Research evidences have also established the mRNA expression of vaspin (adipokine) in human adipose tissues and its association with obesity and insulin resistance and glucose metabolism (Klöting et al. 2006).

12.9 Obesity and Hypertension

Obesity is also associated with hypertension, which contributes to kidney-related disorders, mainly by increasing tubular reabsorption and activation of sympathetic nervous system (SNS) and RAS (renin-angiotensin system) (Jiang et al. 2016). Carbohydrate rich diet, increased consumption of alcohol, tobacco, sedentary lifestyle, and stress constitutes high risk for build-up of fat, cholesterol, and other substances on the arterial walls, hence causing restricted blood flow favoring the formation of atherosclerosis (Ross 1999). Studies have established an increased SNS activity might play an essential role in maintaining high blood pressure levels in the obese. Whereas they found in association with weight loss a decrease in plasma-renin-activity (PRA), blood pressure, and norepinephrine levels (NE) in both hypertensive and a normal obese individual (Sowers et al. 1982). Moreover, increase in sodium retention causing renal- reabsorption-aldosterone system (RAAS), the renin-angiotensin system, and increase in visceral fat stored in abdominal cavity are considered to have

primary role in hypertension related to obesity (Jiang et al. 2016). Increase in blood pressure is a result of local production of angiotensin II by various cell types, hence further increasing peripheral vascular resistance (vasoconstriction), SNS activity (Yiannikouris et al. 2012). White adipose tissues secrete a 16 kDa protein leptin having an essential role in the regulation of energy expenditure and food intake. There is an increase of plasma leptin concentration in obese individuals. Studies have also suggested, apart from obesity, leptin is also involved in complications related to cardiovascular and hypertension (Bell and Rahmouni 2016). Moreover, an influence of leptin on nitric oxide, chronic SNS activation, and sodium excretion through urine (natriuresis) might lead to sodium retention, increasing blood pressure, and vasoconstriction. Therefore, in obese individuals, leptin is also considered to play an essential role in the development of hypertension (Bravo et al. 2006).

Additionally, ACE2 (angiotensin-converting enzyme II) receptor and RAAS (renin-angiotensin-aldosterone system) are both considered as the key regulators of blood pressure. Hence the SARS-CoV 2 infection via ACE2 has shown a direct link between hypertension and COVID-19 in human cells, further resulting into severe clinical outcomes (Schiffrin et al. 2020).

12.10 Obesity and Cardiovascular Diseases

There is a direct relation between obesity and cardiovascular disorders. With increase in BMI, the chances of developing cardiovascular diseases (CVD) and coronary heart diseases are increased (Csige et al. 2018). For the cardiovascular system, the adipose tissues are often considered as crucial bioactive regulators, as they stimulate to secrete several adipokines (leptin, adiponectin, and apelin) which exert an endocrine or paracrine effect (Oikonomou and Charalambos 2019). With evidences, these adipokines are suggested to have direct and diverse effect on the myocardial metabolism, by regulating multiple cell signaling pathways (Karmazyn et al. 2008). The increase of fat deposition in adipose tissues of individuals might result in mortality and affect mobility due to cardiovascular disorders (Akil and Ahmad 2011). There is also an increased level of free fatty acids in obese individuals (Boden 2008). Due to deposition of triglycerides in their blood vessels, there is restricted blood flow in obese persons. These restricted blood vessels also accumulate toxic compounds (e.g., diacyglycerol and ceramide) over a prolonged period of time, thereby increasing the chances of programmed cell death of cardiac cells (Csige et al. 2018). This further might lead to various risk factors such as heart attack and strokes. Studies have established that buildup of abdominal fat leads to production of pro-inflammatory cytokines and adipokines, which thereby leads to major risks of cardiometabolic dysfunction such as atherosclerotic plaques (Carbone et al. 2019).

Studies suggest high frequency of pre-existing cardiovascular disease in patients suffering from COVID-19, further resulting in higher mortality. Moreover, CVDs such as arrhythmias, myocardial injury, thromboembolism, and acute coronary syndrome (ACS) have been found to be more stimulated by the SARS-CoV2. Together, these studies suggest a direct interaction between the cardiovascular system and COVID-19 (Nishiga et al. 2020).

12.11 Obesity and Respiratory Disorder

During the lack of noticeable lung disease, obese and overweight individuals have a greater chance of developing respiratory symptoms when compared to normal BMI individuals. Obstructive sleep apnea syndrome (OSAS), asthma, pulmonary embolism, and obesity hyperventilation syndrome are a few respiratory diseases which have direct association with increasing BMI and obesity (Zammit et al. 2010). There is an indirect relationship between lung volume and BMI. A decrease in lung volume is linked with increase in the BMI as well as overweight (McClean et al. 2008). With increase in BMI, longitudinal studies related to respiratory mechanics have shown a reduction in the volume of exhaled air in one sec-

ond after maximum inspiration (forced expiratory volume in 1 second—FEV1), forced vital capacity (FVC), which is forcible exhalation of the amount of air after a deep inspiration, usually measured by spirometry and functional residual capacity (FRC) (Wannamethee et al. 2005). Due to reduced physical activity during daytime, frequency of carbohydrate intake is resulting in weight gain. Hence this irregular lifestyle and increase in BMI makes an individual more inclined to develop obstructive sleep apnea. This is a very common syndrome caused by breakdown of upper airways due to which breathing cessation occurs which is repetitively nocturnal (Spicuzza et al. 2015). Evidences of reports suggest that due to exertion, obese individuals acquire symptoms like breathlessness and excessive sweating (Zammit et al. 2010). Studies observed patients with chronic cough, dyspnea, frequent smokers, or those prone to allergies have higher risk of developing asthma and COPD. In the above situation, patients are recommended to go for spirometric clinical assessment. Results indicate a decrease in the FEV1/FVC ratio (>70%) together with decrease in FEV1 and FVC levels (Poulain et al. 2006). Studies have also researched that air obstruction in dyspnea being directly associated with obesity. Moreover, obese patients having symptoms of dyspnea often are given wrong treatment without proper diagnosis and pulmonary function testing (Sin et al. 2002) a proper evaluation and diagnosis of the disease also act as a primary role for development of therapeutic strategies.

12.12 Obesity and Inflammation

Several researchers have found association of obesity with low grade systemic inflammation in which pro-inflammatory cytokines are released by activating innate immunity in the adipose tissues. Low-grade inflammation is determined by a condition of adipocyte hypoxia and dysfunction that results in an exuberant secretion of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin (IL) 1β and IL-6 and the recruitment of immune cells macrophage, T-cell, and B-cell, creating an auto-regenerating inflammation loop. It is well reported that pro-inflammatory cytokines, such as TNF-α, IL-6 or C-reactive protein, are increased in overweight and obese adults (Muscogiuri et al. 2020)

Adipose tissues are classified as brown and white adipose tissues (Cypess et al. 2009). These tissues have adipocytes which secrete various adipokines, peptides, and pro-inflammatory cytokines such as Interleukin- 4, IFN- γ, Tumor necrosis factor α (TNF- α), Interlukine-6 (IL-6), visfatin, and resistin, which are known to exert an endocrine effect, hence communicating with other organs. Further the regulation of food intake and energy usage is controlled by these adipokines (Lafontan 2005). A hypothesis proposed by many studies suggests nutrient buildup in the adipocytes encourages cellular and metabolic stress, hence resulting in activation of various inflammatory signaling pathways (Gregor and Hotamisligil 2011). Usually folding, maturation, and storage of proteins occur in endoplasmic reticulum. Under the cellular stress induced by nutrient overload, the accumulation of misfolded proteins take place in ER, leading to activation of the UPR pathway (unfolded protein response). Furthermore, the activation of UPR pathway leads to activation of NFκB pathway (nuclear factor kappa-light-chain-enhancer of activated B cells) with increased expression of cJUX NH- terminal kinase (JNK) which is also a stress-activated protein kinase (Urano 2000) and IKK-β, IRS-1. This in turn leads to enhanced release of pro-inflammatory cytokines and induce insulin resistance (Lee et al. 2013) (Fig. 12.4).

12.13 Obesity and COVID-19

Recently adipose tissue has been recognized as an endocrine organ that secretes adipokines, which can play a major role in the metabolism as well as immune response. Altered immune response in the obese individuals may be associated with significant alteration of immune cells in the adipose tissues leading to a state of chronic inflammation both at local as well as at the systemic levels. Inflammation is at the forefront of COVID-19 research and major complications of COVID-19 infection are directly associated with

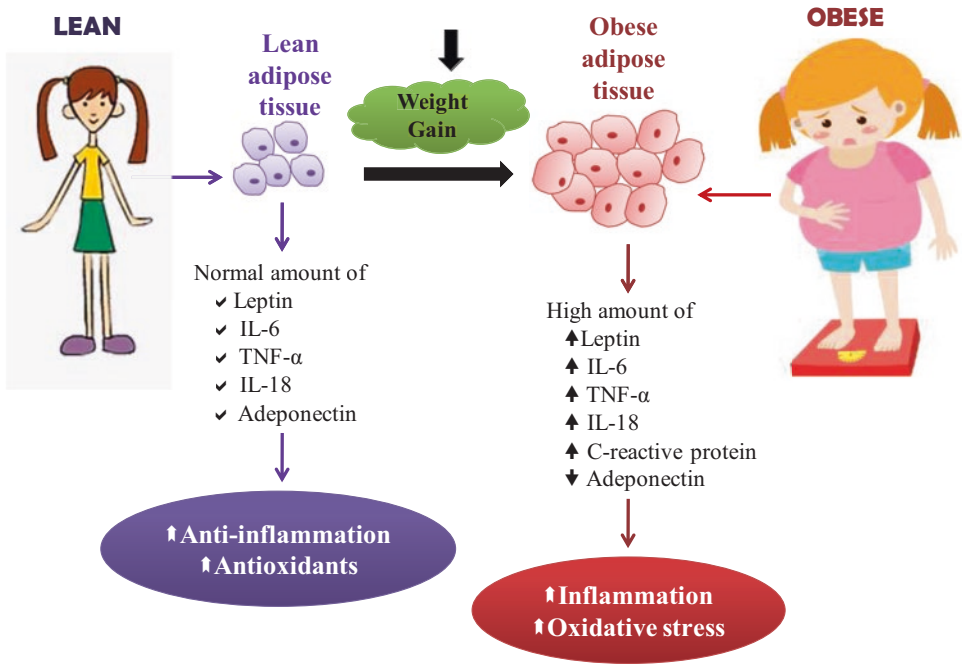


Fig. 12.4 Obesity and inflammation

systemic inflammation (Chiappetta et al. 2020; Inciardi et al. 2020).

Adipose tissue comprises a population of 3 anti-inflammatory cell types allied with normal adipose function. M-2 macrophages, T helper (Th2) cells, and regulatory T-cells (Treg) play a vital role in downstream regulation of the inflammation. Recent studies suggest adipose tissues having higher expression of ACE-2 as compared to other tissues and therefore being directly targeted by SARS-CoV-2 and SARS-CoV-2 RNA. This increased expression of ACE 2 in obese as well has been associated to the worse outcomes with COVID-19 (O'Rourke and Lumeng 2021). This increase in adipocytes in people with obesity might lead to a greater viral load as well as prolonged viremia.

Disease severity and outcome of COVID-19 patients are directly associated with dysregulation of pro-inflammatory cytokines. Increase in visceral adiposity results in obesity with low grade systemic inflammation with the pro-inflammatory cytokines by activating innate immunity in the adipose tissues. Low-grade inflammation results in an exuberant secretion of pro-inflammatory cytokines that may in some cases contribute to the "cytokine storm" of COVID-19 (Dicker et al. 2020).

Additionally, due to excessive fat, the unbalanced regulation of fatty acid metabolism, ER stress, mitochondrial dysfuncton, and hypoxia may lead to a significant alteration of cellular architecture of adipose tissues. Indeed, this favors a pro-inflammatory environment as well as maintains local and systemic inflammation in the body. Moreover people with higher BMI have impaired T and B cells' responses which causes increased vulnerability and delay in viral resolutions (Thus the inflammation ascending from COVID-19, may strengthen prevailing chronic inflammation in obese individuals and further enable viral growth and its spread leading to worst clinical outcomes (Mohammad et al. 2021). Obesity associated with comorbidities such as reduced pulmonary function, diabetes mellitus (Type 2), cardiovascular dysfunction, and hypertension may place people with obesity at high risk of the viral infection (COVID-19) and affect the overall health (Simonnet et al. 2020). Reports suggested an increase in C reactive protein (CRP) and decrease in lymphocyte count was observed in COVID-19 patients (Chen et al. 2020; Qin

et al. 2020; Zilong et al. 2020; Li et al. 2020). One of the interesting studies described that multiple types of chemokines such as G-CSF, GM-CSF, IP-10, MCP-1, MIP-1a, MIP-1b, RANTES, and IL-8 with higher level was observed in COVID-19 patients (Huang et al. 2020). Tang et al. (2020) reported the increase of IL-6 and IL-10 in COVID-19 patients. Not only this, the group also emphasized the crucial role of IL6 in the pathology of COVID-19 that results into chemotaxis of neutrophils and lymphocyte necrosis. Importantly, COVID-19 is more able to cause cytotoxic lymphocytes exhaustion.

Another important finding is obesity-induced chronic inflammation and impaired fibrinolysis which enhances the risk of thrombosis. That could be a probable mechanism potentially involved in lung injury and severe COVID-19 infection. These adverse effects are potentially involved in lung injury and severe COVID-19 infection. COVID-19 patients showed a marked increase in thrombotic complications due to hypercoagulability. Latest study described the acute lung injury in COVID-19 patients which involve fibrin deposition in the pulmonary microcirculation and formation of microthrombi (Paramo 2020). Another study reported that high fibrinogen and a high D-dimer are characteristic of patients with COVID-19 (Hayiroglu et al. 2020; Levi et al. 2020; Blasi et al. 2020).

These are the plausible mechanisms which could explain the increased risk of severe complications of COVID-19 for subjects with obesity.

The current pandemic situation of Coronavirus Disease 19 has focused worldwide research on its life-threatening risk factors. The initial data suggest, older individuals (age >60) suffering from any diabetes, cardiovascular disorders, kidney, or respiratory disorders are highly vulnerable to this viral infection. Table 12.2 describes the available data on the incidence of obesity and risk of COVID 19 fatality.

The very first report to focus on BMI data with small number of patients was conducted by Simonnet et al. 2020 from February 27 to 5 March 2020 at Lille, France and found high frequency of obesity among individuals admitted to intensive care (ICU) for COVID-19. Out of 124 patients independent of their age, sex, hypertension, and diabetes admitted in intensive care unit, 47.6% were obese (BMI > 30 kg/m^2), including class II obesity (BMI 35–39.9 kg/m^2) in 13.7% together with class III obesity (BMI ≥ 40 kg/m^2) in 14.5%.and 28.2% were under severe obesity (BMI > 35 kg/m^2). Identically, the requirement of invasive mechanical ventilation (IMV) in patients with BMI > 35 kg/m^2 was higher. Correspondingly, in New York, another study was conducted with a large number of obese adults. Researchers established a study on 3615 individuals with PCR positive cases for COVID-19 during 4 March 2020–4 April 2020 and found that 21% of individuals had BMI >35% and patients aged less than 60 are 2.0–1.8 times more potentially to be admitted to ICU with BMI between 30–34 kg/m^2 (Lighter et al. 2020). Similar study on 393 COVID positive patients in the same city was conducted in two different hospitals by Goyal et al in 2020. The author found that out of 393, 35.8% were obese during their admission at the hospital. He had also discussed about various serious symptoms, including hypertension, diabetes, COPD, asthma, as a few comorbidities in patients. Likewise, another investigation by Stefan et al. 2020 was conducted with gradually less no. of patients in Seattle region with 24 ill COVID-19 positive patients. The data indicated, among them three patients, were reported to lay under normal BMI category, whereas seven were overweight and 13 fall under obesity category. Further mechanical ventilation was required by 85% patients with obesity and 36% of the same were reported to be dead. Similarly, a study on admitted patients of COVID-19 positive was conducted in China, and they found that, out of 383 patients, 53.1% had normal weight with BMI 18.5–23.9 kg/m^2, 32.0% were found to be overweight with a BMI of 24–29.7 kg/m^2 and 10.7% were found to be obese with BMI ≥ 28 kg/m^2. Further the researchers also suggested that on comparison with non-obese patients, obese patients are more inclined to have cough and fever symptoms as well as overweight patients are 1.84-fold more likely to have severe symptoms of COVID-19 when compared with normal weight patients; however, obese individuals have

Table 12.2 The incidence of obesity and risk of COVID 19 fatality

S.no	Number of positive COVID-19 cases	Country/ city	BMI (kg/m^2)	Requirement of ventilator	Mortality	Reference
1.	124	Lille (France)	47.6% (BMI > 30 kg/m^2), 13.7% (BMI 35–39.9 kg/m^2), 28.2 (BMI > 35 kg/m^2)	68.6%		Simonnet et al. (2020)
2.	3615	New York	21% (BMI 30–34 kg/m^2), 16% (BMI ≥ 35 kg/m^2)	1.8 times 2.2–3.6 times	12%	Lighter et al. (2020)
3.	393	New York	35.8 %	43.4%	–	Goyal et al. (2020)
4.	24	Seattle	7 (Overweight) 13 (obese)	85%	36%	Stefan et al. (2020)
5.	383	China	32% (BMI 24.0–27.9 kg/m^2), 10.7% (BMI ≥ 28 kg/m^2)	3.40-fold		Cai et al. (2020)
6.	5566	New York	23.7% (BMI < 25 kg/m^2), 34.2% (BMI 25–29 kg/m^2), 32.8% (BMI 30–39 kg/m^2), 6.8% (BMI > 40 kg/m^2)	26.9% (BMI < 25 kg/m^2), 32.7% (BMI-25–29 kg/m^2), 30.7% (BMI 30–39 kg/m^2), 7.7% (BMI ≥ 40 kg/m^2)		Petrilli et al. (2020)
7.	5700	New York	41.7% (BMI ≥ 30)	12.2%	19.0 % (BMI ≥ 35)	Richardson et al. (2020)
8.	463	Detroit	57.6% 19.2% severe obese	2.0	–	Suleyman et al. (2020)
9.	51,633	Mexico	20.7%	5.0%	13.5%	Bello-Chavolla et al. (2020)

3.40-fold chances of developing severe disease (Cai et al. 2020), hence suggesting obese individuals are prone to develop this viral disease.

Gradually, after some awareness on this issue, more investigations worldwide are in process. Researchers are finding obesity to play a crucial factor in aggravating the COVID scenario. Subsequently, a large number of cohort studies was carried out in New York, which is also known to have a large number of obese adults. Petrilli et al. (2020) established a similar study in New York with larger number of patients; 5566 adults were found to be positive for COVID-19 and 2741 were needed to be admitted in hospital. Out of which, 650 (23.7%) had BMI < 25 kg/m^2, 939 (34.30%) had BMI ranging from 25–29 kg/m^2, 899 (32.8%) had a BMI between 30–39 kg/m^2, and 185 (6.8%) had a BMI > 40 kg/m^2. Whereas out of 990, 266 (26.9%) having BMI < 25 kg/m^2, 324 (32.7%) with BMI-25–29 kg/m^2, 304 (30.7%) with BMI 30–39 kg/m^2, and 70 (7.7%) having BMI ≥ 40 kg/m^2 were reported to be critically ill or in requirement of mechanical ventilator or intense critical care. Furthermore, an alternative case study in the same city was conducted among patients hospitalized with positive COVID-19, where obesity was regarded as a common comorbidity with hypertension and diabetes. Among 5700 patients, 41.7% (1737) had BMI ≥ 30 and 12.2% received Intensive mechanical ventilation, with 19.0% (BMI ≥ 35) mortality rate (Richardson et al. 2020). Recent research has also given more evidence on obesity being associated with COVID-19. A study was conducted on 463 and 50,633 hospitalized COVID-19 patients, where obesity was considered as comorbidity in Detroit and Mexico, respectively. In Detroit, 57.6% were obese and 19.2% were reported as severely obese, and severe obesity was associated with the need of intensive care admission and mechanical ventilation (Suleyman et al. 2020). Whereas in Mexico, obesity was mediated through diabetes with 20.7% obese patients, where 5% were

shifted to intensive care unit and a 13.5% mortality (Bello-Chavolla et al. 2020). Sattar et al. (2020) suggests excess fat deposition might be a unifying risk factor for COVID-19 that most likely dysregulated the immune system and reduced protective cardiorespiratory reserve, which majorly appears to be a part of this critical illness, thereby increasing mortality. Severity and vulnerability of COVID-19 in association with cardiac, nutritional alteration, and other comorbidities is not only limited to adults but also to children and adolescents being more susceptible to SARS-CoV-2 infection (Nogueira-de-Almeida et al. 2020). A new study in the medical journal The Lancet by Kass et al. (2020) reported an inverse correlation between BMI and age among 265 individuals, further suggesting the younger population with high BMI more likely to get affected by COVID-19.

India currently has the third-highest number of overweight or obese individuals among all the countries. A study on trends of overweight/obesity in India shows rise from 9% to 21% in case of women and 11% to 19% for men during the period between 2005 and 2016 (International Institute of Population Sciences 2017). On the one hand there is malnutrition in India and, on the other, the increase in overweight/obesity in the population of poor socioeconomic status may further increase vulnerability to infections as well as non-communicable diseases (Luhar et al. 2018). However, till date, no data are available on the risk of obesity and COVID 19 patient severity. Clinicians treating the disease in India have reported that a large number of COVID patients is overweight and obese and there is an urgent need to understand the link.

In conclusion, obesity in childhood and adolescence can be considered a risk factor for greater susceptibility and severity of COVID-19 and is associated with nutritional, cardiac, respiratory, renal, and immunological alterations, which may potentiate the complications of SARS-CoV-2 infection.

12.14 Lifestyle Modification in the Global Pandemic

The global pandemic has influenced and modified our lifestyle to a great extent. The lockdown imposed all over the world has a negative impact on diet, sleep, and physical activity. Maintaining a healthy lifestyle, while being isolated and safe has become the need of the hour. There are various reports in which the author has addressed the psychological impact of this pandemic situation, instead of lifestyle issues which mainly include increased screen-time, reduced physical activity, high fat diet intake (Balanzá-Martínez et al. 2020) further being inclined towards weight gaining. Being quarantined with unscheduled routine has created a monotonous lifestyle, and continuous hearing about the stressful pandemic situation might result in sleep disturbance, overeating of foods rich in fat, proteins, carbohydrates, and mostly the sugary comfort foods (Muscogiuri et al. 2020). All these activities are creating a risk factor for various health issues and chronic diseases, especially in young children and adults.

People all over the world must be encouraged to improve their lifestyle to lessen risk both in the current and subsequent waves of COVID-19. Better diet plans and sustainable lifestyle changes must be ensured so as to promote positive well-being. To ensure a healthy lifestyle, one must incorporate proper balanced diet and regular physical exercise, yoga, or meditation. The diet should include a variety of fresh fruits, vegetables, legumes, nuts, and whole grain to get the required amount of macronutrients (carbohydrates, fat, and protein), micronutrients (vitamins, water and minerals), and antioxidants. Vitamin C, vitamin E, zinc, copper, and Selenium are a few antioxidant nutrients, with fruits and vegetables as the primary source which help to prevent cell damage by free radicals, further boosting the immune system. Foods rich in probiotics and prebiotics such as yogurt, fermented,

and fibrous foods are known to support a healthy colony of microorganism in the gut and ease the bowel movement (Miller et al. 2017).

Sleep also plays an important role in maintaining a healthy lifestyle. Getting proper and quality sleep also helps in maintaining our physical, mental health. A regular sleep of 7–8 h is important to keep us healthy as it also boosts immunity.

Apart from a healthy diet, one must also include some sort of physical exercise in their schedules**.** Yoga and meditation are also considered as easy and inexpensive tools with long-term benefits on both physical health and mental condition. This being the pressing priority, it is able to improve overall fitness level, posture, and flexibility. Apart from improving physical health, yoga and meditation also clears our mind from regular stress.

12.15 Implications for Future Research

Although the risk of obesity with several chronic diseases is well known, emerging data indicate that obesity may have a link with the severity of COVID-19 infection. There is an increased prevalence of diseases such as diabetes, hypertension, cardiovascular diseases, and kidney dysfunction in obese individuals. All these conditions are major risk factors for disease severity and mortality associated with COVID-19. Hence, obesity is an additional risk factor associated with worse outcomes in COVID-19 patients. Insulin resistance is an important outcome of obesity which may play a key role in the altered immune response in the obese individuals. Hence it may be helpful if markers of insulin resistance are assessed to look at the association between insulin resistance and the severity of CoVID-19 disease. Most research data that were published about comorbid conditions, which may be associated with increased risk of severe COVID-19, did not provide data about body fat mass or metabolic health. It is imperative that data about fat mass and body composition be collected and further research be carried out. Further, it's time that we tackle and prevent obesity in our society not only for this viral pandemic but also for the prevention of various chronic diseases (Fig. 12.5).

Conflict of Interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

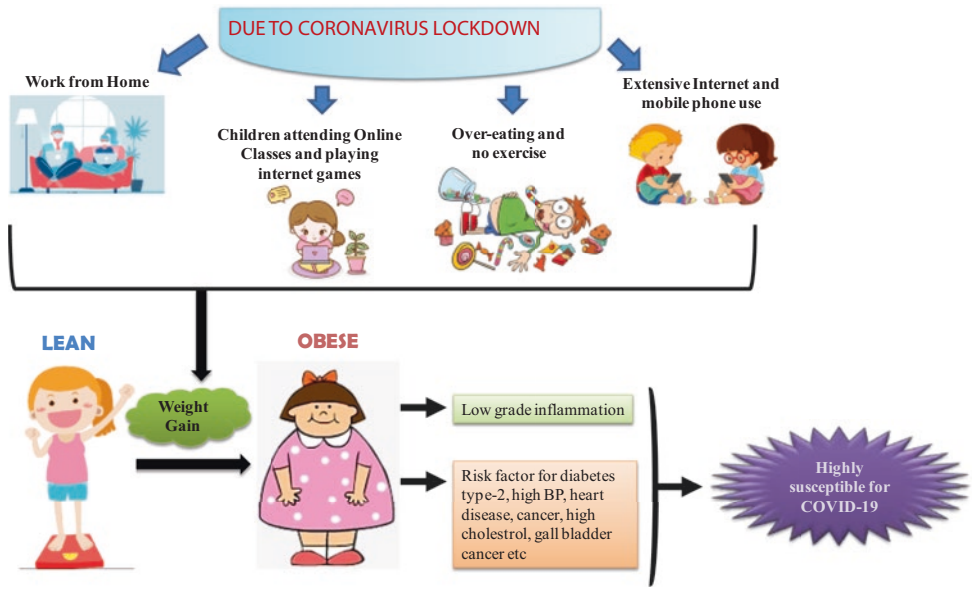


Fig. 12.5 Obesity-related comorbidities and high risk and severity of COVID-19 infection

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13 Cytokine Storm and Failed Resolution in COVID-19: Taking a Cue from Multiple Sclerosis

Insha Zahoor, Yue Li, Ramandeep Rattan, and Shailendra Giri

Abstract

Introduction: Excessive inflammatory responses and failed resolution are major common causes of tissue injury and organ dysfunction in a variety of diseases, including multiple sclerosis (MS), diabetes, and most recently, COVID-19, despite the distinct pathoetiology of the diseases. The promotion of the natural process of inflammatory resolution has been long recognized to improve functional recovery and disease outcomes effectively. To mitigate the excessive inflammation in MS, scientific investigations identified a group of derivatives of omega fatty acids, known as specialized pro-resolving lipid mediators (SPM) that have been significantly effective in treating preclinical disease models of MS.

Methods: This chapter is based on our observations from MS. It is being increasingly deliberated that the ongoing COVID-19 infection induces severe cytokine storm that ultimately triggers rampant inflammation. The impact of infection and associated mortality is much higher in patients with co-morbid diseases. Also, reports suggest a better outcome in diabetic patients with reasonable glycemic control, which certainly hints towards a hidden role of anti-hyperglycemic drugs such as metformin in alleviating disease pathology through its anti-inflammatory feature. Notably, SPM and metformin share common therapeutic features in exerting a broad-spectrum anti-inflammatory activity in human patients with a superior safety profile.

Results: When there is an immediate need to encounter the fast-rampant infection of COVID-19 and control the viral-infection associated morbid inflammatory cytokine storm causing severe organ damage, SPM and metformin should be seriously considered as a potential adjunctive treatment.

Conclusion: Given the fact that current treatment for COVID-19 is only supportive, global research is aimed at developing safe and effective therapeutic options that can result in a better clinical course in patients with comorbid conditions. Accordingly, taking a cue from our experiences in controlling robust inflammatory response in MS and diabetes by simultaneously inhibiting inflammatory process and stimulating its resolution, combinatorial therapy of metformin and SPM in COVID-19

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holds significant promise in treating this global health crisis.

Keywords

Combination therapy · COVID-19 · Cytokine storm · Inflammation resolution · Multiple sclerosis · Resolution mediators

Abbreviations

17-HDHA	17-hydroxydocosahexaenoic acid
ACE2	angiotensin-converting enzyme 2
AMPK	adenosine monophosphate activated protein kinase
ARB	angiotensin II type I receptor blockers
ARDS	acute respiratory distress syndrome
ATLXA4	aspirin-triggered 15-epi-lipoxin A4
BBB	blood brain barrier
COPD	chronic obstructive pulmonary disease
COVID-19	coronavirus disease 2019
CRP	C-reactive protein
EAE	experimental autoimmune encephalomyelitis
FDA	Food and Drug Administration
GPCR	G-protein coupled receptors
ICU	intensive care unit
IFN-γ	interferon-gamma
IL	interleukin
IP-10	IFN-γ–induced protein 10
LDH	lactic acid dehydrogenase
LXA4	lipoxin A4
LXB4	lipoxin B4
MCP-1	monocyte chemoattractant protein 1
MERS	middle east respiratory syndrome
MS	multiple sclerosis
NIH	National Institutes of Health
NPD	neuroprotectin D1
NSAID	non-steroidal anti-inflammatory drugs
PDX	protectin DX
PMN	polymorphonuclear neutrophils
PUFA	polyunsaturated fatty acids
RvD	resolvin D
SARS	severe acute respiratory syndrome
SARS-Co-V-2	severe acute respiratory syndrome coronavirus 2
SPM	specialized pro-resolving lipid mediators
STAT3	signal transducer and activator of transcription 3
TNF-α	tumor necrosis factor-alpha

13.1 Introduction

Inflammation is considered as a physiologically protective in-built immune response to external and internal insults such as injury (burn, trauma, and wound), infection by pathogenic attack (bacteria, fungi, and viruses), and surgical procedure (Kumar et al. 2014). It is a desirable self-limited defense mechanism used by the body to retaliate against any damage due to harmful stimuli, eliminating the threat to the normal functioning of the body. The immunologic players involved in the activation and sustenance of physiologic inflammatory response include classical lipid-derived pro-inflammatory mediators known as eicosanoids, cytokines, chemokines, and immune cells (Duffy et al. 2014; Nathan 2002). Under normal conditions, inflammation is actively resolved in a timely manner by a highly coordinated process of resolution, mediated by an endogenously synthesized family of omega fatty acid-derived specialized pro-resolving lipid mediators (SPM), including resolvins, lipoxins, maresins, and protectins (Serhan 2004; Serhan et al. 2004; Serhan and Chiang 2004). Interestingly, this transition is governed by switch-over within the resolution window, by shifting synthesis of pro-inflammatory lipid mediators to resolution mediators, which shows how onset signals termination (Levy et al. 2001) (Fig. 13.1). During the process of inflammation and its resolution, there occur a plethora of changes affecting the cellular permeability and immune cell trafficking (Nathan 2002). SPM pro-

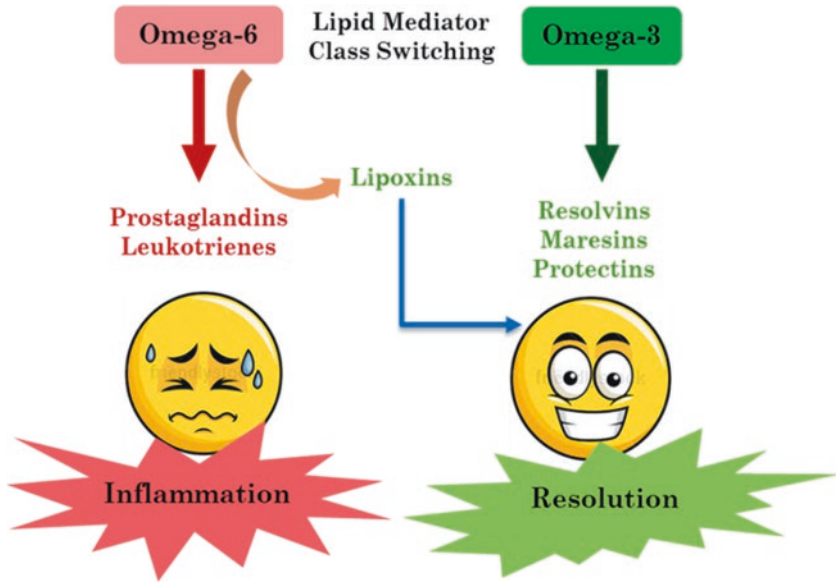

Fig. 13.1 Inflammation-driven resolution showing how onset signals end
While inflammation is governed by the classical pro-inflammatory mediators such as chemokines, cytokines, and omega-6 derived eicosanoids in the initial phase, the transition from the synthesis of pro-inflammation to pro-resolution mediators, mediated by class switching of lipid mediators marks the resolution phase

mote inflammation resolution by acting through specific G-protein coupled receptors (GPCR), which subsequently results in the regulation of pro-inflammatory cytokine and chemokine production, macrophage-based phagocytosis of dead cells and pathogens, leukocyte trafficking, and recruitment of polymorphonuclear neutrophils (PMN) and monocytes (Serhan 2014). Resolution is a restorative process meant to clear the inflammatory exudate and repair the damage caused by inflammation and hence re-establish the normal cellular homeostasis essential for maintaining a healthy state (Serhan 2014; Serhan and Savill 2005). However, its failure leads to uncontrolled and unresolved inflammation, causing disturbance in the homeostatic status that gives rise to a pathological state of chronic inflammation (Nathan and Ding 2010). This makes the timely resolution of inflammation critical in health and disease.

Dysregulated immune response and failed resolution pose the biggest challenge in treating inflammatory conditions. Multiple reports have shown altered levels of resolution mediators in various disease models owing to the defects in their biosynthesis or their receptors through which they act, causing catastrophic inflammation as the trigger for several inflammatory diseases (Dalli 2017; Serhan 2017; Zahoor and Giri 2020). Consequently, SPM have shown beneficial effects in preclinical models of several human diseases, including multiple sclerosis (MS), neuromyelitis optica, Alzheimer's disease, diabetes, asthma, stroke, sepsis, obesity, rheumatoid arthritis, atherosclerosis, and cancer (Nathan and Ding 2010; Abdolmaleki et al. 2020; Kooij et al. 2019a; Poisson et al. 2015; Shang et al. 2019; Wang et al. 2015, 2019) (Fig. 13.2). Ongoing research is primarily focused on promoting inflammation resolution by using these intrinsic resolution mediators as prospective therapeutic candidates for treating multiple inflammatory and infectious conditions (Dalli 2017; Serhan 2017; Zahoor and Giri 2020; Kooij et al. 2019a; Poisson et al. 2015; Russell and Schwarze 2014). There are also some ongoing clinical trials exploring the impact of SPM on the inflammation parameters and resolution in human inflammatory conditions such as acute tissue inflammation and MS (ClinicalTrials.gov 2018; ClinicalTrials.

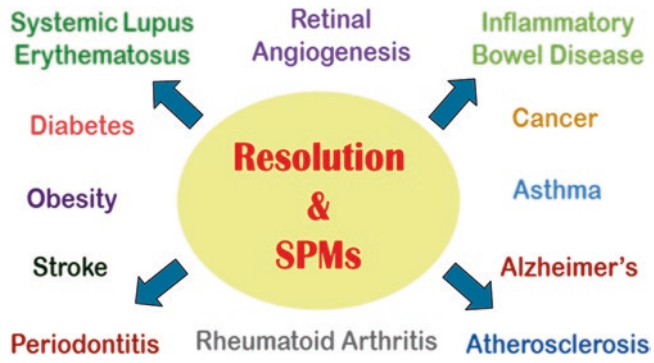

Fig. 13.2 Resolution and SPM in the backdrop of inflammation
Defective resolution and altered levels of resolution mediators (SPM) due to dysfunction in their biosynthetic pathway or receptors are involved in the pathogenesis of several inflammatory diseases

gov 2020a). Based on our work on inflammation resolution in MS, we put forth our perspective in treating COVID-19-induced robust inflammation. Further discussion is based on unresolved inflammation common to MS and COVID-19.

13.2 Metformin and SPM Therapy in COVID-19: A Perspective from MS

Multiple sclerosis (MS) is the leading non-traumatic neuroinflammatory disease that occurs due to hyperimmune response against components of nervous system, mediated primarily by autoreactive T cells (Reich et al. 2018). There is mounting evidence to suggest the role of environmental (non-infectious or infectious) and genetic factors in altering disease risk, with exact trigger behind disease development still unknown, which complicates its accurate diagnosis, prognosis, and management. Owing to the immune-mediated nature of this chronic debilitating disease, the ongoing research is aimed at mitigating the inflammation by promoting endogenous mechanisms of resolution. As a result, there have been few metabolomics studies showing an imbalance in the levels of immunoresolvent mediators (SPM) and their precursor omega polyunsaturated fatty acids (PUFA) in patient-derived samples (Kooij et al. 2019a; Poisson et al. 2015; Aupperle et al. 2008; Bjornevik et al. 2017; Bjornevik et al. 2019; Kooij et al. 2019b; Pruss et al. 2013). The altered resolution mediators include, resolvin D (RvD1, 3, 5), lipoxin A4 (LXA4), lipoxin B4 (LXB4), aspirin-triggered 15-epi-lipoxin A4 (ATLXA4), neuroprotectin D1 (NPD1), and protectin DX (PDX). Interestingly, the current MS studies are based on resolving inflammation using SPM treatment on preclinical cellular and animal models of the disease (Zahoor and Giri 2020). Based on few studies of SPM treatment in experimental autoimmune encephalomyelitis (EAE) model of MS, the possible mechanism of amelioration in disease progression and improved pathology involves protective actions of resolution mediators on various aspects of immune response that result in modification of cellular response to promote resolution phase of inflammation (Kooij et al. 2019a; Poisson et al. 2015; Kooij et al. 2019b) (Fig. 13.3). The key resolution mechanisms involve the polarization of T cells and macrophages towards anti-inflammatory phenotype, reduction in the overall synthesis of pro-inflammatory mediators, attenuation of monocyte activation and transendothelial migration, and inhibition of inflammation-induced blood brain barrier (BBB) dysfunction. Like MS, the ongoing global pandemic of coronavirus disease 2019 (COVID-19) also has excessive inflammation as a key element in its pathophysiology. The findings from MS research suggest potential role of inflammation resolution

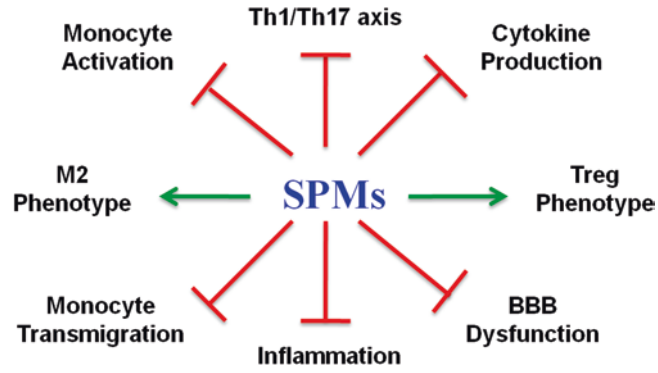

Fig. 13.3 Several mechanisms mediate the protective effects of SPM treatment on cellular and animal models of MS, resulting in overall amelioration of disease pathology
SPMs, in relation to MS, have been found to inhibit autoreactive T cells, pro-inflammatory cytokine production, monocyte activation, inflammation-induced blood brain barrier (BBB) dysfunction, and transendothelial migration of monocytes. They also induce T regulatory cells and promote polarization of macrophages and microglia towards anti-inflammatory M2 phenotype, resulting in overall inhibition of inflammation

in combating this recent uncontrolled inflammatory condition.

COVID-19 is caused by infection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), with its first outbreak reported in December 2019 in Wuhan, China (Lai et al. 2020). It has already led to over 100,000 deaths across the United States. The number of infected cases and mortality are still rising throughout the world due to its high transmission rate and lack of effective treatment. Currently, our knowledge of this novel infectious disease is minimal. However, a prominent hyperinflammatory response induced by the viral infection has been observed in patients of all age groups and strongly associated with disease severity and death. It has been found that the infection primarily starts from the respiratory system and causes pneumonia and acute hypoxia due to massive pulmonary hyperinflammation that often induces life-threatening chemokine/cytokine response, often termed as the 'cytokine storm' syndrome (Mehta et al. 2020). While some patients have mild symptoms and recover on minimal medical intervention, many others show rapid deterioration and develop complications like cardiac inflammation, lung failure, blood clots, kidney failure, sepsis, brain inflammation, and even death (Chen et al. 2020a; Chen et al. 2020b; Huang et al. 2020). It has been observed that people of any age having uncontrolled comorbid conditions are at the highest risk for COVID-19 due to their impact on disease outcome and prognosis. These underlying conditions mainly include hypertension, cardiovascular disease, diabetes, chronic respiratory disease, cancer, renal disease, and obesity (Cai et al. 2020; Guan et al. 2020; Centers for Disease Control and Prevention 2020; Wu et al. 2020; Yang et al. 2020a; Zhang et al. 2020). Also, age has a substantial impact on the disease course, clinical characteristics, comorbidities, outcomes, and prognosis due to the effect of ageing on the functional status of biological system that apparently results in immunological and physiological changes (Miller and Linge 2017; Du et al. 2020; Liu et al. 2020; Zhou et al. 2020). There are reports that have found worse outcomes and higher mortality associated with COVID-19 in aged patients (Wu et al. 2020; Yang et al. 2020a; Du et al. 2020; Liu et al. 2020; Zhou et al. 2020). These findings make it very important to identify risk groups and take into account age factor while evaluating and treating such patients.

The robust hyperimmune response observed in infected patients leads to the activation of inflammasomes, which induce eicosanoid storm due to elevated levels of pro-inflammatory mediators and hypercytokinemia that fuel multiple organ damage and acute respiratory distress syn-

drome (ARDS) in critically ill patients (Fung et al. 2020; Yuki et al. 2020). Serum profiling of patients has revealed that the inflammatory rampage is characterized by disproportional activation of tumor necrosis factor-alpha (TNF-α), interleukins 1, 2, 6, 7, 8 (IL-1, IL-2, IL-6, IL-7, IL-8), and monocyte chemoattractant protein 1 (MCP-1) (Mehta et al. 2020; Huang et al. 2020; Coperchini et al. 2020; Ye et al. 2020). Studies from China have identified biomarkers of fatality and progression in patient-derived samples that included elevated levels of ferritin, IL-6, IL-10, TNF-α, and several other inflammatory markers which certainly point towards the virus-induced hyperinflammatory response; however, plasma levels of IFN-γ–induced protein 10 (IP-10) and MCP-3 were highly associated with severity and disease progression (Ruan et al. 2020; Yang et al. 2020b). There are also reports of immune dysregulation in COVID-19 that primarily affects T lymphocytes by causing a significant reduction in counts of CD4+ and CD8+ T cells and decrease in IFN-γ production by CD4+ T cells, particularly in severe cases requiring intensive care unit (ICU) than moderate cases (Chen et al. 2020a; Diao et al. 2020). Further, there is a case report of overactivation of T cells that resulted in an increase of Th17 cells and high cytotoxicity of CD8 T cells, which in part accounted for severe immune injury observed in the infected patient (Xu et al. 2020). This highly derailed immunological profile of patients suggests compromised resolution pathways in infected individuals, which results in loss of ability to restore cellular homeostasis.

To date, the overall treatment of COVID-19 is supportive, as there are no approved effective vaccines or antiviral medications against this highly contagious virus with current research efforts aimed at finding the one. This necessitates the need for multi-center research collaborations to find a rapid and effective treatment for the disease that could lower the magnitude of inflammatory response and cytokine storm in the initial stage to combat the fatality of the disease. At present, there are precisely no treatment options with absolute safety profile; however, some of the treatment strategies which are being investigated come from the theoretical knowledge of past experiences on viral infections such as, severe acute respiratory syndrome (SARS) and middle east respiratory syndrome (MERS), and other inflammatory conditions. Commonly used treatment options include antiviral therapy, immunomodulators, antithrombotic therapy, and cytokine antagonists (COVID-19 Treatment Guidelines Panel 2019). The National Institutes of Health (NIH) has maintained an exclusive website on COVID-19 treatment guidelines based on the outcome from ongoing studies, to provide the most updated and refined recommendations on different treatment modalities (COVID-19 Treatment Guidelines Panel 2019). A very recent report has evaluated the clinical efficacy of antiviral drug remdesivir in a double-blind, randomized, placebo-controlled trial in 1063 coronavirus infected patients with lower respiratory tract involvement (Beigel et al. 2020). Their preliminary findings showed that intravenous remdesivir was efficient in improving the outcome in patients with severe disease by shortening the recovery time compared to placebo. At the same time, the mortality rate was still higher, which accentuates the use of combinatorial therapy to augment the efficacy of known antivirals. Based on the evidence to control cytokine storm in rheumatoid arthritis by using IL-6 antagonists, tocilizumab, a monoclonal antibody has been shown to be effective in treating severely ill patients with higher serum levels of IL-6 (Biggioggero et al. 2019; Tanaka et al. 2016). On that basis, a randomized controlled trial has been started to test the safety and efficacy of tocilizumab on Chinese COVID-19 patients with pneumonia and raised IL-6 (Chinese Clinical Trial Registry 2020). However, there is still lack of concrete evidence on the use of IL-6 inhibitors (sarilumab, siltuximab, tocilizumab) (COVID-19 Treatment Guidelines Panel 2019). The much-hyped hydroxychloroquine is another drug that has been tried in few cases but lacks scientific acceptance due to poor quality of data, which has forced clinical trials in the United States and other parts of the world to test its safety and efficacy in combination with azithromycin (ClinicalTrials.gov 2020b). Altogether, there is insufficient evidence

in support of any treatment; however, management strategies being used are decided on the basis of the severity of patients. The need of the hour is to identify the strategy aimed at controlling the disproportionally activated inflammatory responses in COVID-19 infection using protective therapies with proven effectiveness and safety profile to address the immediate need to fight off the virus-induced pathology.

The endogenous resolution pathways have been recognized in the cessation of infectious pathologies like influenza due to the regulation of antiviral B lymphocytic activity of resolution mediators (Morita et al. 2013; Ramon et al. 2014; Tam et al. 2013). There is prior evidence to support the role of SPMs or their precursors to confer protection against influenza by promoting adaptive immune response that has led to the recognition of their potential as vaccine adjuvants, especially for 17-hydroxydocosahexaenoic acid (17-HDHA) (Morita et al. 2013; Ramon et al. 2014). This indirectly provides compelling evidence of exploiting SPM or their precursors as potential therapeutic interventions for suppressing immune response and hypercytokinemia to achieve a better clinical outcome in infectious diseases like COVID-19. Also, there is evidence of protectin D1 to significantly attenuate replication of the influenza virus and reduce its severity in a mice model, even when regular antiviral drugs do not come to the rescue (Morita et al. 2013), implicating the therapeutic potential of SPM in lethal COVID-19 infection. There is a previous report that has studied the effect of exercise on pro-inflammatory and pro-resolving lipid mediators, and they have suggested against the use of non-steroidal anti-inflammatory drugs (NSAID) such as ibuprofen due to its ability to block exercise-induced prostaglandin increase and more specifically it resulted in a decrease in the levels of resolution mediators after recovery from exercise (Markworth et al. 2013). This could severely affect the outcome in COVID-19 individuals, which supports the use of resolution mediators as promising therapeutic options for promoting the resolution of inflammation. SPM therapy has an added advantage over conventional anti-inflammatory agents, which completely block the inflammation in its initial phase rather than downregulating by promoting its resolution and are associated with adverse effects. Based on the past evidence of inflammation resolution with SPM treatment in preclinical disease models, reports are strongly suggesting using SPM or their precursors in treating COVID-19 infection in conjunction with other supportive therapies (Panigrahy et al. 2020; Regidor 2020).

Moreover, human coronavirus enters into cells by binding to angiotensin-converting enzyme 2 (ACE2), which is a receptor expressed on cellular epithelia of lungs, kidneys, and blood vessels (Wan et al. 2020). Some studies have reported the upregulation of ACE in hypertensive and diabetic patients, and that treatment with ACE inhibitors and angiotensin II type I receptor blockers (ARB or angiotensin II inhibitors) further stimulates its expression (Li et al. 2017). Also, there is evidence of its increased expression by thiazolidinediones and ibuprofen. These findings suggest the possibility of higher infectivity of COVID-19 in patients with underlying conditions of diabetes and hypertension because the higher expression of ACE2 fairly eases entry of the virus into the system, and that somehow explains higher complications and fatality in such cases (Fang et al. 2020). With higher infectivity, there is a comparatively massive inflammatory response that induces marked increase in blood sugar level leading to diabetic ketoacidosis, which confers poor outcomes in such cases. A recent study found that COVID-19 patients with pre-existing type 2 diabetes are at increased risk of a poor outcome, especially those with poorly controlled blood glucose (Zhu et al. 2020). Moreover, the fast deterioration in these patients was associated with higher serum levels of neutrophil counts and inflammatory markers, including IL-6, C-reactive protein (CRP), and lactic acid dehydrogenase (LDH). Metformin has been recently found to inhibit IL-6 signaling and reduce CRP and LDH in patients (Kahn et al. 2010; Karam and Radwan 2019; Mishra and Dingli 2019), vouching for its use as a potential therapy to improve the outcome of COVID-19 infection through an anti-inflammatory mechanism.

Metformin is currently the single most prescribed, oral anti-diabetic drug in the US and worldwide, with long history of being safely used in humans (~ 60 years) and about 100 million patients taking it every day (Holman 2007). Apart from the glucose-lowering property, it has several pleiotropic therapeutic effects, including anti-inflammatory and antioxidant properties, and the ability to improve endothelial function (Karam and Radwan 2019; Forouzandeh et al. 2014; Hattori et al. 2015). Our previous work on EAE model of MS treated with metformin provides direct evidence in support of the therapeutic role of metformin in attenuating inflammation by down-regulating pro-inflammatory cytokine production, promoting expression of anti-inflammatory cytokine IL-10, and overall inhibition of T cell-mediated immune response in EAE (Nath et al. 2009). Similarly, we have reported its beneficial effects in reducing cancer growth and promoting better survival in ovarian cancer, which further emphasizes its therapeutic potential, making it a budding treatment option for several inflammatory conditions (Al-Wahab et al. 2015; Kumar et al. 2013; Tebbe et al. 2014). Its role has also been implicated in mitigating mortality associated with diabetes in patients with chronic obstructive pulmonary disease (COPD) (Zhu et al. 2019; Ho et al. 2019). Metformin drives its anti-inflammatory effects by targeting macrophages via regulating monocyte-macrophage differentiation and macrophage polarization involving stimulation of adenosine monophosphate activated protein kinase (AMPK) activation and signal transducer and activator of transcription 3 (STAT3) inhibition (Hattori et al. 2015; Vasamsetti et al. 2015). Keeping in view high virulence, infectivity, pathogenicity, and fatality of COVID-19, it is highly recommended to identify and control the disproportionally activated lethal inflammatory responses using therapies with proven effectiveness and safety profile to address the immediate need to reduce the rising global mortality. The cumulative outcome from above-discussed findings suggests that both SPM and metformin are relatively safe for human use and have a significant role in regulating inflammation. Metformin is already an approved drug by US Food and Drug Administration (FDA), which will save both time and money related to the drug development, and will be easy to move into clinical trials for targeting COVID-19. Based on the current data and considering the urgency in finding rapid and effective drug combinations with a good safety profile, a combination of metformin and SPM seems to be an effective approach to encounter the COVID-19 infection as both have a different mode of action. Metformin treatment will abrogate inflammation and oxidative stress, and SPM will resolve inflammation by inducing efferocytosis and possibly reduce virus replication (Fig. 13.4).

13.3 Conclusions

Inflammation resolution has become the core of recent therapeutic approaches for targeting inflammation as underlying cause of several inflammatory conditions, such as autoimmune, metabolic, neurodegenerative, respiratory, and cardiovascular diseases. In recent years, the logistics of diabetes treatment has evolved beyond merely glycemic control to a stage that anti-inflammation became essential in improving the dire macrovascular and microvascular complications. As a proven first-line drug for blood glucose control in type 2 diabetes, metformin has been increasingly recognized to have pleiotropic therapeutic properties, including anti-inflammation and improve vascular endothelial functions. On that basis, our work on MS involving simultaneous inhibition of inflammation and promoting its resolution in cellular and animal models emphasizes the utility of metformin and resolution mediators (SPM) in treating hyperinflammatory conditions. Both SPM and metformin have a fairly good safety profile for human use. Considering overwhelming inflammatory response in form of ‘cytokine storm’ in COVID-19, there is an urgent need to combat that process and its associated damage, which leads to multiple organ failure and even death. Altogether, taking perspective from MS and diabetes, there is a possible role of metformin and SPM as a novel

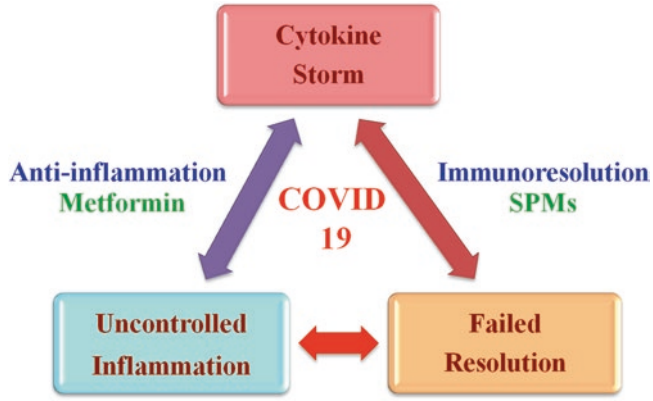

Fig. 13.4 Metformin and SPM as adjunctive therapy for COVID-19
A novel combinatorial therapeutic approach using metformin and SPM as potential intervention in COVID-19, which would possibly abrogate robust inflammatory response by simultaneously inhibiting it and promoting its resolution

combinatorial therapy that should be tested on COVID-19 patients under present crisis situation.

Acknowledgments This chapter is a very special contribution from our research based on inflammation resolution in multiple sclerosis. It serves as a dedication to all precious lives that have been and are continually being affected by the invisible viral enemy of humankind until its existence in the population, urging world communities to develop effective treatment strategies to contain the infection. We hope that our small endeavor through therapeutic interventions would make some difference in managing COVID-19. Through this chapter, we would like to send our message across the world that let us pledge to come together, breaking all human-made barriers, and fight this humanitarian crisis through collaborative research efforts and make this planet a better place to live. This work is in part supported by the National Multiple Sclerosis Society (US) Research Grant (RG-1807-31964 and RG-1508-05912), the US National Institutes of Health Grant (NS112727 and AI144004), and Henry Ford Hospital Internal Grant (A10270 and A30967) to SG. The funders had no role in study design, data collection, and interpretation, or the decision to submit the work for publication.

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Ethical Approval for Studies Involving Human This article does not contain any studies with human participants performed by any of the authors.

Ethical Approval for Studies Involving Animals This article does not contain any studies with animals performed by any of the authors.

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