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Lost in deletion: The enigmatic ORF8 protein of SARS-CoV-2

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ABSTRACT

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) genome contains nine open reading frames (ORFs) that encode for accessory proteins which, although dispensable for viral replication, are important for the modulation of the host infected cell metabolism and innate immunity evasion. Among those, the *ORF8* gene encodes for the homonymous multifunctional, highly immunogenic, immunoglobulin-like protein that was recently found to inhibit presentation of viral antigens by class I major histocompatibility complex, suppress the type I interferon antiviral response and interact with host factors involved in pulmonary inflammation and fibrogenesis. Moreover, the *ORF8* is a hypervariable gene rapidly evolving among SARS-related coronaviruses, with a tendency to recombine and undergo deletions that are deemed to facilitate the virus adaptation to the human host. Intriguingly, SARS-CoV-2 variants isolated in the beginning of the coronavirus disease 2019 (Covid-19) pandemic that were deleted of the *ORF8* gene have been associated to milder symptoms and better disease outcome. This minireview summarizes the current knowledge on the SARS-CoV-2 ORF8 protein in perspective to its potential as antiviral target and with special emphasis on the biochemical, biophysical and structural aspects of its molecular biology.

1. Introduction

At the time of writing, the pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), for which the notorious coronavirus disease 2019 (Covid-19) term was coined, has counted more than thirty-seven million infected cases and more than one million victims worldwide [1]. Together with the SARS-CoV and the Middle East respiratory syndrome coronavirus (MERS-CoV), the Covid-19 etiologic agent is the third highly pathogenic coronavirus that emerged in the last two decades [2]. Therefore, not only such death toll is a bitter reminder of the burden posed to global health by the spillover of zoonotic viruses into human population, but is also a warning that members of this viral group may undergo cross-species transmission and cause pandemics with high mortality rates even in the future. SARS-related coronaviruses (SARSr-CoVs) are enveloped, non-segmented, positive-sensed, single stranded RNA (ssRNA) viruses that belong to the *Sarbecovirus* subgenus (or lineage B) of the genus *Betacoronavirus* of the family *Coronaviridae*, which groups in the order *Nidovirales* of the realm *Riboviria* [3]. In the SARS-CoV-2 species, the long (≈30 kb) ssRNA genome is organized into 15 open reading frames (ORFs), which encode for up to 29 proteins. Of those, four structural ones, namely the spike (S), the envelope (E), the membrane (M) and the nucleocapsid (N) proteins are encoded by homonymous ORFs and are primarily important for viral entry, virion integrity, immune evasion and genome packaging, respectively. Sixteen non-structural proteins (Nsp) are encoded by the *ORF1a* (Nsp1-11) and *ORF1ab* (Nsp12-16) genes, and are involved in replication and transcription of the viral genome, immune evasion as well as in processing of viral proteins and nucleic acids [4,5]. Nine accessory proteins - termed as ORF3a, 3b, 6, 7a, 7b, 8, 9a, 9b and 10 - are encoded by homonymous ORFs and, although deemed as non-essential for the virus replication, are thought to exert important functions in modulating the host infected cell metabolism and antiviral immunity [4,6,7]. Moreover, whereas the SARS-CoV-2 genome organization follows the pattern shared by other members in the *Coronaviridae* for the genes encoding the structural and non-structural proteins, those encoding for the accessory ones vary among coronaviral species by number, location, denomination and display low sequence similarity [8,9]. Since the beginning of the Covid-19 pandemic, profound endeavors have been made by the scientific community, aimed at either the development of a vaccine or the identification of drugs targeting SARS-CoV-2 proteins [10,11]. In this effort, the homologs of well characterized antiviral targets in SARS-CoV and MERS-CoV - such as

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the S glycoprotein, the Nsp5 3-chymotrypsin-like main protease, the Nsp3 papain-like protease, the Nsp13 helicase and the Nsp12 RNA-dependent RNA polymerase catalytic subunit – have thus far gained major attention [11,12]. Nevertheless, given their involvement in virulence and pathogenesis, the set of accessory proteins encoded by the SARS-CoV-2 genome may represent promising and attractive options as novel targets for therapeutic intervention. Within this picture, the ORF8 protein is one of particular interest and very challenging to investigate, given that it is the most variable accessory protein among those encoded by SARSr-CoVs and because of its tendency to undergo mutations that may correlate to the epidemic trend and to the adaptation to new host species [13,14]. This minireview summarizes the current state of knowledge on the SARS-CoV-2 ORF8 protein. Moreover, the origin and the evolution of the SARS-CoV-2 *ORF8* gene, together with a comparative analysis of its translated product with those of the SARS-CoV, have been extensively discussed in a recent review article [15]. The focus here is on recent findings on the SARS-CoV-2 ORF8 structure and functions, with the aim to describe the features of this protein from a biophysical point of view with an emphasis on its role in the subversion of the innate immune system and the potential as antiviral target for drug development or repurposing.

2. The functionally elusive, yet dispensable, SARS-CoV-2 *ORF8* gene

The SARS-CoV-2 *ORF8* gene spans 366 nucleotides (nt), is located between position 27,894 and 28,259 of the virus genome, following the *ORF7b* and preceding the *N* genes, respectively, and encodes for a 121 amino acid-long ORF8 protein (NCBI reference sequence NC_045512.2, Gene ID 43740577 and Protein ID YP_009724396.1) (Fig. 1A) [16]. The *ORF8* gene is part of a hypervariable genomic region of ~ 430 bp in length that has been recognized as a recombination hotspot, also highly susceptible to deletions and nt substitutions [13]. Such region was found in SARS-CoV as well as in SARSr-CoVs of bat and pangolin origin, among which the *S* and *ORF8* are the most divergent genes. Noteworthy, this well correlates with the proposed theory of SARS-CoV-2 originating from recombination events between horseshoe bat coronaviruses, as well as with the plausible role of pangolins as intermediate hosts that preceded SARS-CoV-2 spillover into human population [4,17]. The SARS-CoV-2 genome evolved early during human-to-human transmission and diverged into at least three major phylogenetic groups across the world. One of those was characterized by the emergence of single point mutations at genomic positions 28077 and 28144 of the *ORF8* gene, resulting in a Valine to Leucine substitution of residue 62 and a Leucine to Serine substitution of residue 84 in the ORF8 protein, respectively [18–21]. Another point mutation, found in co-presence with the L84S, substitutes a Histidine at residue 112 with a Glutamine [22]. Deletions affecting the ORF8 amino acid length have been also reported. The one of a single nt at position 28254 (Δ 1) causes, by introducing a frameshift, the loss of the last Isoleucine residue and the addition of five new C-terminal residues (Ser-Lys-Arg-Thr-Asn), whereas the one of 6 nt between positions 28090–28095 (Δ 6) results in the substitution of three internal residues (Gly66-Ser67-Lys68) with a Glutamic acid [16]. Besides, a cluster of SARS-CoV-2 mutant strains bearing a 382 nt deletion that overlaps the *ORF7b* and *ORF8* genes by 366 nt (namely Δ382 *ORF8*, located between genomic positions 27848 and 28229) was identified from clinical specimens of patients hospitalized in Singapore and Taiwan. Furthermore, similar deletions in the *ORF7b/8* region were observed in viral genome sequences from Australia (Δ138), Bangladesh (Δ345) and Spain (Δ62) (Fig. 1B) [23–25]. The phylogenetic analysis of the Δ382 *ORF8* related genomes, and the travel history from Wuhan reported by at least one of the screened patients in Taiwan and Singapore, allowed to trace back the Δ382 *ORF8* mutation to the Covid-19 epicenter in the Hubei province capital. This demonstrates that such deletion emerged already in mid-December 2019, hence at the beginning of the pandemic [23,24]. The Δ382 mutation removes 40 nt from the end of the *ORF7b* gene, 6 nt from the intergenic region and 336 nt from the *ORF8* gene, including its transcription regulatory element. As a result, expression of the ORF8 is abolished, and a hybrid protein consisting of an ORF7b that lacks its last 12 amino acids and is

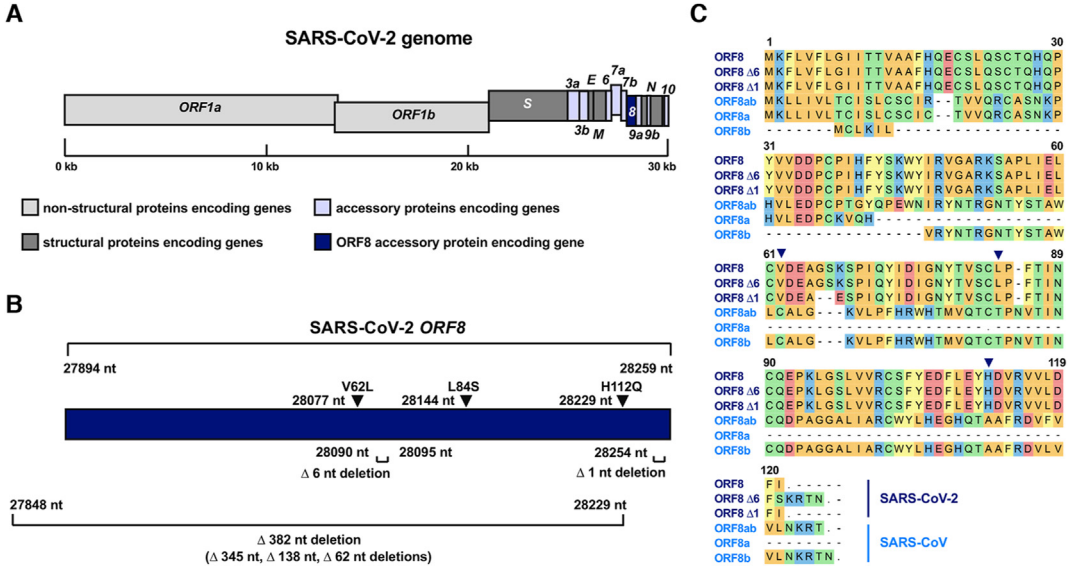

Fig. 1. Hypervariability of the SARS-CoV-2 *ORF8* gene. (A) Schematic organization of the SARS-CoV-2 genome; genes encoding non-structural proteins and structural ones are highlighted in light and dark gray, respectively; genes encoding accessory proteins are highlighted in light pink, whereas the *ORF8* gene is highlighted in dark blue. (B) Currently identified mutations affecting the SARS-CoV-2 *ORF8* gene; nt positions and type of mutation are indicated above and below the gene bar. (C) Amino acid sequence alignment between SARS-CoV-2 and SARS-CoV ORF8 protein variants; WT SARS-CoV-2 ORF8, Δ6 nt and Δ1 nt deletion mutants are marked in dark blue, whereas SARS-CoV ORF8ab, 8a and 8b are marked in light blue; positions of amino acid substitutions in SARS-CoV-2 ORF8 are marked with black triangles.

fused to the C-terminal 5 residues of ORF8 is putatively translated [24]. Nevertheless, such genetic loss did not affect the Δ382 *ORF8* virus spreading capability, given that - initially transmitted as co-infection with the WT - this strain has become the dominant found in a single patient and the one more frequently detected among secondary cases descending from the original cluster. Also, no differences in replication kinetics, gene expression and viral load were observed between the WT and the Δ382 *ORF8* SARS-CoV-2 in vitro, which supports the notion that the mutant virus is unaffected in its replicative fitness [23,25]. On the other hand, a phenotypical analysis in a cohort study reported that, although leading to clinically important illness with pneumonia, infection with the Δ382 *ORF8* variant was associated to milder symptoms with later onset, lower probability to develop hypoxia and a better disease outcome with respect to WT. Possibly, this is due to a more efficiently elicited immune response in the absence of ORF8. Consistently, hallmarks of severe Covid-19, such as high levels of systemically-released pro-inflammatory cytokines, chemokines and growth factors associated to lung injury were all found at lower concentrations in Δ382 *ORF8* infected patients with respect to WT-infected ones, whereas interferon gamma (IFN-γ) and other cytokines responsible for T-cells activation were instead upregulated [25]. Therefore, those observations corroborate the hypothesis that the *ORF8* gene deletion has conferred to SARS-CoV-2 some advantage towards the adaptation to humans, as the result of a mixed pattern of recombination events and immune-driven purifying selection occurred on SARSr-CoVs in different reservoir and intermediate host species [26]. An adaptive change favoring transition from the zoonotic host to the human one was also inferred during the SARS-CoV 2002–2003 epidemic, in which the emergence of similar deletions at the *ORF8* genomic region were detected [27]. Viral isolates collected early in the outbreak from wildlife species in Chinese wet markets were all retaining an intact *ORF8*, whereas those obtained from traders in the same markets and from hospitalized patients were almost identical but showed a 29 nt deletion in the genomic tract between positions 27869–27897 [28]. Such deletion caused the split of the gene into two *ORFs*, namely *8a* and *8b*, after which in place of the 122 residues long WT ORF8 (lately termed as ORF8ab), two proteins of 39 (ORF8a) and 84 (ORF8b) amino acids were expressed, respectively (Fig. 1C) [29]. The mutation became dominant among isolates collected during the peak of the outbreak, and even more extended deletions of 82 nt, 386 nt and 415 nt - all of which resulted in total loss of the *ORF8* gene products expression - were found in the late epidemic phase [27,30]. Several studies aimed to elucidate the differences in the functional properties of the ORF8ab, 8a and 8b proteins (briefly discussed later in this minireview), leading to hypothesize that the Δ29 *ORF8ab* deletion would modulate SARS-CoV pathogenesis and facilitate adaptation to human host. However, contrary to what has been observed for the SARS-CoV-2 Δ382 *ORF8*, the SARS-CoV Δ29 *ORF8ab* deletion was shown to have no advantageous impact compare to WT in viral load persistence and cytopathic effect [31]. Rather, it was associated with much decreased virus replication kinetics in vitro as well as in animal models, and this regardless to the effectiveness of the elicited IFN response and the cellular system assayed, either consisting of primate, bat or human cell lines [32]. This is why, alternative to explain the origin of the Δ29 *ORF8ab* deletion as the result of positive selection, other processes have been called into question. These include relaxed-purifying selection that removed a dispensable ORF8ab protein, or a founder effect resulting from transmission bottlenecks, which allowed a genotype with a randomly-emerged and slightly-deleterious mutation to keep circulating in spite of the reduced viral fitness [32,33]. Indeed, whatever the nature of the evolutionary driving forces that shaped the SARS-CoV-2 *ORF8* and the SARS-CoV *ORF8ab*, the picture is made more complex by the fact that those genes recombine and undergo deletions also in their natural hosts. In fact, recombination breakpoints flanking the *ORF8* gene were found in the genome of coronaviruses isolated from *Rhinolophus ferrumequinum* (Rf) and *Rhinolophus sinicus* (Rs) horseshoe bats, and *ORF8* gene recombination between the corresponding SARSr-Rf-BatCoVs and SARSr-Rs-BatCoVs was determined as one of the events at the origin of the SARS-CoV ancestor discovered in Himalayan palm civets (*Paguma larvata*) and raccoon dogs (*Nyctereutes procyonoides*) [34,35]. Also, various deletions in the *ORF8* gene of SARSr-BatCoVs were reported, including the *ORF8* loss in a European strain isolated from *Rhinolophus blasii* [36], a 26 nt deletion causing the *ORF8* split into three distinct *ORFs* in a Guangdong strain isolated from Rs bats [37] and a 5 nt deletion in a Yunnan strain isolated from Rs bats that resembled the split into *ORF8a* and *8b* detected in SARS-CoV [38]. Extensive recombination events among SARSr-CoVs from bat species of the *Rhinolophus* genus and Malayan pangolins (*Manis javanica*) played a key role also in the origin of the SARS-CoV-2 [26,39]. Moreover, the recombination breakpoints flanking the SARS-CoV-2 *ORF8* gene as well as the deletion breakpoints within it, were all found to overlap with sites of perfect nt repeats and predicted hairpin formation, a kind of RNA secondary structure that is often associated to genomic instability [16]. Therefore, it is clear that the *ORF8* gene of SARSr-CoV is constantly evolving already in the reservoir species and that its encoded protein(s) may be dispensable for viral fitness in those as well as in intermediate and final hosts. Yet, it remains plausible that new viral phenotypes may arise upon *ORF8* loss or from new *ORF8* gene products, which can either lay the foundations for host switching and/or result in attenuation of virulence and pathogenicity in humans.

3. Structure and functions of the SARS-CoV-2 ORF8 protein

Computational analysis of the SARS-CoV-2 ORF8 amino acid sequence revealed that its structural organization resembles the one observed among members of the immunoglobulin (Ig)-like domains containing protein superfamily (IgSF). Typically, such organization comprehends an N-terminal signal peptide for transmembrane (TM) import and secretion, an internal β-sandwich core and a C-terminal TM region followed by a stretch of basic residues [40]. Encoded by a variety of viruses, IgSF proteins seem to evolutionary descend by host-acquired genes, and to have evolved to mimic the original host function – which consist in cell-to-cell adhesion or ligand-receptor recognition processes - thereby interfering with that and acting as molecular traps with immunomodulatory properties [41,42]. In spite of a low sequence similarity, the IgSF architecture is shared by all the ORF8, ORF7a and ORF8/ORF7a-like proteins (variously annotated as ORF9 or ORF10) from SARS-CoV-2, SARS-CoV and several SARSr-CoVs. However, the SARS-CoV-2 ORF8 stands out by differing from its homologs for the lacking the C-terminal TM domain and the presence of a long insertion within the core. Moreover, in the SARS-CoV-2 ORF8 the N-terminal TM signal peptide spans residues 1–17 and the β-strands core comprises residues 18–121, respectively (Fig. 2A) [40]. The predicted modular organization has been confirmed by the recently solved SARS-CoV-2 ORF8 crystal structure (PDB code 7JTL), which also reveals a covalently-bound dimer held by an intermolecular disulfide bridge between the two Cysteine residues at position 20. Within each monomer, a β-sheet core of eight antiparallel β-strands is held together by three intramolecular disulfide bridges, while two β-strands from each core are involved in hydrophobic interactions with their counterparts in the other monomer to further stabilize the dimer interface (Fig. 2B) [43]. Dimerization brings the two N-termini to interact, making plausible that their respective

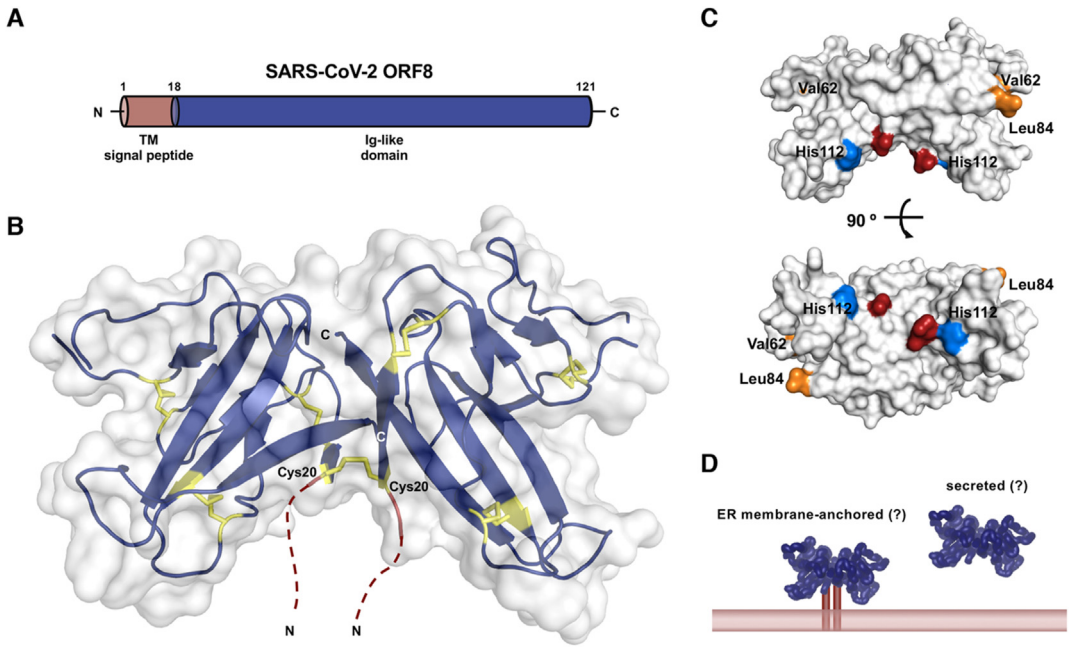

Fig. 2. Structure of the SARS-CoV-2 ORF8 protein. (A) Structural organization of the SARS-CoV-2 ORF8 protein; the TM signal peptide and the Ig-like domain are colored in dark red and dark blue, respectively. (B) Crystal structure of dimeric SARS-CoV-2 ORF8 (PDB: 7JTL) shown as superimposed surface and cartoon representations; di-sulfide bridges are highlighted in yellow; missing sequences of N-terminal TM signal peptide are represented as dark red dashed lines. (C) Surface representation of dimeric SARS-CoV-2 ORF8 shown in two orientations; residues affected by mutations are indicated and colored in orange (Val62 and Leu84) and light blue (His112), respectively; N-terminal residues adjacent to the missing TM signal peptide are colored in dark red. (D) Schematic diagram showing the two hypothetical states (membrane-anchored and secretory) of SARS-CoV-2 ORF8.

TM signal peptides (which are absent in the crystal structure) would point out from each monomer like stems towards the membrane. Val62, Leu84 and His112, residues whose mutations are responsible for the ORF8 isoforms thus far reported [22], as well as those affected by minor deletions such as Ile121 and the Gly66-Ser67-Lys68 tract [16], are all exposed to the solvent and therefore unlikely to cause major structural perturbations when substituted or deleted (Fig. 2C). The relatively high number of disulfide bridges - that would require an oxidative environment to be established and maintained - and the presence of a TM signal peptide at the N-terminus, are reminiscent of a protein that is either extracellularly secreted or resident in the endoplasmic reticulum (ER), two features that are well in agreement with an ORF8 role as immunomodulation decoy (Fig. 2D) [40,43]. Indeed, a secretory status for the ORF8 is supported by the fact that, together with the N and ORF3b proteins, ORF8 is the one to elicit the strongest and more specific antibody response among SARS-CoV-2 antigens, either during the acute phase as well as the convalescent one and the long-term period [44]. However, consistently with the notion of its accumulation in the ER, the SARS-CoV-2 ORF8 was shown able to potently downregulate the expression of the class I major histocompatibility complex (MHC-I) and promote its degradation in several cell lines [45]. Specifically, upon heterologous overexpression of ORF8 or infection with SARS-CoV-2, MHC-I was captured during its transit through the ER and re-routed to the auto-phagosome and auto-lysosome degradation pathway. By contrast, treatment with autophagy inhibitors or knockdown of autophagy cargo proteins could restore MHC-I expression at the cell surface (Fig. 3) [45]. Of note, while both are known for being ER resident proteins [29], neither the SARS-CoV ORF8ab nor the ORF8a were found capable to exert any MHC-I downregulation. Therefore, even though those preliminary findings will need validation from further studies to be conducted on in vivo models, the observed phenotype in vitro may indicate a strategy unique to SARS-CoV-2 among coronaviruses, through which it prevents the presentation of viral antigens at the cell surface by MHC-I and, consequently, hampers the recognition and lysis of virus-infected cells by cytotoxic T-lymphocytes [45]. Dysregulation of T-cell response and adaptive immunity during SARS-CoV-2 infection has been correlated with pathogenesis and disease outcome. However, in some patients an overaggressive immune response was observed, whereas exhaustion or dysfunction of immune cells was a prominent feature in others, which depicts a complex scenario where different immunotypes vary greatly in the way they respond to the infection [46]. In turn, T-cells impairment manifesting as lymphocytopenia may be the consequence of deficiency in type I IFNs, whose timely production is essential for T-cells proliferation and response during viral infections [47]. In this regard, it is worth to note that subversion of the innate immunity leading to suppression of type I IFNs was associated to persistent viral load and exacerbated inflammation among Covid-19 patients. Moreover, the loss of IFN-β production was observed in cases displaying all grades of disease-severity, and low IFN-α levels were shown by those with the most critical prognosis [48]. SARSr-CoVs are known for employing strategies to counter the host innate immune antiviral response. They target either the pattern recognition receptors (PRRs) sensing of pathogen-associated molecular patterns (PAMPs) that leads to type I IFNs production, or the signaling cascade that, from the type I IFNs interaction with its cognate interferon-α/β receptor (IFNAR), leads to the production of IFN-stimulated genes (ISGs) [49]. In SARS-CoV-2, several proteins have been identified as type I IFNs antagonists, including the Nsp1, Nsp3, Nsp12, Nsp13, Nsp14, Nsp15, S, ORF3b, M, ORF6, ORF8 and N [50–52]. In particular, SARS-CoV-2 ORF8 was found able to inhibit the induction of the IFN-β and the interferon-stimulated response element (ISRE) promoters, which were stimulated upon infection with Sendai virus (SeV) or by overexpression of the PRRs retinoic acid-inducible gene I (RIG-I) and melanoma differentiation gene 5 (MDA5), the PRRs

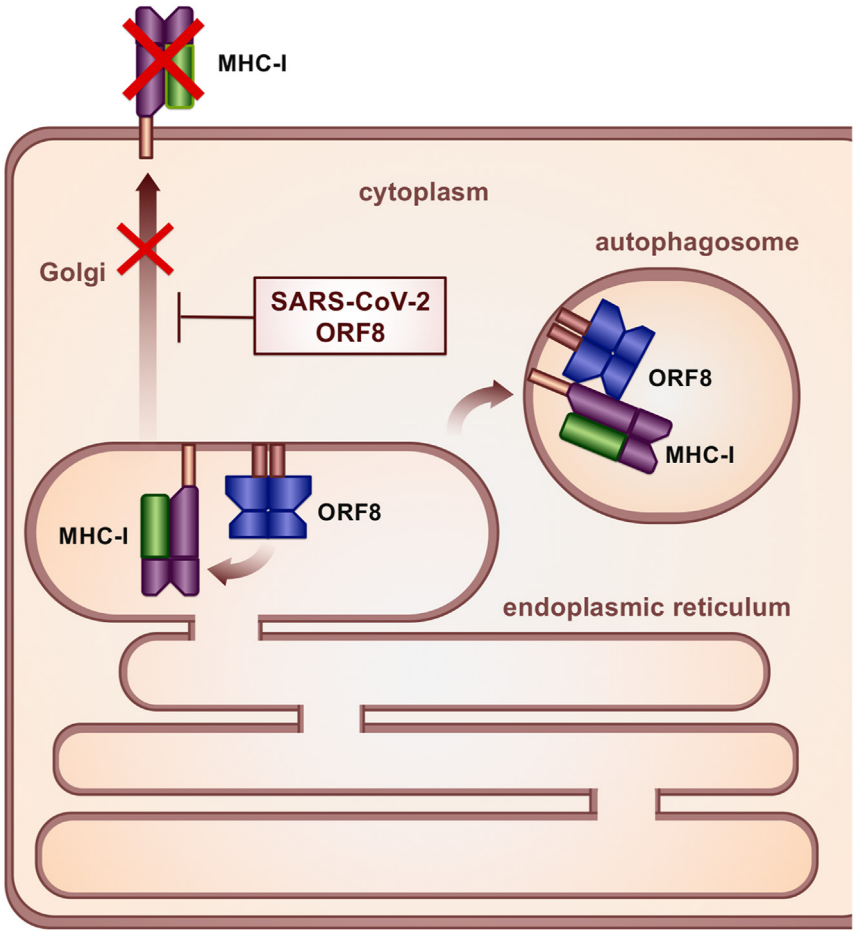

Fig. 3. Downregulation of the MHC-I-mediated antigen presentation by SARS-CoV-2 ORF8. Schematic diagram describing how the SARS-CoV-2 ORF8 mediates the MHC-I degradation via an autophagy-dependent pathway. Diagram is an original reinterpretation of what graphically shown in Zhang et al. (2020) [45].

adaptor mitochondrial antiviral signaling protein (MAVS) and the transcription factor IFN regulatory factor 3 (IRF-3). Furthermore, ORF8 was able to suppress the ISRE promoter induction and the mRNA expression of two ISGs, namely ISG54 and ISG56, upon cell treatment with IFN-β (Fig. 4) [51,52]. Of note - possibly due to differences in experimental conditions - one early study reported as negligible the type I IFN antagonism activity of ORF8 and other SARS-CoV-2 proteins [50]. In a subsequent comparative study, the antagonistic IFN-β promoter activity of ORF8 was reported as weaker with respect to that of ORF6 and N [51]. Nevertheless, two different studies clearly showed that ORF8 inhibited the ISRE promoter induction and the ISGs expression in cells under IFN-β treatment, at concentrations even lower than the one tested in the study where the *anti*-IFN activity of ORF8 and other SARS-CoV-2 proteins resulted difficult to detect. These data demonstrate that the SARS-CoV-2 ORF8 is a type I IFN antagonist that exerts its functions by targeting both the PRRs-mediated pathway that induces the IFN-β promoter, and the signaling cascade downstream the interaction between IFN-β and IFNAR that induces the ISGs (Fig. 4) [51,52]. Overall, the functional studies conducted thus far have started to unveil a role of SARS-CoV-2 ORF8 in evading the host innate immunity processes of antigen presentation and type I IFN-mediated antiviral response. However, understanding the mechanistic details through which such evasion is exerted requires further investigations, and whether the above described as well as other yet to be discovered functions are shared between the SARS-CoV-2 ORF8 and its homologs in SARS-CoV, needs to be elucidated.

Indeed, SARS-CoV ORF8ab, 8a and 8b have been shown to differently modulate viral pathogenesis, with the two truncated proteins exhibiting diverse cellular localization and functional properties with respect to each other and to their full-length ancestor [53,54]. Like the SARS-CoV-2 ORF8, SARS-CoV ORF8ab was identified as an ER luminal surface resident protein, and shown to modulate the unfolded protein response (UPR) by upregulating expression of ER chaperons to facilitate protein folding [29,55]. Specifically, ORF8ab causes the proteolytic cleavage-dependent maturation of the activating transcription factor 6 (ATF6) and promotes its translocation to the nucleus, where ATF6 induces the expression of ER stress response element (ESRE)-containing genes that encode for ER chaperons. As a result, ER stress coming from the accumulation of unfolded or misfolded proteins - an otherwise unavoidable consequence of the high protein translation rates reached during viral infection - is counteracted by upregulation of ER chaperons [55]. Regarding the subversion of type I IFNs, SARS-CoV ORF8ab and 8b were found able to inhibit the IFN-β promoter induction in cells stimulated with polyinosinic:polycytidylic acid (poly (I:C)) through the interaction with phosphorylation-activated IRF-3, thereby hampering its dimerization and subsequent nuclear translocation [56]. Additionally, both ORF8ab and 8b were ubiquitinated and could promote the degradation of IRF-3 through the ubiquitin-proteasome system [53,56]. The SARS-CoV ORF8b has been localized in punctuate vesicle-like structures throughout the cytosol and in the nucleus [54,57]. Ectopically expressed SARS-CoV ORF8b markedly increased DNA synthesis, a phenotype reminiscent of a

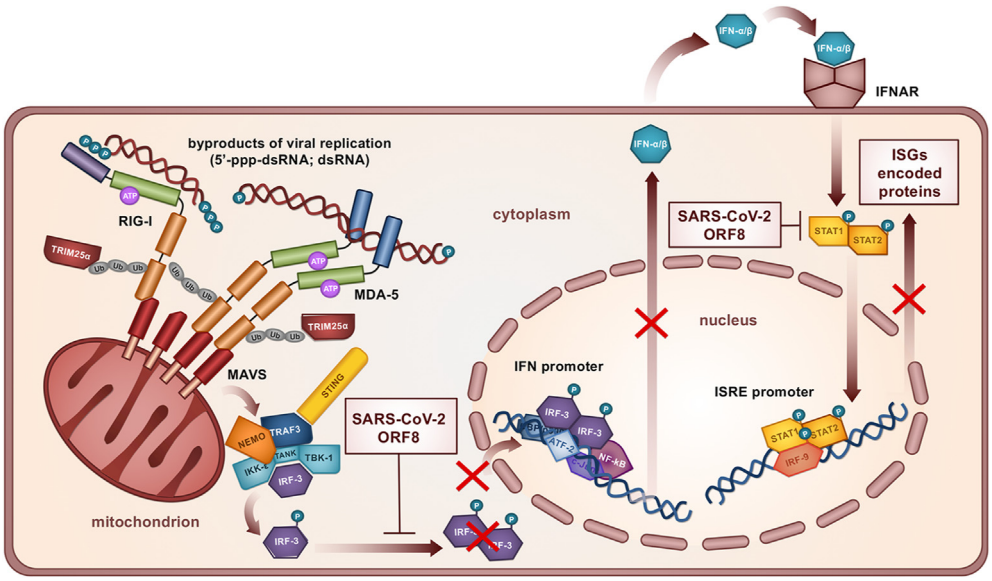

Fig. 4. Inhibition of the type I IFN antiviral response by SARS-CoV-2 ORF8. Schematic diagram describing how the SARS-CoV-2 ORF8 suppresses the IRF-3-mediated induction of the IFN-β promoter and the IFN-β-stimulated expression of ISGs. Diagram is an original implementation and simplified reinterpretation of what graphically shown in Zinzula and Tramontano (2013) [49].

cell-proliferation effect similar to the one observed in the lung epithelium of SARS-CoV patients, which may represent a strategy to facilitate viral spread early in the infection [57]. Moreover, SARS-CoV ORF8b was able to post-translationally downregulate the SARS-CoV E protein and mediate its degradation via a ubiquitin-independent proteasome pathway [53,54,58]. Since over-expression of SARS-CoV E was found to induce apoptosis in T-cells, and given that ORF8b overexpression had a negative effect on SARS-CoV replication, downregulation of E by ORF8b could reflect a strategy for attenuating pathogenesis by tuning viral replication [54,58]. Conversely, to both SARS-CoV ORF8a and ORF8b have been ascribed functions that result in the activation of the innate immune response and the enhancement of viral pathogenesis. In macrophages, SARS-CoV ORF8b was shown to form insoluble aggregates that activate the nucleotide-binding domain leucine-rich repeat (NLR)-family pyrin domain containing 3 (NLRP3) inflammasome. In turn, this triggers the nuclear translocation of the transcription factor EB (TFEB) and the subsequent induction of target genes related to autophagy, release of pro-inflammatory cytokines and cell death [59]. Similarly, overexpression of ORF8a enhanced viral replication and cytopathic effect in SARS-CoV infected cells. In particular, it was found that ORF8a localizes to mitochondria via its N-terminal signal peptide, where it triggers the hyperpolarization of the transmembrane potential and thus induces cell apoptosis in a caspase 3 dependent pathway [60]. Taken together, the described findings show that - while dispensable for virus replication - the protein encoded by the SARS-CoV-2 *ORF8* gene and those encoded by the SARS-CoV *ORF8ab* gene, all exert functions influencing viral pathogenesis. For SARS-CoV-2, the advantage on viral fitness that comes from the absence of ORF8 may be intuitive, whereas such inference is not immediate in the case of SARS-CoV. This is because the functional properties of ORF8ab are not fully recapitulated by 8a and 8b, into which the ORF8ab split give rise to new and often opposite phenotypes when 8a and 8b are taken individually. Nevertheless, it is conceivable that, since during SARS-CoV infection ORF8a and 8b are concomitantly expressed, their synergistic functions would result in a fine tuning of viral pathogenesis more coherent with the logic of an adaptive process towards the replication and persistence in the human host.

4. Potential of SARS-CoV-2 ORF8 as antiviral target

The milder Covid-19 phenotype associated to the SARS-CoV-2 *ORF8* gene full deletion and the functions ascribed to the SARS-CoV-2 ORF8 protein in the context of host innate immunity evasion, highlight the ORF8 potential as hotspot of SARS-CoV-2 vulnerability. On the other hand, the hypervariable nature of the *ORF8* gene and the rapid evolution it undergoes represent a limit that can compromise the ORF8 protein suitability as antiviral target. Nevertheless, rather than directly the viral protein, targeting the host factors with which it establishes critical interactions may represent a valid alternative strategy [61]. Within this picture, opportunities for the development of therapeutic interventions against Covid-19 come from the SARS-CoV-2 host-pathogen protein-protein interactions (PPI) network, which started to be deciphered by studies using affinity purification coupled to mass spectrometry (AP-MS) analysis and statistical modeling [62,63]. In a first study, the SARS-CoV-2 proteins were heterologously expressed in human embryonic kidney 293T/17 cells, and 332 human proteins interacting with the viral baits were identified. Of those, 66 interactors turned out to be targetable by small-molecule compounds based on chemoinformatic and knowledge-based analysis. In particular, 47 human proteins were found to interact with the SARS-CoV-2 ORF8, of which 15 are known targets of drugs that are either in preclinical phase, clinical trial or have been already approved by the United States Food and Drug Administration (FDA) [62]. Those proteins are involved in various processes including glycosylation, glycosaminoglycan synthesis, organization of the extra-cellular matrix (ECM) and ER protein quality control. Representative ORF8-interactors associated to ECM modification and to the ER stress and ER-associated degradation (ERAD) pathway are, for example, the lysyl oxidase (LOX), the UDP-glucose/glycoprotein glucosyltransferase 2 (UGGT2), the ER degradation enhancing alpha-mannosidase like protein 3 (EDEM3), the N-glycanase 1 (NGLY1), the osteosarcoma amplified 9 (OS9) and the FAD-dependent oxidoreductase domain-containing protein 2 (FOXRED2). Noteworthy, some of the identified ORF8-interactors

are implicated in pulmonary fibrogenesis, an exacerbation of lung inflammation that leads to respiratory distress in most severe Covid-19 cases. Among these, are the FK506-binding protein 10 (FKBP10), the interleukin 17 receptor A (IL17RA), neuraminidase 1 (NEU1), growth/differentiation factor 15 (GDF15) and heparan sulfate 6-O-sulfotransferase 2 (HS6ST2) [62]. In a second, AP-MS-based comparative study, the SARS-CoV-2 and SARS-CoV proteins were expressed in the lung-derived A549 carcinoma cell line, leading to the identification of 1484 host-pathogen interactions that involve 1086 different human proteins. Also, changes in the host proteome profile upon expression of each viral protein were analyzed. Relative to ORF8, the SARS-CoV-2 protein (but none of its SARS-CoV homologs 8a and 8b) was found to interact with the complex formed by transforming growth factor-β 1 (TGFβ1), latency associated peptide (LAP) and latent TGFβ binding protein 1 (LTBP1), and with the complex formed by integrin subunit alpha 3 (ITGA3) and serpin family E member 1 (SERPINE1). Notably, also these complexes are significantly involved in the progression of lung fibrosis, which well correlates with the notion that, by interacting with them, the ORF8 functions as a modulator of SARS-CoV-2 pathogenesis [63].

5. Conclusions

The ORF8 is one of the less known among the gene products encoded by the genome of the Covid-19 etiologic agent, yet the recently published information summarized in this minireview allow to glimpse a pivotal role for this accessory protein in determining SARS-CoV-2 virulence and pathogenesis. Therefore, it is anticipated that functional studies will be conducted in order to elucidate the mechanistic details on how ORF8 acts as evader of the host innate immune system and modulator of cellular pathways. In particular, from a biochemical and biophysical perspective and without any claim to exhaustiveness, some research directions are foreseen as a priority. A first one, given the low sequence similarity among ORF8 proteins of SARSr-CoVs, is to find differences and commonalities in the functional properties displayed by the ORF8 of viruses from bat reservoirs, from putative intermediate species such as pangolins and from SARS-CoV-2. This could help to clarify what is the ORF8 contribution to viral fitness and where the borderline between dispensability and functional importance in a given host is drawn. A second one is to determine what, if any, are the implications in the context of the different functions ascribed to SARS-CoV-2 ORF8 of mutations that have been positively selected during the course of the pandemic, such as the L84S substitution. Finally, a third research direction should aim at solving new structures of ORF8 from SARSr-CoVs, isolated as well as in complex with already known host interactors or with newly discovered ones. This would shed light on common structural constraints needed by different ORF8 for the exertion of their functions, which in turn could unveil antiviral targets with pan-SARSr-coronaviral valence. Furthermore, those structural complexes would provide novel frameworks for the development of therapeutics that are specifically directed against either the ORF8 protein or its host interactors.

Declaration of competing interest

The author declares to have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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