Coronaviruses post-SARS: update on replication and pathogenesis

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Abstract | Although coronaviruses were first identified nearly 60 years ago, they only received notoriety in 2003 when one of their members was identified as the aetiological agent of severe acute respiratory syndrome. Previously these viruses were known to be important agents of respiratory and enteric infections of domestic and companion animals and to cause approximately 15% of all cases of the common cold. This Review focuses on recent advances in our understanding of the mechanisms of coronavirus replication, interactions with the host immune response and disease pathogenesis. It also highlights the recent identification of numerous novel coronaviruses and the propensity of this virus family to cross species barriers.

Prothrombinase Molecule that cleaves thrombin, thereby initiating the coagulation cascade.

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Coronaviruses, a genus in the Coronaviridae family (order Nidovirales; FIG. 1), are pleomorphic, enveloped viruses. Coronaviruses gained prominence during the severe acute respiratory syndrome (SARS) outbreaks of 2002-2003 (REF. 1). The viral membrane contains the transmembrane (M) glycoprotein, the spike (S) glycoprotein and the envelope (E) protein, and surrounds a disordered or flexible, probably helical, nucleocapsid^{2,3}. The viral membrane is unusually thick, probably because the carboxy-terminal region of the M protein forms an extra internal layer, as revealed by cryo-electron tomography². Coronaviruses are divided into three groups, and further subdivided into subgroups (TABLE 1), based initially on serologic, and more recently on genetic, analyses. With the identification of more distantly related viruses, the taxonomy of these viruses is likely to undergo further changes.

Coronaviruses contain a single stranded, 5'-capped, positive strand RNA molecule that ranges from 26-32 kb and that contains at least 6 open reading frames (ORFs). The first ORF (ORF1a/b) comprises approximately twothirds of the genome and encodes replicase proteins (FIG. 2a). Translation begins in ORF1a and continues in ORF1b after a -1 frameshift signal. The large ORF1a and ORF1ab polypeptides, commonly referred to as pp1a and pp1ab, respectively, are processed primarily by the virally encoded chymotrypsin-like protease 3CLpro (also called Mpro or main protease) with additional cleavage performed by one or two viral papain-like proteases (PLPs), depending on the species of coronavirus⁴. The majority of the remaining one-third of the genome encodes four structural proteins: S, E, M and nucleocapsid (N) proteins. A subset of group 2 coronaviruses encode an additional haemagglutinin-esterase (HE) protein (FIG. 2a,b). The HE protein, which may be involved in virus entry or egress, is not required for replication, but appears to be important for infection of the natural host⁵.

Receptors for several coronaviruses have been identified (TABLE 1). The prototypical coronavirus, mouse hepatitis virus (MHV), uses CEACAM1a, a member of the murine carcinoembryonic antigen family, to enter cells. Deletion of this protein makes mice resistant to infection⁶. Several group 1 coronaviruses use aminopeptidase N to adhere to host cells, consistent with their respiratory and enteric tract tropisms (reviewed in REF. 7). SARS-CoV, a group 2 coronavirus, enters host cells through an interaction of the S protein with human angiotensin converting enzyme 2 (ACE2)8. Strikingly, human coronavirus-NL63 (HCoV-NL63), which causes mild disease, also uses ACE2, although it binds to a different part of the protein than does SARS-coronavirus (SARS-CoV)9,10. ACE2 is postulated to have a protective role in the inflamed lung, and SARS-CoV S protein binding to ACE2 is thought to contribute to disease severity^{11,12}. As infection with HCoV-NL63 produces mild disease, however, binding to ACE2 by itself cannot be sufficient for this process.

The N protein is important for encapsidation of viral RNA and acts as an interferon (IFN) antagonist (see below). Additionally, it causes upregulation of FGL2, a prothrombinase that contributes to fatal hepatic disease in mice that are infected with MHV-3 (REF. 13) and that modifies transforming growth factor- β (TGF β) signalling in SARS-CoV-infected cells¹⁴.



Figure 1 | **The Nidoviruses.** Phylogenetic relationship of viruses in the order Nidoviruses.

The E proteins are small integral membrane proteins with roles in virus morphogenesis, assembly and budding. In the absence of E proteins, virus release is inhibited completely (in the case of transmissible gastroenteritis virus (TGEV)) or partially (in the case of SARS-CoV and MHV)¹⁵⁻¹⁷. The E protein also possesses ion channel activity, which is required for optimal virus replication^{18,19}.

Interspersed between and in these structural genes are one to eight genes that encode accessory proteins, depending on the virus strain. These show no sequence similarity with other viral or cellular proteins and are not required for virus replication in cultured cells^{20–22}. However, they are conserved in virus species isolated at different times and locales (for example, for SARS-CoV²³), which suggests that these proteins have an important role in replication in the natural host. Several accessory proteins are virion-associated^{24–27}, although whether these proteins are truly structural is controversial²⁸.

The genes that encode non-replicase proteins are expressed from a set of 'nested' subgenomic mRNAs that have common 3' ends and a common leader that is encoded at the 5' end of genomic RNA. Proteins are produced generally only from the first ORF of subgenomic mRNAs, which are produced during minus strand RNA synthesis. Transcription termination and subsequent acquisition of a leader RNA occurs at transcription regulatory sequences (TRS), located between ORFs. These minus strand subgenomic RNAs serve as templates for the production of subgenomic mRNAs (FIG. 3), an efficient process that results in a high ratio of subgenomic mRNA to minus strand subgenomic RNA²⁹.

Coronavirus replication

One consequence of the SARS epidemic was an increase in efforts to understand coronavirus replication and identify additional possible targets for anti-viral therapy. ORF1 of most coronaviruses encodes 16 proteins that are involved in viral replication (FIG. 2a); ORF1 of group 3 coronaviruses lacks nsp1 and thus encodes only 15 proteins. The structure of many of these proteins has been solved by X-ray crystallography or nuclear magnetic resonance, facilitating structure–function studies^{30–40}. Functions were predicted⁴¹ and later confirmed for many of these proteins (TABLE 2), including PL1^{pro} and PL2^{pro} (papain-like proteases) contained in nsp3 and 3CL^{pro} or M^{pro} contained in nsp5, the RNA-dependent RNA polymerase nsp12 and the helicase nsp13. A second RNA polymerase, nsp8, may function as a primase⁴². The nsp3 protease has additional roles in the assembly of virus replication structures (see below) and possesses poly(ADP-ribose) binding capabilities, and deubiquitylating activity in its protease domain, although the role of the latter in virus replication is not yet known⁴³.

Nsp7, nsp8, nsp9 and nsp10 are postulated to have a role in subgenomic and genomic RNA replication, and all four proteins are essential for viral replication⁴⁴. Nsp7 and nsp8 form a hexadecameric structure, with RNA binding activity³¹. The structure of nsp9 also suggests that it binds RNA⁴⁵. Mutations in nsp10 inhibit minus strand RNA synthesis, but this effect may be indirect, as studies have showed that nsp10 is required for proper function of the main viral protease (M^{pro})⁴⁶.

Nsp14, a bifunctional protein, is a $3' \rightarrow 5'$ exonuclease, with a role in maintaining fidelity of RNA transcription⁴⁷, and a (guanine-*N*7)-methyl transferase (*N*7-MTase), involved in RNA cap formation⁴⁸. Coronaviruses also encode a novel uridylate-specific endoribonuclease (NendoU), nsp15, that distinguishes nidoviruses in general from other RNA viruses and that is crucial for virus replication⁴⁹. Cleavage of RNA by NendoU results in 2'-3' cyclic phosphate ends, but its function in the virus life cycle remains unknown. Nsp16 is an S-adenosyl-Lmethionine-dependent RNA (nucleoside-2'O)-methyl transferase (2'O-MTase) and, like nsp14, is involved in cap formation⁵⁰. Nsp15 has been postulated to function with nsp14 and nsp16 in RNA processing or cap production, but this remains to be proven.

RNA replication is thought to occur on doublemembrane vesicles (DMVs)⁵¹ (FIG. 4). Newly synthesized genomic RNA is then incorporated into virions on membranes that are located between the endoplasmic reticulum (ER) and the Golgi apparatus (ER–Golgi intermediate compartment (ERGIC); reviewed in REF. 52). Initial studies suggested that these DMVs assemble using components of the autophagy pathway⁵³, but other studies showed replication proceeded normally and that DMVs were produced in macrophages lacking ATG5, a key component of autophagosomes⁵⁴. Thus, whether autophagy is involved at all or whether its involvement is cell-specific remains uncertain. In addition, the unfolded protein response (UPR) is induced during coronavirus infections and may contribute to DMV formation⁵⁵.

Recent results show that DMVs are likely to originate from the ER. Using electron tomography of cryo-fixed SARS-CoV-infected Vero E6 cells and three-dimensional reconstruction imaging, Knoops et al. showed that DMVs are not isolated vesicles, but rather are part of a reticulovesicular network of modified ER membranes⁵⁶. At later times after infections, these networks appear to merge into large single-membrane vesicles. Proteins involved in virus replication (nsp3, nsp5 and nsp8; TABLE 2) are located mainly outside of DMVs, in adjacent reticular structures. Double-stranded RNA, representing either replicative intermediates or 'dead end' double-stranded RNA, was detected primarily in DMVs and, surprisingly, no obvious connections between the interior of these vesicles and the cytosol were detected⁵⁶. Thus, it

Primase

In the case of nsp8, an RNA-dependent RNA polymerase that produces RNA primers that are required for initiation of RNA synthesis by the main viral RNA polymerase, nsp12.

Double-membrane vesicle

A structure that is observed in electron micrographs of infected cells and that is thought to be the site of virus replication.

Table 1 Representative coronavirus species and their receptors				
Group	Host	Virus	Cellular receptor	
Group 1a	Bat‡	BtCoV	Unknown	
	Cat	FCoV	APN	
	Cat	FIPV	APN	
	Dog	CCoV	APN	
	Pig	TGEV	APN	
Group 1b	Human	HCoV-229E	APN	
	Human	HCoV-NL63	Angiotensin-converting enzyme 2 (ACE2)	
	Pig	PEDV	Unknown	
Group 1*	Rabbit	RbCoV	Unknown	
Group 2a	Cattle, ruminants, alpaca	BCoV and related viruses	9-O-acetylated sialic acid	
	Dog	CRCoV	Unknown	
	Human	HCoV-HKU1	Unknown	
	Human	HCoV-OC43	9-O-acetylated sialic acid	
	Mouse	MHV	Carcinoembryonic antigen adhesion molecule 1	
	Pig	PHEV	Unknown	
Group 2b	Bat‡	BtCoV (multiple species)	Unknown	
	Human	SARS-CoV	ACE2	
Group 2*	Manx shearwaters	PCoV	Unknown	
	Rat	RtCoV	Unknown	
	Rat	SDAV	Unknown	
Group 3a	Chicken	IBV	Unknown	
	Pheasant	PhCoV	Unknown	
	Turkey	TCoV	Unknown	
Group 3b	Beluga whale	SW1	Unknown	
Group 3c	Bulbul	BuCoV-HKU11	Unknown	
	Thrush	ThCoV-HKU12	Unknown	
	Munia	MuCoV-HKU13	Unknown	
	Asian leopard cat, Chinese ferret badger	ALCCoV	Unknown	

*Due to a lack of sequence data, subgroup has not been assigned. [‡]More than 60 bat coronavirus species have been identified and tentatively classified as members of group 1 or group 2 (REF. 91). ALCCoV, Asian leopard cat coronavirus; APN, aminopeptidase N; BCoV, bovine coronavirus; BtCoV, bat coronavirus; BuCoV, bulbul coronavirus; CCoV, canine coronavirus; CRCoV, canine respiratory coronavirus; FCoV, feline coronavirus; FIPV, feline infectious peritonitis virus; HCoV, human coronavirus; IBV, infection bronchitis virus; MHV, mouse hepatitis virus; MuCoV, munia coronavirus; PCoV, puffinosis coronavirus; PEDV, porcine epidemic diarrhoea virus; PhCoV, pheasant coronavirus; PHEV, porcine hemagglutinating encephalomyelitis virus; SbCoV, rabbit coronavirus; SARS-CoV, severe acute respiratory syndrome-associated coronavirus; SDAV, sialodacryoadenitis virus; TCoV, turkey coronavirus; ThCoV, thrush coronavirus; TGEV, transmissible gastroenteritis virus.

remains unknown how newly synthesized RNA might be transported to sites of virus assembly, assuming that RNA transcription occurs in DMVs.

Formation of DMVs requires membrane curvature, and this may be initiated by insertion of specific viral proteins into membranes. Based on studies of equine arteritis virus⁵⁷, a non-coronavirus member of the nidovirus order (FIG. 1), nsp3 and nsp4 are probably sufficient for DMV formation. Mutations in nsp4 result in aberrant formation of DMVs, further supporting a role for this protein in establishing sites of virus replication⁵⁸. Nsp6, like nsp3 and nsp4, also contains multiple transmembrane regions and may be involved in membrane modification^{59–61}. Notably, nsp3 and nsp6 encode an odd number of hydrophobic domains, but both the amino and carboxyl termini of these proteins are in the cytoplasm, suggesting that one hydrophobic region does not span the membrane⁶⁰; whether this region contributes to membrane curvature or has another function requires further investigation.

Coronavirus-mediated diseases

Before the SARS epidemic of 2002–2003, two human coronaviruses, HCoV-OC43 and HCoV-229E, were recognized as important causes of upper respiratory tract infections and were occasionally associated with more severe pulmonary disease in the elderly, newborn and immunocompromised⁶². SARS-CoV, unlike HCoV-OC43 and HCoV-229E, causes a severe



Figure 2 | **Structure of coronavirus genome and virion. a** | Schematic diagram of representative genomes from each of the coronavirus groups. Approximately the first two-thirds of the 26–32 Kb, positive-sense RNA genome encodes a large polyprotein (ORF1a/b; green) that is proteolytically cleaved to generate 15 or 16 non-structural proteins (nsps; nsps for severe acute respiratory syndrome coronavirus (SARS-CoV) are illustrated). The 3'-end third of the genome encodes four structural proteins — spike (S), membrane (M), envelope (E) and nucleocapsid (N) (all shown in blue) — along with a set of accessory proteins that are unique to each virus species (shown in red). Some group 2 coronavirus express an additional structural protein, haemagglutinin-esterase (not shown). b | Schematic diagram of the coronavirus virion. 2'OMT, ribose-2'-O-methyltransferase; ExoN, 3' \rightarrow 5' exonuclease; Hel, helicase; IBV, infection bronchitis virus; NendoU, uridylate-specific endoribonuclease; RDRP, RNA-dependent RNA polymerase; ssRBP, single-stranded RNA binding protein; ssRNA, single-stranded RNA; TGEV, transmissible gastroenteritis virus.

respiratory disease, and nearly 10% mortality was observed in 2002–2003 (REF. 1). Notable features of the disease were an apparent worsening of symptoms as the virus was cleared (suggesting the disease had an immunopathological basis), and a lack of contagion until lower respiratory tract symptoms were apparent. This latter feature made control of the epidemic by quarantine feasible, as it simplified identification of infected patients. Unlike HCoV-OC43 and HCoV-229E, SARS-CoV also caused systemic disease, with evidence of infection of the gastrointestinal tract, liver, kidney and brain, among other tissues⁶³. Although the virus spread primarily via respiratory droplets, infection of the gastrointestinal tract may have facilitated other routes of spread.

The recognition that SARS was caused by a coronavirus intensified the search for other pathogenic coronaviruses associated with human disease, which led to the identification of HCoV-NL63 and HCoV-HKU1. These viruses were isolated from hospitalized patients, either young children with severe respiratory disease (HCoV-NL63)^{64,65} or elderly patients with underlying medical problems (HCoV-HKU1)65,66. HCoV-NL63 has infected human populations for centuries, as phylogenetic studies show that it diverged from HCoV-229E nearly 1,000 years ago67. HCoV-NL63 and HCoV-HKU1 have worldwide distributions and generally cause mild upper respiratory tract diseases, with the exception that HCoV-NL63 is also an aetiological agent of croup⁶⁸. HCoV-NL63 can be propagated in tissue culture cells, and an infectious cDNA clone of this virus was recently engineered, facilitating future studies⁶⁹. By contrast, HCoV-HKU1 cannot be grown in tissue culture cells, which makes it imperative that an infectious cDNA clone be developed for future studies.

Although the severe disease forming capabilities of human coronaviruses were only recognized because of the SARS epidemic, it was well known that animal coronaviruses could cause life-threatening disease. TGEV, which causes diarrhoea in piglets, infectious bronchitis virus (IBV), a cause of severe upper respiratory tract and kidney disease in chickens, and bovine coronavirus (BCoV), which causes respiratory tract disease and diarrhoea in cattle ('winter dysentery' and 'shipping fever'), are all economically important pathogens. Feline infectious peritonitis virus (FIPV), a virulent feline coronavirus (FCoV), causes an invariably fatal systemic disease in domestic cats and other felines. Unlike most strains of FCoV, which are endemic causes of mild diarrhoea, FIPV arises sporadically, most likely by mutation or deletion in felines persistently infected with enteric strains of FCoV⁷⁰, and is macrophage-tropic.

Perhaps the most convincing explanation for FIPVmediated disease was suggested by the observation that progressive waves of virus replication, lymphopenia and ineffectual T cell responses occurred in feline infectious peritonitis (FIP)71. In conjunction with previous studies, these results raised the possibility that FIPV infection of macrophages and dendritic cells caused aberrant cytokine and/or chemokine expression and lymphocyte depletion, resulting in enhanced virus loads and, consequently, a fatal outcome. Although this explanation is appealing, additional work is needed to prove its validity. Notably, anti-FIPV antibody-mediated enhancement has been implicated in pathogenesis, but this has been shown only after immunization with S protein expressing vaccines⁷²; it has not been shown to play a role in a natural feline infection.



Figure 3 | **Mechanism of coronavirus replication and transcription.** Following entry into the cell and uncoating, the positive sense RNA genome is translated to generate replicase proteins from open reading frame 1a/b (ORF1a/b). These proteins use the genome as a template to generate full-length negative sense RNAs, which subsequently serve as templates in generating additional full-length genomes (a). Coronavirus mRNAs all contain a common 5' leader sequence fused to downstream gene sequences. These leaders are added by a discontinuous synthesis of minus sense subgenomic RNAs using genome RNA as a template (reviewed in REF. 29). Subgenomic RNAs are initiated at the 3' end of the genome and proceed until they encounter one of the transcriptional regulatory sequences (TRS; red) that reside upstream of most open-reading frames (b). Through base-pairing interactions, the nascent transcript is transferred to the complementary leader TRS (light red) (c) and transcription continues through the 5' end of the genome (d). These subgenomic RNAs then serve as templates for viral mRNA production (e).

Cross-species transmission

A striking feature of the 2002–2003 SARS epidemic was the ability of the SARS-CoV to cross species from Himalayan palm civets (*Paguma larvata*), raccoon dogs (*Nyctereutes procyonoides*) and Chinese ferret badgers (*Melogale moschata*) to infect human populations⁷³ (FIG. 5a). Transmission occurred in live animal retail (wet) markets, where animal handlers became infected. In retrospect, it seems that variants of SARS-CoV related to the epidemic strain infected human populations in the wet markets fairly frequently, as is shown by the high seropositivity rate detected in animal handlers who did not develop SARS-like illnesses⁷³. The epidemic began when a physician who was treating personnel in the wet markets became infected and subsequently infected multiple contacts⁷⁴.

Genetic analyses of virus isolates from infected palm civets and humans during the epidemic showed that the virus underwent rapid adaptation in both hosts^{75,76}, primarily in the receptor binding domain (RBD) of the S protein, to allow more efficient infection of human cells⁷⁷. In particular, mutations K479N and S487T in the RBD of the S protein were key to adaptation to the human receptor (ACE2). These results were recently confirmed using cell lines expressing civet ACE2 or human ACE2 (REF. 78).

The observation that SARS-CoV could not be detected in either farmed or wild palm civets⁷⁹, together with evidence of adaptive changes detected in virus isolated from infected animals, suggested that palm civets and other animals in wet markets were not the primary reservoir for the virus. As SARS-like CoV were isolated

from Chinese horseshoe bats (*Rhinolophus* spp.)^{23,80}, which were also present in the live animal markets, the virus may have recently spread from bats to other mammals, such as palm civets, and then to humans (FIG. 5a). Consistent with a recent spread, antibodies to SARS-CoV were detected at extremely low levels (0.008%) in population studies in Hong Kong⁸¹. Bat SARS-like CoV cannot replicate in cells that express bat ACE2, although productive infection of cells expressing human ACE2 occurs if the RBD of the bat S protein is replaced with that of a human isolate^{82,83}. Collectively, these observations suggest that virus spread from bats to other species. Host cell entry does not occur via ACE2 in bats, although it does in palm civets and humans.

Besides SARS-CoV, there are other examples of coronavirus cross-species transmission. BCoV and HCoV-OC43 are similar and the virus may have crossed from bovine to human hosts approximately 100 years ago⁸⁴. BCoV has continued to cross species, as a related virus (99.5% similarity) has been isolated from an alpaca with enteritis and from captive wild ruminants^{85,86} (FIG. 5b), Furthermore, canine coronavirus (CCoV), feline and porcine viruses show evidence they have recombined with each other, indicating that they were present in the same host. Recombination events between early CCoV and FCoV strains (CCoV-I and FCoV-I) and an unknown coronavirus resulted in two sets of novel viruses - CCoV-II and FCoV-II. Sequence data suggest that TGEV resulted from a cross-species transmission of CCoV-II from an infected canine⁸⁷ (FIG. 5c).

Molecular surveillance studies have identified at least 60 novel bat coronaviruses in China⁸⁸, North America⁵⁰, Europe^{89,90} and Africa⁹¹. These bat CoVs may have originated from a common source and then subsequently diverged as they adapted to growth in different species of bat; they are now only distantly related to other coronaviruses. These studies also identified several novel avian group 3 coronaviruses92 that were related to a novel coronavirus isolated from Asian leopard cats (Prionailurus bengalensis) and Chinese ferret badgers sold in illegal wild animal markets in China93, suggesting that this virus, like SARS-CoV, can cross species. Another novel group 3 coronavirus, isolated from a deceased beluga whale (Delphinapterus leucas), is only distantly related to IBV-like and novel avian coronaviruses, suggesting that it comprises a third subgroup94. Thus group 3 coronaviruses, which formerly included only avian viruses, now consist of at least 3 subgroups and include viruses that infect mammalian hosts.

Immunopathology in coronavirus infections

It is generally accepted that the host response is responsible for many of the disease manifestations in infections caused by coronaviruses^{95,96}. This was shown initially in mice infected with the neurotropic strains of mouse hepatitis virus (the JHMV and MHV-A59 strains). Many attenuated strains of JHMV cause a subacute or persistent infection in the central nervous system, with persistence in glia, especially oligodendrocytes. A consequence of host efforts to clear the virus is myelin destruction (demyelination). However, JHMV infection

Table 2 Coronavirus non-structural proteins and their functions			
Protein	Functions		
Nsp1	Host mRNA degradation; translation inhibition; cell cycle arrest; inhibition of IFN signaling		
Nsp2	Unknown		
Nsp3	Papain-like proteases (PL1 ^{pro} , PL2 ^{pro}) (polyprotein processing); poly(ADP-ribose) binding; DMV formation (?); IFN antagonist; nucleic acid binding; deubiquitinating activity		
Nsp4	DMV formation (?)		
Nsp5	Main protease (M^{pro} , $3CL^{pro}$); polyprotein processing		
Nsp6	DMV formation (?)		
Nsp7	Single-stranded RNA binding		
Nsp8	Primase		
Nsp9	Part of replicase complex		
Nsp10	Part of replicase complex		
Nsp11	Unknown		
Nsp12	RNA-dependent RNA polymerase		
Nsp13	Helicase; nucleoside triphosphatase activity; RNA 5'-triphosphatase activity		
Nsp14	$3' \rightarrow 5'$ exoribonuclease; RNA cap formation (guanine-N7)-methyltransferase		
Nsp15	Endonuclease		
Nsp16	RNA cap formation (2'O-methytransferase)		

DMV, double-membrane vesicle; IFN, interferon.

of mice that lack T or B cells (sublethally irradiated mice or mice with severe combined immunodeficiency or genetically deficient in recombination activating gene 1 (RAG1^{-/-})) results, eventually, in death in all mice, but without demyelination. Adoptive transfer of CD4⁺ or CD8⁺ T splenocytes 7 or 30 days after immunization with JHMV to infected RAG1-/- or SCID mice results in virus clearance and demyelination95-97. Myelin destruction is also observed if anti-JHMV antibody is transferred to infected RAG1-/- mice in the absence of T cells98, or if mice are infected with virus expressing the macrophage chemoattractant CCL2 in the absence of other interventions⁹⁹. In all cases, infiltrating macrophages appear to be crucial for virus clearance and subsequent demyelination; these results suggest that the process of macrophage infiltration can be initiated by T cells, anti-JHMV antibody or overexpression of a single macrophage chemoattractant. These results have been extended to mice with encephalitis caused by virulent strains of JHMV. Although CD4+ and CD8+ T cells are both required for virus clearance¹⁰⁰, partial abrogation of the CD4+ T cell response (by mutating the immunodominant CD4+ T cell epitope rJ.M_{Y1350}) results in disease amelioration, and virulence is regained if another CD4+ T cell epitope is reintroduced into the rJ.M_{Y1350} genome¹⁰¹. Thus acute encephalitis, like chronic demyelination, is at least partially mediated by the immune system.

Similar processes may occur in SARS-CoV-infected humans, as pulmonary disease often worsens at 1–2 weeks after onset of respiratory symptoms, concomitant with the onset of virus clearance¹. Although worsening clinical disease occurring as a consequence of virus clearance has not been duplicated in any animal model of SARS, the severe disease observed in older patients can be mimicked in SARS-CoV-infected aged mice¹⁰²⁻¹⁰⁴. This has been attributed, in part, to a suboptimal T cell response resulting in delayed kinetics of virus clearance. A suboptimal T cell response, occurring as a consequence of infection of macrophages or dendritic cells, may also be critical for the immunopathological lethal disease that is observed in FIPV-infected felines⁷¹. Thus, in many instances, host efforts to clear a coronavirus infection result in some tissue destruction.

Evasion of the innate immune response

Although anti-viral T cells and antibodies are crucial for virus clearance and for the prevention of recrudescence (reviewed in REF. 96), the efficacy of the innate immune response determines the extent of initial virus replication and thus the load that the host must overcome to clear the infection (FIG. 6a). Coronaviruses, like all other successful viruses, have developed strategies to counter the innate immune response (FIG. 6b-d). IFN expression is a crucial component of this initial response, and coronaviruses have developed 'passive' and 'active' tools to prevent IFN induction and signalling. Interferon is not induced in fibroblasts that are infected with either SARS-CoV or MHV¹⁰⁵⁻¹⁰⁷. However, in both instances, treatment of cells with polyinosinic:polycytidylic acid or with other IFN-inducing agents, results in activation of IFN regulating factor 3 (IRF3) and IFN induction^{105,106}. Thus, in these cells, viruses appear to be invisible to intracellular viral sensors (such as RIG-I, MDA5 and TLR3), perhaps because double stranded RNA, a potent stimulator of the innate immune system, is buried in a DMV (FIG. 6b).

Additionally, viral proteins, in particular nsp1, nsp3, N protein and the SARS-CoV accessory proteins ORF6 and ORF3b, also prevent IFN induction¹⁰⁸⁻¹¹³. The N protein of MHV inhibits activator protein 1 (AP1) signalling and protein kinase R (PKR) function, whereas the N protein of SARS-CoV also inhibits nuclear factor-ĸB activation¹⁰⁸⁻¹¹⁰ when expressed in transfection assays. Whether these inhibitory functions of the N protein are coronavirus or cell-type specific, and whether they occur in infected cells, remains to be determined. The ORF6 protein inhibits IFN signalling by binding to karyopherin- α 2, thereby tethering karyopherin- β to cytoplasmic membranes¹¹⁴. This, in turn, prevents nuclear translocation of proteins containing classical nuclear import signals¹¹⁵, including STAT1, a crucial component of IFNa, IFNB and IFNy signalling pathways. Of note, deletion of ORF6 does not increase the IFN sensitivity of SARS-CoV¹¹⁶, probably because mechanisms of IFN antagonism are redundant.

SARS-CoV and MHV nsp1 function, at least in part, by degrading host cell mRNA and inhibiting translation^{111-113,117}. Nsp1 also inhibits IFN signalling in both SARS-CoV- and MHV-infected cells, in part by inhibiting STAT1 phosphorylation^{112,113}. Mutation of nsp1 attenuates SARS-CoV and MHV growth in mice and

tissue culture cells in the presence of an intact IFN system, but not when IFN function is deficient¹¹¹⁻¹¹³. Nsp3 is also an IFN antagonist, and it inhibits phosphorylation and nuclear importation of IRF3 (REF. 118).

Both MHV and SARS-CoV inhibit IFN α and IFN β induction and signalling. However, IFN α and/or IFN β are detected in infected mice and humans^{119,120} and mice deficient in IFN α and/or IFN β receptor expression are exquisitely sensitive to MHV infection^{113,121}, showing that IFN α and/or IFN β has a major role in the antivirus immune response. Reconciling these disparate results, recent studies showed that IFN α is produced in large amounts in SARS-CoV- and MHV-infected plasmacytoid dendritic cells, via a <u>TLR7</u>-dependent mechanism¹²². Furthermore, IFN β is expressed by macrophages and microglia, but not by dendritic cells after MHV infection¹²³. Macrophages, and to a lesser extent dendritic cells, are the major targets for IFN α and/or IFN β in MHV-infected mice¹²⁴.

In addition to IFN, multiple chemokines and cytokines are also induced as part of the host response to coronaviruses such as MHV, SARS-CoV and FIPV. Cytokines such as interleukin 1 (IL-1), IL-6 and IL-12 and chemokines such as IL-8, CCL2 and CXCL10 are elevated in SARS patients. Using genomics and proteomics, Cameron *et al.* found that IFN α and/or IFN β and IFN γ , as well as chemokines such as CXCL10 and CCL2, are elevated at early times post infection in all patients and diminished in those who recovered, accompanied by a robust anti-virus antibody response¹¹⁹. However, levels of CXCL10, CCL2 and other proinflammatory mediators remained elevated and anti-SARS-CoV antibody titres were low in those patients who developed severe disease. SARS-CoV-infected pulmonary epithelial cells were the source of at least some of the cytokines and/or chemokines, such as CCL2, IL-6, IL-1ß and tumour necrosis factor (TNF)¹²⁵. Others have suggested that a strong T_H2 (IL4, IL-5 and IL-10) response correlated with a poor outcome¹²⁶. It has been postulated that an over-exuberant cytokine response contributed to a poor outcome in patients with SARS in 2002-2003 (reviewed in REFS 95,127,128). Collectively, these results do not strongly prove or disprove a role for an exuberant cytokine and chemokine response in severe SARS, in part because virus titres could not be determined concomitantly and also because serum levels, but not pulmonary cytokine or chemokine levels were measured.

Animal models for SARS

As human SARS has disappeared, the role of an exuberant (but perhaps appropriate for the titre of the virus) immune response will need to be addressed using animal models of SARS. Mice, cats, ferrets, macaques and civet cats are all susceptible to SARS-CoV, but none, with the exception of aged mice, develop severe disease (reviewed in REF. 129). In efforts to develop models that closely mimic human disease, mice that are transgenic for the expression of human ACE2 were developed and infected with SARS-CoV^{130,131}. Although these mice develop more severe pulmonary disease than nontransgenic mice, they also develop an overwhelming



Figure 4 | Coronavirus-induced membrane alterations as platforms for viral replication. Coronavirus infection induces the formation of a reticulovesicular network of modified membranes that are thought to be the sites of virus replication. These modifications, which include double-membrane vesicles (DMVs), vesicle packets (VPs, single-membrane vesicles surrounded by a shared outer membrane) and convoluted membranes (CMs), are all interconnected and contiguous with the rough endoplasmic reticulum (RER). Viral double-stranded RNA is mostly localized to the interior of the DMVs and inner vesicles of the VPs, whereas replicase proteins (that is, nsp3, nsp5 and nsp8) are present on the surrounding CM. Some nsp8 can be detected inside the DMVs. All membranes are bound by ribosomes. (Figure based on data from REFS. 56,141,142.)

neuronal infection, accompanied by high cytokine and/ or chemokine expression and minimal cellular infiltration in the brain¹³². Although the severity of the brain infection observed in human ACE2 transgenic mice is greater than that seen in human patients, infection of this organ has been detected in some studies and patients who survived SARS had a greater incidence of neurological and psychiatric sequelae than anticipated^{63,133,134}. The high susceptibility of these mice to infection with SARS-CoV makes them useful for vaccine and therapeutic trials. Another approach to developing an animal model for SARS was to adapt the virus by passage 10–15 times through the lungs of BALB/c mice or rats^{103,135,136}. Three to six mutations were detected in the adapted viruses, with changes most commonly observed in the S protein and in nsp5 (3CL^{pro}).



Figure 5 | **Cross-species transmission of coronaviruses. a** | Severe acute respiratory syndrome (SARS)-like bat coronavirus (BtCoV) spread and adapted to wild animals such as the Himalayan palm civet that was sold as food in Chinese wet markets. The virus frequently spread to animal handlers in these markets, but caused minimal or no disease. Further adaptation resulted in strains that replicated efficiently in the human host, caused disease and could spread from person to person. **b** | Human coronavirus OC43 (HCoV-OC43) and bovine coronavirus (BCoV) are closely related and it is thought that the virus originated in one species and then crossed species. BCoV has also spread to numerous other animals, such as alpaca and wild ruminants. **c** | Feline coronavirus I (FCoV-I) and canine coronavirus I (CCoV-I) are thought to share a common ancestor. CCoV-I underwent recombination with an unknown coronavirus to give rise to canine coronavirus II (CCoV-II). CCoV-II in turn underwent recombination with FCoV-I (in an unknown host) to give rise to feline coronavirus II (FCoV-II). CCoV-II probably also spread to pigs, resulting in transmissible gastroenteritis virus (TGEV).

The adapted virus caused extensive pulmonary infection and disease was most severe in aged animals. These viruses will be useful for studies of pathogenesis and for vaccine and therapeutic trials.

Some models have been tested on the genomic and proteomic level. Studies of SARS-CoV infected macaques showed that several chemokines and/or cytokines, such as IL-6, IL-8, CXCL10 and CCL2, as well as IFN α , IFN β and IFN γ , were upregulated¹³⁷. These animals recovered, showing that the same inflammatory mediators that are associated with severe human disease are also produced as part of the inflammatory response in animals that mount an appropriate response. Genomics studies of mice infected with the Urbani strain of SARS-CoV showed continued expression of inflammatory mediators, such as IL-6, TNF, CXCL10 and CCL2, accompanied by slower kinetics of virus clearance and worse outcomes in aged compared to young animals¹³⁸, paralleling disease patterns in patients with SARS¹¹⁹. These two studies also showed changes in expression of proteins that are involved in cell growth, cycling, cell-to-cell signalling and development and death. It will be important to determine whether these changes are useful as a 'fingerprint' for SARS or whether they represent generalized responses to pulmonary stress.

Future directions

Perhaps the most important insight made over the past several years is that coronaviruses have and will likely continue to cross between species and cause



Figure 6 | Inefficient activation of the type 1 interferon response, and immunopathological disease, in coronavirus infections. a | Coronaviruses, as exemplified by severe acute respiratory syndrome coronavirus (SARS-CoV) and mouse hepatitis virus (MHV), induce a type 1 interferon (IFN) response in plasmacytoid dendritic cells (pDC) and macrophages, via TLR7- and MDA5-dependent pathways, respectively. b | IFN α and/or IFN β is not produced in either SARS-CoV fibroblasts or DCs, partly because coronavirus macromolecules appear to be invisible to immune sensors. Additionally, coronaviruses encode proteins that actively inhibit IFN α and/or IFN β expression (such as nucleocapsid (N) protein, nsp3, ORF6 and ORF3b) or signalling through the type 1 IFN receptor (such as N, nsp1, ORF6 and ORF3b). c | Consequently, the kinetics of virus clearance is delayed, with subsequent robust T and B cell and cytokine and/or chemokine responses. d | This pro-inflammatory response results in immunopathological disease that occurs during the process of virus clearance. In MHV-infected mice, virus clearance involves recruitment of activated macrophages and microglia to sites of virus infection, leading to demyelination. Similar mechanisms with exuberant cytokine production may function in the lungs of SARS-CoV-infected humans, leading to severe pulmonary disease (adult respiratory distress syndrome, ARDS). AP1, activator protein 1; DMV, double-membrane vesicle; dsRNA, double-stranded RNA; NF- κ B, nuclear factor- κ B; ssRNA, single-stranded RNA.

disease in unrelated hosts. This disease may be mild, like the disease caused by the SARS-like CoV that was transmitted to animal handlers in wet markets in China, but it may be severe, as illustrated by the transmission that triggered the SARS epidemic. Further, SARS-CoV appeared to use an entirely new receptor when it crossed species from bats to palm civets and humans. As part of this transmission to a new species, the virus also needed to evolve strategies to evade the innate immune response of the new hosts. One future goal will be to further delineate how the virus evades the immune response and better understand its interaction with the T and B cell responses, both in the original host (bats), in which disease appears to be mild, and in humans and experimentally infected animals.

Collaborative cross mice

A panel of 1,000 recombinant inbred mouse strains derived from 8 genetically diverse founder strains. The crosses were designed for complex trait analysis and will be useful for identifying and establishing the role of host genes in SARS pathogenesis. Although coronaviruses use host proteins as part of their replication strategies, it has also become clear that immune, metabolic, stress, cell cycling and other pathways are activated by infection. Assessing the biological function of these pathways in virus replication and in disease outcome will be critical. Determining the extent to which virus-host interactions are coronavirus-specific and organ-specific will be possible, using genomics and proteomics, as well as new reagents and collaborative cross mice. The collaborative cross, a panel of approximately 1,000 recombinant inbred mouse strains derived from 8 founder strains, will be useful for analyses of complex genetic traits¹³⁹.

Using sophisticated microscopy and biochemical approaches, details of coronavirus replication in infected cells have been revealed. However, these new results have led to a new set of questions about the relationship between sites of viral RNA replication and virus assembly. Furthermore, although putative functions have been assigned to many of the proteins encoded by the large ORF1 replicase gene, the precise roles of these proteins in virus replication still require additional investigation. Progress in these fields will take advantage of new methodologies that allow detailed observations of both fixed and living cells at high resolution.

Finally, no effective treatments exist for any coronavirus infections, including SARS¹⁴⁰; vaccines, even for animal coronaviruses, are not effective; and live attenuated vaccines are prone to recombination with circulating coronaviruses. One future goal will be to translate new information about the structure and function of coronavirus proteins into specific antivirus therapies. Also, development of live, attenuated, safe vaccines that do not recombine in the wild is another goal, made more feasible as more is learned about basic coronavirus biology. Over the past few years, the development of new technologies has simplified the identification of novel coronaviruses; the next major goals will be to understand viral pathogenesis and to design effective coronavirus vaccines and therapies.

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DATABASES

UniProtKB: http://ca.expasy.org/sprot ACE2 | CCL2 | CXCL10 | EGL2 | IL-6 | IL-8 | IRE3 | MDA5 | RAG1 | STAT1 | TLR7

FURTHER INFORMATION

Stanley Perlman's homepage: http://www.uiowa.edu/ microbiology/perlman.shtml

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