Coronaviruses post-SARS: update on replication and pathogenesis

Stanley Perlman and Jason Netland

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.

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 [ref. 1]. 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 tomography [fig. 1]. 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 two-thirds 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 coronavirus [fig. 2a]. 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 host [fig. 2b].

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 infection [ref. 7]. 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) [fig. 1]. 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) [fig. 1]. 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]. 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 (TGFβ) signalling in SARS-CoV-infected cells [14].

Department of Microbiology and Interdisciplinary Program in Immunology, University of Iowa, Iowa City, Iowa 52242, USA. Correspondence to S.P. e-mail: stanley-perlman@uiowa.edu doi: 10.1038/nrmicro2147 Published online 11 May 2009
RNA polymerase, nsp8, may function as a primase\textsuperscript{42}. 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 known\textsuperscript{43}.

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 replication\textsuperscript{44}. Nsp7 and nsp8 form a hexadecameric structure, with RNA binding activity\textsuperscript{31}. The structure of nsp9 also suggests that it binds RNA\textsuperscript{44}. 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\textsubscript{pro})\textsuperscript{46}.

Nsp14, a bifunctional protein, is a 3'→5' exonuclease, with a role in maintaining fidelity of RNA transcription\textsuperscript{47}, and a (guanine-N7)-methyl transferase (N7-MTase), involved in RNA cap formation\textsuperscript{48}. Coronavirus also encode a novel uridylicate-specific endoribonuclease (NendoU), nsp15, that distinguishes nidoviruses in general from other RNA viruses and that is crucial for virus replication\textsuperscript{49}. 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-l-methionine-dependent RNA (nucleoside-2'-O')-methyl transferase (2'O-MTase) and, like nsp14, is involved in cap formation\textsuperscript{50}. 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 double-membrane vesicles (DMVs)\textsuperscript{51} (Fig. 4). Newly synthesized genomic RNA is then incorporated into virions 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 pathway\textsuperscript{53}, but other studies showed replication proceeded normally and that DMVs were produced in macrophages lacking ATG5, a key component of autophagosomes\textsuperscript{54}. 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 formation\textsuperscript{55}.

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, Knoop and al. showed that DMVs are not isolated vesicles, but rather are part of a reticulovesicular network of modified ER membranes\textsuperscript{56}. 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 detected\textsuperscript{58}. Thus, it
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 virus, 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 replication. Nsp6, like nsp3 and nsp4, also contains multiple transmembrane regions and may be involved in membrane modification. 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 membrane; 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 immunocompromised. SARS-CoV, unlike HCoV-OC43 and HCoV-229E, causes a severe

---

**Table 1 | Representative coronavirus species and their receptors**

| Group | Host | Virus | Cellular receptor |
|-------|------|-------|-------------------|
| Group 1a | Bat⁴ | 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 | Bat⁴ | 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 | ALCoV | 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). ALCoV, 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; RbCoV, rabbit coronavirus; RCoV, rat coronavirus; SARS-CoV, severe acute respiratory syndrome-associated coronavirus; SDAV, sialodacryoadenitis virus; TCoV, turkey coronavirus; ThCoV, thrush coronavirus; 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 tissues64. 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)65,66 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 ago65. 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 group48. HCoV-NL63 can be propagated in tissue culture cells, and an infectious cDNA clone of this virus was recently engineered, facilitating future studies49. 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 FIPV-mediated disease was suggested by the observation that progressive waves of virus replication, lymphopenia and ineflectual 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.
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 populations29 (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 illnesses29. The epidemic began when a physician who was treating personnel in the wet markets became infected and subsequently infected multiple contacts30.

Genetic analyses of virus isolates from infected palm civets and humans during the epidemic showed that the virus underwent rapid adaptation in both hosts31,32, primarily in the receptor binding domain (RBD) of the S protein, to allow more efficient infection of human cells31. 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 civets, 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.)33,34, 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 Kong35. 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 isolate32,33. 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 ago36. 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 ruminants37,38 (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 canine39 (FIG. 5c).

Molecular surveillance studies have identified at least 60 novel bat coronaviruses in China40, North America41, Europe42,43 and Africa44. These bat CoVs may have originated from a common source and then subsequently diverged as they adapted to grow in different species of bat; they are now only distantly related to other coronaviruses. These studies also identified several novel avian group 3 coronaviruses45 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 China42, 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 subgroup42. 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 coronaviruses46,47. 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
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 demyelination. Myelin destruction is also observed if anti-JHMV antibody is transferred to infected RAG1−/− mice in the absence of T cells, or if mice are infected with viruses expressing the macrophage chemoattractant CCL2 in the absence of other interventions. 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 clearance, partial abrogation of the CD4+ T cell response (by mutating the immunodominant CD4+ T cell epitope rM113Q) results in disease amelioration, and virulence is regained if another CD4+ T cell epitope is reintroduced into the rM113Q genome. 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 clearance. 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 mice. 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 felines. 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 MHV. 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. 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.

Additionally, viral proteins, in particular nsp1, nsp3, N protein and the SARS-CoV accessory proteins ORF6 and ORF3b, also prevent IFN induction. 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-κB activation 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-α2, thereby tethering karyopherin-β to cytoplasmic membranes. This, in turn, prevents nuclear translocation of proteins containing classical nuclear import signals, including STAT1, a crucial component of IFNα, IFNβ and IFNγ signalling pathways. Of note, deletion of ORF6 does not increase the IFN sensitivity of SARS-CoV, 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. Nsp1 also inhibits IFN signalling in both SARS-CoV- and MHV-infected cells, in part by inhibiting STAT1 phosphorylation. Mutation of nsp1 attenuates SARS-CoV and MHV growth in mice and
Both MHV and SARS-CoV inhibit IFNα and IFNβ induction and signalling. However, IFNα and/or IFNβ are detected in infected mice and humans119,120 and mice deficient in IFNα and/or IFNβ receptor expression are exquisitely sensitive to MHV infection113,121, showing that IFNα and/or IFNβ has a major role in the antiviral immune response. Reconciling these disparate results, recent studies showed that IFNα is produced in large amounts in SARS-CoV- and MHV-infected plasmacytoid dendritic cells, via a TLR7-dependent mechanism122. Furthermore, IFNβ 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 IFNα and/or IFNβ 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 IFNα and/or IFNβ and IFNγ, 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-1β and tumour necrosis factor (TNF)125. Others have suggested that a strong TLR-2 (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-CoV130,131. Although these mice develop more severe pulmonary disease than non-transgenic mice, they also develop an overwhelming 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 anticipated63,133,134. 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 rats103,135,136. Three to six mutations were detected in the adapted viruses, with changes most commonly observed in the S protein and in nsp5 (3CLpro).
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 IFNα, IFNβ and IFNγ, were upregulated. 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 animals, paralleling disease patterns in patients with SARS. 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
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.

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 | IFNα and/or IFNβ 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 IFNα and/or IFNβ 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-κB, nuclear factor-κB; ssRNA, single-stranded RNA.
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 traits.

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 SARS\(^{146}\), 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 anti-virus 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.
Syndrome (Non-SARS)-related human coronavirus
the clinical impact of non-severe acute respiratory
Garbino, J.

Non-structural proteins 2
Knoops, K.

Syndrome-coronavirus replicative protein nsp9 is a
A59 replication.

103

Biol. Chem.

REVIEWS

G., Yoshimori, T., Mizushima, N., A. Memory CD4

169

18–26 (2007).

This report—REF. 106 are the first to suggest that
coronaviruses are invisible to the host innate
immune response in some cells.

102, 845–895 (2001).

Clementz, M.A., Kanjanahunlaha, A., Obrien, T.E. & Baker, S.C. Mutation in murine coronavirus
replication complex protein 4a decreases the stability of double-stranded
vesicles. Virology 357, 118–129 (2008).

Kanjanahunlaha, A., Chen, Z., Jukneliene, D. & Baker, S.C. Membrane topology of murine
coronavirus replicase nucleoprotein 3. Virology 361, 391–391 (2007).

Oostra, M. et al. Topology and membrane anchoring of the coronavirus replication complex. Proc. Natl. Acad. Sci. USA 105, 12392–12405 (2008).

Oostra, M. & de Zwette. Membrane topology of coronavirus nonstructural protein 4: involvement of the early secretory pathway in replication. J. Virol. 81, 13466–13474 (2007).

Garbino, J. et al. A prospective hospital-based study of the clinical impact of non-severe acute respiratory syndrome (Non-SARS)-related human coronavirus infection. Emerg. Infect. Dis. 13, 1009–1010 (2007).

Gu, J. et al. Multiple organ infection and the pathogenesis of SARS. J. Exp. Med. 202, 415–426 (2005).

Fourrier, R. A. et al. A previously undescribed coronavirus associated with respiratory disease in humans. Proc. Natl. Acad. Sci. USA 101, 6212–6216 (2004).

van der Hoek, L. et al. Identification of a new human coronavirus. Nat. Med. 10, 368–375 (2004).

Woo, P.C. et al. Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. J. Virol. 79, 884–895 (2005).

Pyrc, K. et al. Mosaic structure of human coronavirus NL63, one of the fastest-evolving of human coronaviruses. J. Mol. Biol. 364, 963–976 (2007).

This manuscript delineates molecular evolution of HCoV-229E and HCoV-NL63.

van der Hoek, L. Severe acute respiratory syndrome-coronavirus replicative protein nsp10 is a critical regulator of viral RNA synthesis. J. Virol. 81, 6356–6365 (2007).

Eckert, A.D., Lu, X., Sperry, S.M., Choi, L. & Denison, M.R. High fidelity of murine hepatitis virus RNA replication is decreased in nsp 14 eorxibonucleotidases. J. Virol. 81, 1215–12144 (2007).

Chen, Y. et al. Functional screen reveals SARS coronavirus nonstructural protein 14 as a novel cat N7 methyltransferase. Proc. Natl. Acad. Sci. USA 102, 8007–8012 (2005).

Ikino, K.A. et al. General marker of nidoviruses encodes a replicative endoribonuclease. Proc. Natl. Acad. Sci. USA 106, 25199–25204 (2009).

Decroly, E. et al. Coronavirus nonstructural protein 16 is a cap-0 binding enzyme possessing (nucleoside-2’0)-transferrase activity. J. Virol. 82, 4055–4064 (2008).

Snijder, J. et al. Ultrastructure and origin of membrane vesicles associated with the severe acute respiratory syndrome coronavirus replication complex. J. Virol. 80, 5927–5940 (2006).

de Haan, C.A. & Rotter, P.J. Molecular interactions in the assembly of corona viruses. Adv. Virus Res. 64, 165–205 (2007).

Prentice, E., Jerome, W.G., Yoshimori, T., Mizushima, N., Imai, T. & Enjuanes, L. Coronavirus replicase complex formation utilizes components of cellular autophagy. J. Biol. Chem. 279, 10136–10141 (2004).

Zhao, Z. et al. Coronavirus replication does not require the autophagy gene ATG5. Autophagy 3, 581–585 (2007).

Bechill, J., Chen, Z., Brewer, J.W. & Baker, S.C. Coronavirus infection modulates the unfolded protein response and mediates sustained translational repression. J. Virol. 82, 4492–4501 (2008).

Kremer, J. et al. Coronavirus replication complex is supported by a reticulovesicular network of modified endoplasmic reticulum. PLoS Biol. 6, e226 (2008).

This is an elegant electron microscopic study that uses 3D imaging of SARS-CoV infected cells to
delineate the relationship between virus replication and virus-induced membranous changes.

Snijder, E.J., N.J. & Pedersen, K.W. Non-structural proteins 2 and 3 interact to modify host cell membranes during the formation of the arterivirus replication complex. J. Gen. Virol. 82, 985–994 (2001).

Clementz, M.A., Kanjanahunlaha, A., Obrien, T.E. & Baker, S.C. Mutation in murine coronavirus replication protein 4a decreases the stability of double-stranded membrane vesicles. Virology 357, 118–129 (2008).

Kanjanahunlaha, A., Chen, Z., Jukneliene, D. & Baker, S.C. Membrane topology of murine coronavirus replicase nucleoprotein 3. Virology 361, 391–391 (2007).

Oostra, M. et al. Topology and membrane anchoring of the coronavirus replication complex. Nature 437, 409–413 (2005).

Gilead, I. et al. Nidovirid hydropic domains of nsP5 and nsP6 are membrane spanning. J. Virol. 82, 12392–12405 (2008).

Oostra, M. et al. Membrane topology of coronavirus nonstructural protein 4: involvement of the early secretory pathway in replication. J. Virol. 81, 13466–13474 (2007).

Garbino, J. et al. A prospective hospital-based study of the clinical impact of non-severe acute respiratory syndrome (Non-SARS)-related human coronavirus infection. Emerg. Infect. Dis. 13, 1925–1930 (2007).

Glück, Rausch. P. et al. Detection and prevalence patterns of group 1 coronaviruses in bats, northern Germany. Emerg. Infect. Dis. 14, 626–631 (2008).

Tong, S. et al. Detection of novel SARS-like and other coronaviruses in samples from Kenya. Emerg. Infect. Dis. 15, 482–485 (2009).

Woo, P.C. et al. Comparative analysis of complete genome sequences of the two unique coronavirus species HKU1, HKU2 and HKU4 from humans. J. Virol. 83, 4586–4597 (2009).

Ye, Y., Hauns, K., Langland, J.O., Jacobs, B.L. & Hogue, B. Mouse hepatitis coronavirus AS9 nucleocapsid protein is a type I interferon antagonist. J. Virol. 81, 2546–2565 (2007).
Narayanan, K. et al. Severe acute respiratory syndrome coronavirus nsp1 suppresses host gene expression, including that of type 1 interferon, in infected cells. *J. Virol.* **82**, 4471–4479 (2008).

Wathelet, M. G., Orr, M., Rennick, M. B. & Baric, R. S. Severe acute respiratory syndrome coronavirus evades antiviral signaling: role of nsp1 and rational design of an attenuated strain. *J. Virol.* **81**, 11620–11633 (2007).

Zust, R. et al. Coronavirus non-structural protein 1 is a major pathogenicity factor: implications for the rational design of coronavirus vaccines. *PLoS Pathog.* **3**, e109 (2007).

Frieden, M. et al. Severe acute respiratory syndrome coronavirus ORF6 antagonizes STAT1 function by sequestering nuclear import factors on the rough endoplasmic reticulum/Golgi membrane. *J. Virol.* **81**, 9812–9824 (2007).

Hussain, S., Perlman, S. & Gallagher, T. M. Severe acute respiratory syndrome coronavirus protein 6 accelerates murine hepatitis virus infections by more than one mechanism. *J. Virol.* **82**, 7212–7222 (2008).

Zhao, J. et al. Severe acute respiratory syndrome-CoV protein 6 is required for optimal replication. *J. Virol.* **83**, 2568–2573 (2009).

Kamitani, W. et al. Severe acute respiratory syndrome coronavirus nsp1 protein suppresses host gene expression by promoting host mRNA degradation. *Proc. Natl Acad. Sci. USA* **103**, 12885–12890 (2006). Describes a novel mechanism of viral protein-induced inhibition of host gene expression.

Devraji, S. G. et al. Regulation of IRF-5-dependent innate immunity by the papain-like protease domain of the severe acute respiratory syndrome coronavirus. *J. Biol. Chem.* **282**, 32208–32221 (2007).

Cameron, M. J. et al. Interferon-mediated immunopathological events are associated with atypical innate and adaptive immune responses in patients with severe acute respiratory syndrome. *J. Virol.* **81**, 8692–8706 (2007). This report provides a careful description of the changes in cytokine and chemokine expression that occurred in patients during the 2002–2003 epidemic.

Rempel, J. D., Murray, S. J., Meiner, J. & Buchmeier, M. J. Differential regulation of innate and adaptive immune responses in viral encephalitis. *Virology* **318**, 581–592 (2004).

Ireland, D. D., Stohlman, S. A., Hinton, D. R., Atkinson, R. & Bergmann, C. C. Type I interferons are essential in controlling neurotropic coronavirus infection irrespective of functional CD8 T cells. *J. Virol.* **82**, 300–310 (2008).

Cervantes-Barragan, L. et al. Control of coronavirus infection through plasmacytoid dendritic cell-derived type I interferon. *Blood* **109**, 1131–1137 (2007).

Roth-Cross, J. K., Bender, S. J. & Weiss, S. R. Murine coronavirus mouse hepatitis virus is recognized by MDA5 and induces type I interferon in brain macrophages/microglia. *J. Virol.* **82**, 9829–9838 (2008).

Cervantes-Barragan, L. et al. Type I IFN-mediated protection of macrophages and dendritic cells secures control of murine coronavirus infection. *J. Immunol.* **182**, 1099–1106 (2007).

He, L. et al. Expression of elevated levels of pro-inflammatory cytokines in SARS-CoV-infected ACE2 cells in SARS patients: relation to the acute lung injury and pathogenesis of SARS. *J. Pathol.* **210**, 288–297 (2006).

Li, C. K. et al. T cell responses to whole SARS coronavirus in humans. *J. Immunol.* **181**, 5490–5500 (2008).

Gu, J. & Korteweg, C. Pathology and pathogenesis of severe acute respiratory syndrome. *Am. J. Pathol.* **170**, 1136–1147 (2007).

Chen, J. & Subbarao, K. The immunobiology of SARS. *Annu. Rev. Immunol.* **25**, 443–472 (2007).

Subbarao, K. & Roberts, A. Is there an ideal animal model for the SARS? *Trends Microbiol.* **14**, 299–305 (2006).

McCray, P. B. Jr et al. Lethal infection in K18-hACE2 mice infected with SARS-CoV. *J. Virol.* **81**, 813–821 (2007). This paper and REF. 131 show that mice transgenic for the human SARS-CoV develop a lethal infection characterized by extensive injury of the brain.

Tseng, C. T. et al. SARS coronavirus infection of mice transgenic for the human angiotensin-converting enzyme 2 (hACE2) virus receptor. *J. Virol.* **81**, 1162–1173 (2007).

Netland, J., Meyerholz, D. K., Moore, S., Cassell, M. & Perlman, S. Severe acute respiratory syndrome coronavirus infection causes neuronal death in the brain of F344 rats infected with SARS-CoV. *PLoS Pathog.* **3**, e109 (2007).

Lee, D. T. et al. Factors associated with psychosis among patients with severe acute respiratory syndrome: a case-control study. *Clin. Infect. Dis.* **39**, 1247–1269 (2004).

Xu, J. et al. Detection of severe acute respiratory syndrome coronavirus in the brain: potential role of the chemokine mig in pathogenesis. *Clin. Infect. Dis.* **41**, 1089–1096 (2005).

Nagata, N. et al. Mouse-passaged severe acute respiratory syndrome-associated coronavirus leads to lethal pulmonary edema and diffuse alveolar damage in adult but not young mice. *Am. J. Pathol.* **172**, 1625–1637 (2008).

Nagata, N. et al. Participation of both host and virus factors in induction of severe acute respiratory syndrome (SARS) in F344 rats infected with SARS coronavirus. *J. Virol.* **81**, 1848–1857 (2007).

de Lang, A. et al. Functional genomic highlights differential induction of antiviral pathways in the lungs of SARS-CoV-infected macaques. *PLoS Pathog.* **3**, e112 (2007).

Baas, T. et al. Genomic analysis reveals age-dependent innate immune responses to severe acute respiratory syndrome coronavirus. *J. Virol.* **82**, 9465–9476 (2008).

Morahan, G., Balmer, L. & Monley, D. Establishment of “The Gene Mine”: a resource for rapid identification of complex trait genes. *Annu. Genom.* **19**, 390–393 (2008).

Stockman, L. J., Bellamy, R. & Garner, P. SARS systemic review of treatment effects. *PLoS Med.* **3**, e543 (2006).

Gosert, R., Kanjanahaluethai, A., Egger, D., Bienz, K. & Baker, S. C. RNA replication of mouse hepatitis virus takes place at double-membrane vesicles. *J. Virol.* **76**, 5709–5708 (2002).

Simms, A. C., Osterrahn, J. & Denison, M. R. Mouse hepatitis virus replicates proteins associate with two distinct populations of intracellular membranes. *J. Virol.* **74**, 5647–5654 (2000).

**Acknowledgements**

Supported in part by research (PO1 AI060699 and RO1 NS46592) and training (T32 AI007533) grants from the National Institutes of Health (USA).