JGV Papers in Press. Published September 24, 2014 as doi:10.1099/vir.0.069732-0

A Review of Genetic Methods and Models for Analysis of Coronavirus Induced Severe Pneumonitis

Contents Category: Review

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Summary: 182

Main text: 6500

Tables: 2

SUMMARY (Abstract)

Coronaviruses have been studied for over 60 years, but have only recently gained notoriety as deadly human pathogens with the emergence of severe respiratory syndrome coronavirus and Middle East respiratory syndrome virus. The rapid emergence of these viruses has demonstrated the need for good models to study severe coronavirus respiratory infection and pathogenesis. There are, currently, different methods and models for the study of coronavirus disease. The available genetic methods for the study and evaluation of coronavirus genetics are reviewed here. There are several animal models, both mouse and alternative animals, for the study of severe coronavirus respiratory disease that have been examined, each with different pros and cons relative to the actual pathogenesis of the disease in humans. A current limitation of these models is that no animal model perfectly recapitulates the disease seen in humans. Through the review and analysis of the available disease models investigators can employ the most appropriate available model to study coronavirus various aspects of pathogenesis and evaluate potential antiviral treatments that may potentially be successful in future treatment and prevention of severe coronavirus respiratory infections.

INTRODUCTION

Severe acute respiratory syndrome coronavirus (SARS-CoV) is a novel human coronavirus that caused the first major pandemic of the new millennium in 2002-2003 (Baas *et al.*, 2008; Drosten *et al.*, 2003). Bats have been a source of a number of emerging zoonotic diseases, including Nipha and Hindra (Haagmans *et al.*, 2009; Wang *et al.*, 2006), and the animal source of the novel human SARS-CoV is thought to be Chinese horseshoe bats (*Rhinolophus sinicus*) (Lau *et al.*, 2010; Wang *et al.*, 2006). It is believed that a bat coronavirus adapted to infect civet cats and in civet cats the virus further adapted enabling it to infect humans (Lau *et al.*, 2010; Li, 2008). The receptor utilized by these SARS-like coronaviruses was shown to be angiotensin converting enzyme 2 (ACE2) (Li *et al.*, 2003). Recently a bat SARS-like coronaviruses has been recovered from *R. sinicus* that can utilize human ACE2 as a receptor underlining the ongoing threat of re-emergence (Ge *et al.*, 2013). Until the 2003 SARS-CoV pandemic there was little urgency to study coronavirus-related human disease because the disease was usually a self-limiting upper respiratory infection (Abdul-Rasool & Fielding, 2010; Kuri *et al.*, 2011). The SARS-CoV pandemic spurred a search for additional human coronaviruses (HCoV) and several new human respiratory coronaviruses, HCoV-HKU1 and HCoV-NL63 were discovered (Abdul-Rasool & Fielding, 2010; Zhou *et al.*, 2013). These viruses, as well as previously known human coronaviruses HCoV-OC43 and HCoV-229E, can cause significant human respiratory disease in the elderly and in infants and mild upper respiratory infections in otherwise healthy children and adults (Mesel-Lemoine *et al.*, 2012; Zhou *et al.*, 2013). Infection with the four different human coronaviruses typically takes place during childhood (Zhou *et al.*, 2013).

Originally coronaviruses were thought to be limited to individual species and a narrow organ tropism in a given species (Kuo *et al.*, 2000; Li, 2008; Zhang *et al.*, 2006). The spike receptor

protein, a very strong determinant of tissue and species tropism, binds to its cognate receptor and initiates viral entry into a host cell. There are also viral accessory genes that are thought to aid in immune evasion and viral replication in target species and tissues. Since the SARS-CoV outbreak, and the resulting population studies, it has been postulated that cross-species events occur more often than originally hypothesized (Rest & Mindell, 2003). The more recent 2012 emergence of the Middle East respiratory syndrome coronavirus underscores the potential for zoonotic spread of animal coronaviruses to humans. Thus there is a continuing need for animal models of severe coronavirus disease (Assiri *et al.*, 2013; Memish *et al.*, 2013).

There are two overarching aspects in modeling penumopathogenesis: the direct contributions of the virus and the response of the host immune system. The severity of the acute respiratory disease in SARS-CoV infected patients is thought, in large part, to be due to the immune response of the patient more than any predominant contribution of the virus (Frieman & Baric, 2008; Perlman & Dandekar, 2005). Herein we will review the genetic methods that are available to study viral contributions to disease, the animal models that have been analyzed for use as SARS-CoV infection models, and the viruses that are used in studying SARS-CoV biology and disease pathogenesis.

GENETIC APPROACHES TO STUDY CORONAVIRUS PATHOGENESIS

Although Coronaviruses have been studied for over 60 years the methods of evaluating viruses have changed, and scientists are continually developing methods that allow us to rapidly evaluate viruses. To investigate a gene's individual contribution to pathogenesis a method to make predetermined and targeted changes in select genes is required. There are two options for manipulating coronavirus genomes: targeted recombination and a complete reverse genetic system. These methods allow investigators to knock out individual genes or groups of genes and

allow for the generation of chimeric viruses that can be used to investigate the role of individual SARS-CoV genes.

Targeted recombination

Targeted recombination takes advantage of the high natural recombination rate of coronaviruses (Makino *et al.*, 1986). During normal coronavirus replication the coronavirus RNA dependent RNA polymerase (RdRp) employs a mechanism akin to template switching during minus strand RNA synthesis to accomplish leader-body joining and generate templates for subgenomic mRNA synthesis (Plant *et al.*, 2010; Sawicki & Sawicki, 1990; Zuniga *et al.*, n.d.), and this property of the RdRp is thought to contribute to the high recombination rate through template switching (Enjuanes *et al.*, 2006). Targeted recombination takes advantage of this natural event, by introducing *in vitro* transcribed RNA into infected cells by electroporation and recombinant virus is generated (Fischer *et al.*, 1997; de Haan *et al.*, 2002; Leparc-goffart *et al.*, 1998; Masters *et al.*, 1994). It is possible for there to be multiple template switching events, so the distance from the original template switch site is important to consider when using this method. The first targeted recombination system was developed for mouse hepatitis virus (MHV) and used a temperature sensitive trait to select and screen for template switching between the original temperature sensitive virus containing a mutation in the nucleocapsid gene and the new recombinant virus that had lost the temperature sensitive phenotype due to recombination (Koetzner *et al.*, 1992). Later experiments optimized the targeted recombination method by substituting the coding sequence for the ectodomain of the spike protein of MHV-A59 with the corresponding sequences encoding the ectodomain of Feline Infectious Peritonitis virus in the donor RNA (Kuo *et al.*, 2000). This allowed recombination events to be selected based on the host range of the spike protein: mouse or feline, and selected for template switching events that

were 5’ to the S gene rather than recombination events that were 5’ to the temperature sensitive mutation in N. The host range selection was much more stringent: recombinant MHV that expressed the FIPV spike would only grow on feline cells, the non-recombinant MHV would not. The resulting recombinant felinized virus expressing FIPV spike was then used as an acceptor using transcripts of donor RNAs containing the original MHV spike and any additional mutations engineered into the S gene or sequences 3’ of the S gene. Viruses that underwent template switching to the donor RNA would now express the MHV spike and can be selected by their ability grow on mouse cells.

Complete reverse genetic systems

In order to introduce mutants into genes 5’ to the S gene complete reverse genetic systems were developed. Three different approaches have been taken to develop complete reverse genetic systems for coronaviruses: a systematic in vitro assembly of multiple cDNAs (most commonly 7) carried in separate plasmids (Scobey *et al.*, 2013; Yount *et al.*, 2000, 2002, 2003), an infectious cDNA clone that houses the genome in a bacterial artificial chromosome (BAC) (Almazán *et al.*, 2006; Pfefferle *et al.*, 2009), and a recombinant vaccinia virus vector (Casais *et al.*, 2001; Tekes *et al.*, 2008; Thiel *et al.*, 2001). In the BAC the viral genome is housed as a single piece and so unique restriction sites may need to be introduced into the genome in order to facilitate assembly of the clone as well as to facilitate later manipulations of the genome (Almazán *et al.*, 2006; Pfefferle *et al.*, 2009). BACs can be stably maintained for over 200 passages (Almazán *et al.*, 2006). Vaccinia vectors are known for their stability and can house the entire coronavirus genome which can be manipulated by well established systems employing homologous recombination in vaccinia virus (Casais *et al.*, 2001; Lai *et al.*, 1991; Thiel *et al.*, 2001; Vennema *et al.*, 1990). One advantage of these systems is a consistently

higher amount of whole genomic cDNA that can be prepared for *in vitro* transcription since there is no stepwise ligation of cDNA fragments, and loss during this process, to generate the genomic cDNA. The BAC system also can be designed with a CMV promoter and can be transfected into cells to generate recombinant virus without *in vitro* transcription.

The in vitro cDNA ligation approach (Scobey *et al.*, 2013; Youn *et al.*, 2005; Yount *et al.*, 2000, 2002; Weiss lab personal comminuation) comprised of 6 or 7 plasmids that each contain a cDNA fragment corresponding to a portion of the genome (Youn *et al.*, 2005; Yount *et al.*, 2000, 2002, 2003). The plasmids that contain the genomic fragment are digested with type IIS restriction enzymes that have been engineered to flank the genomic cDNA insert. Enzyme digestion can then liberate the cDNA genome fragment without altering the viral genome sequence. These cDNA fragments are ligated together and *in vitro* transcribed to form a viral genome RNA that can now be transfected into cells with the N gene (either independently expressed or as transcribed RNA) and a recombinant virus can be generated. This system requires more *in vitro* manipulation to generate a full length cDNA that can be used for transcription. However, the maintenance of the genome in multiple fragments facilitates the manipulation of the genome.

Betacoronaviruses as Models

By comparing the members of the betacoronavirus group we can identify shared mechanisms of lung injury that occur during betacoronavirus infection. Virus-unique contributions and mechanisms of pathogenesis, such as the contribution of the interaction of the spike protein with its cognate receptor to disease, can also be identified and studied. Both SARS-CoV and MHV are members of the betacoronavirus genus. However, the specific organ tropism of infection of many MHV strains makes them unsuitable as a model for SARS-CoV

infection. The most widely-studied strains, MHV-JHM and MHV-A59, primarily infect the brain (MHV-JHM and MHV-A59) or liver (MHV-A59) (Weiss & Leibowitz, 2007). The brain is considered an immune-privileged site, thus cytokine/chemokine signaling and the cellular response will not be the same as in a less privileged organ, like the lung. However MHV-1 is pneumotropic (Leibowitz *et al.*, 2010) and MHV-1 infected mice can serve as a mouse model for severe respiratory coronavirus infections (see below).

Other betacoronaviruses have been used to dissect the function of SARS-CoV genes *in vitro* and *in vivo* both by the study of homologous genes and by placing SARS-CoV proteins into an MHV virus that does not express a homologue to the SARS-CoV gene (Hussain *et al.*, 2008; Kuri *et al.*, 2011; Pewe *et al.*, 2005; Tangudu *et al.*, 2007). One example is the study of nsp3, which contains multiple functional domains, one of which is called the X domain (Kuri *et al.*, 2011). The X domain is a functional monophosphatase, called ADP-ribose-1”-pase (ADRP). ADRP are important and ubiquitous cellular processing enzyme involved in the tRNA splicing pathway, catalyzing the conversion of ADP-ribose-1 monophosphate to ADP-ribose and are conserved in coronaviruses and in members of the “alphavirus-like supergroup” of phylogenetically related positive-strand RNA viruses that includes viruses of medical importance, such as rubella virus and hepatitis E virus (Eriksson *et al.*, 2008). The enzymatic activity of the X domain is nonessential in HCoV- 229E for replication in cell culture (Kuri *et al.*, 2011), but the ADRP activity has been shown to be important for the development of liver disease during MHV-A59 infection (Eriksson *et al.*, 2008). Another protein conserved amongst lineage one betacoronaviruses, but not SARS-CoV, is the ns2 protein. MHV-A59 ns2 is a cyclic phosphodiesterase, similar to those functioning in tRNA metabolism, but its physiologic role is the hydrolyis of 2-5oligo(A), thus functioning to block the induction of RNaseL during MHV-

A59 infection (Roth-Cross *et al.*, 2009). Ns2 was not essential for infection of continuous cell lines (Roth-Cross *et al.*, 2007), was critical for efficient MHV replication in the liver and the development of hepatitis, but it does not play a significant role in the infection of the brain or the development of CNS disease (Roth-Cross *et al.*, 2009; Zhao *et al.*, 2011). Ns2 greatly enhanced MHV replication in bone marrow derived macrophages (Zhao *et al.*, 2012) suggesting that it plays a similar role in Kupffer cells in the liver, Thus it is possible that ns2, which is present in other MHV strains, is important to the ability of the virus to replicate in specific tissues. In another study the SARS-CoV ORF6 protein was placed into a MHV-JHM variant and it was discovered that ORF6 had a role in replication and pathogenesis that was previously unable to be identified in SARS-CoV (Hussain *et al.*, 2008; Pewe *et al.*, 2005; Tangudu *et al.*, 2007). However, the MHV-JHM strain does not produce pulmonary disease, but rather has the CNS as the primary target of infection. Although these studies were helpful in understanding the role of SARS-CoV ORF6, the role of ORF6 in the lung could not be assessed in the context of a neurotropic virus. When comparing the individual contribution of viral genes to pathogenesis it can become difficult to ascertain the role of individual genes. While SARS-CoV nsp1 has been shown to play a role in cytokine dysregulation (Law *et al.*, 2007), it is important to note that the nsp1 of SARS-CoV is different, by sequence, and is shorter than the MHV nsp1. It is possible that the differences in size are in nonfunctional regions or that the differences are purely host-related. However, it is also possible that these sequence differences reflect important functional differences regarding the role of nsp1 in pathogenesis.

SARS-COV MODELS OF DISEASE

Recently a comparison of transcriptional profiles in human systemic inflammatory diseases and the corresponding mouse models reported that transcriptional responses in murine

models were a poor mimic of the responses in human disease (Seok *et al.*, 2013). This comparison was motivated by the poor success rate of drug trials moving from mouse to human. Responses were similar between humans and mice at 6-12 hours. However, the overall recovery time for genes to return to base line was drastically different in humans and mice. Relevant to models of SARS, different mouse models of acute respiratory disease (ARD) had transcriptional profiles which had R^2 correlations between 0 and 0.8, with 47-61% of the genes shifting in the same direction, approximating that of random occurrence. Despite all the potential causes for inconsistency in human responses (ie. age, different treatments, diseases /trauma severity) the transcriptional profiles of human cases of ARD were highly consistent, with R^2 values of .55, with 84% of the genes changing in the same direction. In the following sections we will examine the validity of the animal model's response to SARS-CoV infection.

Animal Models of SARS-CoV

For some zoonotic diseases the natural host is unknown because these animals show no signs or symptoms of illness, while in others disease in the natural host is mild and transient (Wood *et al.*, 2012). In the case of SARS-CoV the natural animal reservoirs show limited disease (bats and civet cats), whereas the human infection is more severe. To date mice (Coleman *et al.*, 2014), hamsters (de Wit *et al.*, 2013a) and ferrets (Raj *et al.*, 2014) have been shown to not support replication of MERS-CoV, with the exception of mice transduced with a recombinant adenovirus driving the expression of the MERS-CoV receptor (Zhao *et al.*, 2014) .

The ability of the animal model to actually mimic the disease in humans is required, but one must also consider the cost of experimentation and the ease of working with the animals. Different species of animals have differing responses to coronavirus infection, and so the models must be evaluated in terms of fitness compared to human SARS-CoV infection and disease

(Table 1, a more complete review of pathology can be found in (van den Brand *et al.*, 2014)). In this section we will review the models that have been used in studying SARS-CoV disease (Table 2).

Non-transgenic Models

Mice are capable of being infected by human SARS-CoV (Chen *et al.*, 2010). Virus replicates in lungs and nasal turbinates of 4-6 week old BALB/c mice and is cleared by 7 days post infection. However, these mice do not develop significant pulmonary lesions when challenged with a human SARS-CoV isolate, limiting their usefulness (Subbarao *et al.*, 2004). Aged BALB/c mice infected with SARS-CoV show evidence of alveolar damage and interstitial pneumonitis similar to human cases (Roberts *et al.*, 2005a). Recently, a novel non-transgenic approach to creating a mouse model for MERS-CoV utilized transduction of BALB/c mice with adenoviral vectors expressing the human host-cell receptor for MERS-CoV, dipeptidyl peptidase 4 (Zhao *et al.*, 2014). Infection with MERS-CoV was not fatal, but did produce a perivascular and peribronchial lymphoid infiltration, progression to an interstitial pneumonia, and viral clearance occurring 6-8 days post infection.

Transgenic Animals

Use of transgenic mice in studying coronaviruses is twofold: elimination of the need for host adapted viruses and abrogating elements of the host immune response to study changes in the pathology induced by infection and the role of these elements in pathogenesis. Two labs generated transgenic mice that express the human ACE2 receptor so that SARS-CoV could be studied without the requirement of adaptation to a murine host. McCray et al generated a transgenic C57Bl/6 mouse that expresses the human ACE2 receptor (hACE2) under the control of the human cytokeratin 18 promoter which confers transgene expression in airway epithelial

cells (but not in alveolar epithelia), as well as in epithelia of other internal organs (McCray *et al.*, 2007). The transgenic mice expressed similar levels of mouse ACE2 as the non-transgenic counterparts in the lung, but in addition hACE2 was expressed in multiple organs where the mouse ACE2 receptor is not normally found (colon, liver, and kidney). Additionally, the expression of hACE2 in tissues that normally express ACE2 increased the total ACE2 content of those tissues, notably in the brain. Expression of hACE2 did not guarantee SARS-CoV infection of an organ as virus was not detected in the liver, kidney, or ileum at either 2 or 4 days post infection. Mice suffered a lethal disease, with 100% mortality by day 7 in both strains when infected with $2.3x10^4$ PFU. Nontransgenic and K18-hACE2 mice showed evidence of perivascular and peribronchiolar inflammation. There were more widespread inflammatory cell infiltrates, increased inflammatory cell margination, more epithelial cell sloughing, more signs of lung injury, and extensive viral replication in the brain with viral antigen present in neurons throughout the cerebrum, thalamus, and brainstem, with relative sparing of the olfactory bulb and cerebellum in K18-hACE2 mice. Tseng et al (Tseng *et al.*, 2007) generated two lines of transgenic mice, AC70 and AC63, which both expressed hACE2 ubiquitously, but AC70 expressed hACE2 at a higher level. AC70 mice developed clinical illness regardless of the route of inoculation (intranasal or intraperitoneal) and died uniformly within 8 days if infection; whereas AC63 mice developed clinical symptoms but eventually recovered from the infection. Mice also had extensive infection of the CNS during infection. However, not all hACE2 expressing cells in the CNS were susceptible to SARS-CoV infection; SARS-CoV antigen was not detected in endothelial cells of the brain despite their abundant expression of ACE2. While both models may seem extreme in the over-expression of hACE2 throughout the mouse it is important to remember that SARS-CoV has been found in multiple organ sites in human

patients, and that multiorgan involvement is associated with fatal cases of SARS-CoV infection (Farcas *et al.*, 2005; Gu *et al.*, 2005). Transgenic ACE2 mice develop a lethal disease when infected with wild type SARS-CoV, However the development of severe encephalitis, which is not a feature of SARS in humans, likely limits their usefulness to studies of antiviral agents and vaccines on SARS-CoV infection.

Knock-out mice have been used in evaluating the roles of the interferon in controlling coronavirus infection (Frieman & Baric, 2008; Raaben *et al.*, 2009a; See & Wark, 2008; Whitman *et al.*, 2009). SARS-CoV infection of IFNAR$^{-/-}$ mice, lacking the IFN receptor, have demonstrated that IFN signaling is important for control of virus replication and dissemination as well as protection of pulmonary disease (Raaben *et al.*, 2009a, b). Mice were still able to upregulate IFN regulated genes, though to a lesser extent, and so demonstrate that there are secondary mechanisms by which the cell can signal genes that are predominantly regulated by IFN, though mechanisms were not discussed. Mice that have the ACE2 receptor knocked out have confirmed that ACE2 is important in the infection of SARS-CoV, as animals not expressing ACE2 had a 105 fold lower titer in the lungs than wild type animals (Imai *et al.*, 2010). STAT1-/- mice are resistant to antiviral effects of IFN and have more severe pulmonary disease and increased viral load in the lungs (Hogan *et al.*, 2004) with systemic spread of virus to the liver and spleen.

Rodent Adapted Viruses

To generate a disease with a pathogenesis that is similar to SARS-CoV infection of humans SARS-CoV has been serially passaged and adapted to mice or rats (Day *et al.*, 2009; Nagata *et al.*, 2010). Host-adapted viruses are useful in dissecting host-function specific genes. Multiple passages in animals select for mutations that allow the virus to thrive in a specific

environment (Li, 2008; Zhang *et al.*, 2006). Adapted viruses are sequenced and then compared with the parental genome to find mutations that occurred and to attempt to correlate them to the adaptation. Because of adaptation mutations the virus may not utilize the same set of pathogenic mechanisms as the parent virus does in humans. These viruses are also useful in conjunction with transgenic animals. SARS-CoV has been adapted to mice and rats and the adapted viruses can mimic a SARS-CoV like disease (Day *et al.*, 2009; Nagata *et al.*, 2007, 2008; Pfefferle *et al.*, 2009; Roberts *et al.*, 2007).

A mouse-adapted SARS-CoV that produced disease and mortality in young BALB/c mice was first developed in 2007 (Roberts *et al.*, 2007). SARS-CoV Urbani was passaged 15 times through BALB/c mice to generate a virus designated MA15. Subsequently a second mouse adapted strain of SARS-CoV that could be used as a lethal model for SARS-CoV infection in BALB/c mice was developed (Day *et al.*, 2009). Strain V2163 was adapted to mice from SARS-Urbani after 25 serial passages. This strain caused severe illness in 5-6 week old mice. A comparison of MA15 and V2163 found that V2163 had a lower LD_{50} and produced higher virus titers in the lungs of infected animals. MA15 was found to cause more weight loss and had a later mean date of death in older animals. Both strains contained a conserved mutation in the spike protein (Y436H), and both contained non-identical mutations in the membrane proteins, in nsp9, and in nsp13. Both strains elicit expression of IL-12, IL-6, MIP-1α, MCP-1, and RANTES. MA15 and V2163 stimulate low levels of IFN-γ, whereas IFN-γ is not induced in mice infected with SARS-CoV Urbani. V2163 stimulates significantly more IL-6 and MCP-1 than MA15, and conversely MA15 stimulates significantly more MIP-1α and RANTES than V2163. These data are consistent with the idea that IL-6 and MCP-1 can be correlated with clinical outcome.

316 Later studies used MA15 to study protective T-cell responses (Zhao & Perlman, 2010;
317 Zhao *et al.*, 2009). One study found that elimination of alveolar macrophages protected mice
318 challenged with an otherwise lethal dose of MA15, but only in older mice, as depletion of
319 alveolar macrophages in young mice had no effects on disease (Zhao *et al.*, 2009). Mice that
320 were depleted showed an earlier and more robust virus-specific T-cell response, however it is
321 possible that the use of clodronate to deplete the alveolar macrophages has an effect on T-cell
322 responses independent of SARS-CoV infection, as animals that were treated with clodronate
323 show higher pro-inflammatory cytokines pre-infection. Weight loss was similar in infected and
324 uninfected treated mice by day 2 post infection, but it is possible that the priming response may
325 be affecting overall mortality. Further studies with MA15 infected mice found that SARS-CoV
326 specific CD8 T cells were more protective than SARS-CoV specific CD4 T cells purified from
327 lethally infected mice, and that protection is dose dependent in animals in which activated CD4
328 and CD8 T cells were transferred individually or together (Zhao & Perlman, 2010). Both
329 enhance survival in BALB/c mice that are lethally challenged with MA15. Immunizations with
330 dendritic cells coated with a specific spike peptide were almost 100% protective in BALB/c by
331 inducing a specific T cell response in the lung and spleen.
332 A third strain of mouse adapted SARS-CoV, F-musX, was developed from the SARS-
333 CoV Frankfurt strain (Nagata *et al.*, 2008). Clinical disease was observed only in aged animals
334 at day 2 post infection, with a mortality rate of 30-50%. Lungs from aged mice had significantly
335 higher IL-4 and lower IL-10 and IL-13 levels before infection than young mice, whereas lungs
336 from young mice contained not only proinflammatory cytokines but also IL-2, interferon-γ, IL-
337 10, and IL-13.

The major drawback to the use of the MA15 or other mouse adapted SARS-CoV is the requirement of older mice for the development of lethal disease. Aged animals are more difficult to acquire in large numbers and they are more expensive than younger mice.

Rats have been used in ARDS and ACE2 studies, and seem a viable option for an animal model of SARS-CoV infection and disease (Burrell *et al.*, 2004; Chen *et al.*, 2003; Dı *et al.*, 2006). A rat adapted SARS-CoV was developed by serially passaging the SARS-CoV Frankfurt 1 strain, a mixture of the original virus without an ORF7a deletion and a variant virus that did have the ORF7a deletion, ten times through young F334 rats (Nagata *et al.*, 2007). Adult rats (7 to 8 month old males) had more severe acute lung injury with higher level of cytokines expressed than young (4 week old females) rats. Young rats had limited clinical symptoms and lesions were limited to the bronchi, bronchioles, and the alveoli with only mild edema around the blood vessels. Adult rats became lethargic, had ruffled fur, and abdominal breathing. There was no mortality in either young or old animals.

One limitation of the rat model is the lack of mortality. The disease appears to resolve, though researchers do not state when clinical symptoms stop, and virus is still present in the lungs of young and old rats on day 21 (end of study) despite the presence of neutralizing antibodies. This study also does not report if the adapted rat virus contains the ORF7a deletion as a majority or minority of the virus population or address what mutations, other than the spike Y442S mutation, were required to adapt the Frankfurt1 strain to rats.

Golden Syrian hamsters

Syrian hamsters have also been proposed as a model for SARS-CoV infection (Roberts *et al.*, 2005b). Syrian hamsters, 5 week old females, support efficient viral replication that continues to 5 days post infection. The disease resolved in 14 days with no mortality reported.

In hamsters low titers of virus were present in the liver and spleen at days 2 and 3 post infection, but not thereafter. The animals developed a robust protective neutralizing antibody response by day 7, one that the researchers report was more robust than the antibody response in mice.

Other studies used the golden Syrian hamster model to evaluate monoclonal antibody therapy (Roberts *et al.*, 2006) and the immunogenicity of a live attenuated SARS-CoV vaccine (Lamirande *et al.*, 2008). When treated with monoclonal antibodies after infection 5 week old female hamsters showed a reduced viral burden (Roberts *et al.*, 2006). Hamsters also showed reduced lung pathology by virtue of decreased interstitial pneumonitis and decrease lung consolidation by day 7 post infection. Neither response was dose dependent, and 4 mg/kg of antibody was insufficient to protect from infection because not all hamsters had measurable levels of circulating antibodies in the serum. The study evaluating the use of a live attenuated vaccine used 7 week old male hamsters vaccinated with a wildtype recombinant SARS-CoV Urbani strain or a recombinant SARS-CoV lacking the E gene (Lamirande *et al.*, 2008). After 4 weeks the hamsters were challenged with either SARS-CoV Urbani or a recombinant SARS-CoV with the spike protein of the GD03 strain of SARS-CoV. All vaccinated hamsters had no detectable virus in the nasal turbinates by day 5 post infection or the lungs at any time post infection.

While these studies are promising, the use of the Golden Syrian hamster has been limited. These animals do not suffer any type of obvious clinical disease and they completely resolve their lung lesions (Roberts *et al.*, 2005b). To date there is no evaluation of SARS-CoV infection of aged hamsters, so it is possible that, like some mouse strains, pulmonary disease could develop in older animals. There is an immunosuppressed Golden Syrian model in which cyclophosphamide treatment leads to significant weight loss, expanded tissue tropism of SARS-

CoV, and increased pathology in lung, heart, kidney, and nasal turbinates (Schaecher *et al.*, 2008). This model is useful because the hamsters have a longer duration of illness, mortality being at 20-35 days post infection, depending on cyclophosphamide treatment, and have a slower progression of disease. However, cyclophosphamide causes lymphopenia, suppresses B-cell activity and activation, and suppresses regulatory T-cell function limiting the model to the study of viral replication and pathogenesis in the host and cannot be used to evaluate the effectiveness of vaccination or antiviral treatment in SARS-CoV infection.

Medium-sized mammals

Other mammals that can be infected with SARS-CoV include civets, ferrets, and domestic cats (van den Brand *et al.*, 2008; Martina *et al.*, 2003; Nagata *et al.*, 2010). Outbred animals are less expensive and easier to handle than primates. Cats or ferrets are able to transmit virus to uninfected animals that are housed with them (van den Brand *et al.*, 2008; Martina *et al.*, 2003) making them useful for epidemiological and transmission studies. Cats do not show any lethargy or difficulty breathing, but do show multifocal pulmonary consolidation in the lungs. Cats also develop histological lesions in Peyer's patches (van den Brand *et al.*, 2008). Although SARS-CoV replicates in the human GI track, intestinal lesions were rare in SARS patients. Ferrets become lethargic from day 2 post-infection and develop multifocal pulmonary consolidation in the lungs but fail to develop lethal disease (Chu *et al.*, 2010). The ferret model has only studied animals in a single age range and, to date, there have been no published reports of an aged ferret model. Civet cats, the intermediary host when SARS-CoV moved from bats, are capable of being infected with SARS-CoV isolates recovered from humans and civets (Lau *et al.*, 2010; Li, 2008; Nagata *et al.*, 2010; Tu *et al.*, 2004; Wu *et al.*, 2005). They become lethargic, develop fever, leucopenia and an interstitial pneumonitis (Wu *et al.*, 2005). Civet

cats recover and are afebrile by 13 days post infection. The interstitial pneumonitis was less severe than that observed in human cases of SARS, with lesions similar to those seen infected macaques. The pulmonary lesions resolved after day 35.

Primate models

While primates are more closely related to humans than other animals, they are still unique in their responses to infection. Primates are also very expensive to purchase and to house. There is a demarcation between Old World Primates (ie macaques) and New World Primates (ie marmosets) and their responses to disease. Old and New World primates are susceptible to infection by SARS-CoV (Greenough *et al.*, 2005; Smits *et al.*, 2010). However, neither primate group are susceptible to a lethal SARS-CoV disease (Nagata *et al.*, 2010).

Marmosets (*Callithrix jacchus*) infected with SARS-CoV developed clinical disease with diarrhea on day 2 and dyspnea and fever beginning at 4 days after infection (61). Pathologically the disease was characterized by multifocal mononuclear cell interstitial pneumonitis without diffuse alveolar damage (the hallmark of human infection with SARS-CoV) and severe hepatic and gastrointestinal inflammation (Greenough *et al.*, 2005). Marmosets can be used to recapitualte lethal disease when infected with MERS-Co (Falzarano *et al.*, 2014).

Macaque models have yielded mixed results in the study of SARS-CoV infection. One study reports the effects of SARS-CoV infection in rhesus and cynomolgus macaques had a limited disease where symptoms presented 2 or 3 days post infection and quickly resolved (McAuliffea *et al.*, 2004; Rowe *et al.*, 2004). Both rhesus and cynomolgus macaques had a limited disease where symptoms presented 2 or 3 days post infection and quickly resolved. No animals demonstrated signs of respiratory distress, body temperatures remained normal during the study, blood chemistries and hemotologic parameters were largely unchanged. A second

study with cynomolgus macaques demonstrated that infection with SARS-CoV did not produce severe illness, but an illness similar to the milder SARS-CoV infections seen in younger children (Lawler *et al.*, 2006). Infection of aged cynomolgus macaques did produce a disease that was similar to the severe SARS-CoV illness seen in elderly patients (Smits *et al.*, 2010). Innate immune responses in aged macques in response to SARS-CoV infection differed from the innate responses of young animals (Smits *et al.*, 2010). There were only 14 genes differentially regulated, of 518 examined, between the two age groups. In aged macaques there was a more robust induction of NF-κB regulated genes such as IL-6 than in young animals. STAT1 was differentially expressed between the two age groups, with up-regulation in older animals whereas it was not observed in younger animals. Another study used cynomolgus macaques to evaluate pegylated interferon-α treatment of SARS-CoV infection (Haagmans *et al.*, 2004). Researchers do not state the age of animals used in the study, but report infection of type 1 pneumocytes by day 4 post infection, and extensive hyperplasia of type 2 pneumocytes by day 6. Animals pre-treated with pegylated interferon-α showed decreased viral titer in the lungs and the severity of diffuse alveolar damage was reduced by 80%. Animals treated with pegylated interferon-α after SARS-CoV infection also had reduced virus titers in the lungs. Rhesus macaques have been shown to have a mild to moderate disease when infected with MERS-CoV (Munster *et al.*, 2013; de Wit *et al.*, 2013b; Yao *et al.*, 2014). A significant limitation of the macque model is that lethal disease is only seen in older animals, and it is difficult and expensive to obtain an appropriate number of older animals for study.

MHV-1 Infected Mouse Model

In 2006 a study was published that examined that ability of multiple MHV strains to cause a SARS-CoV like disease in various inbred mouse strains after intranasal challenge (de

Albuquerque *et al.*, 2006). MHV-1 infection of 5-6 week old A/J mice induced a lethal pneumonitis that was similar to human SARS-CoV infection in terms of histopathologic changes and levels of type I interferon and cytokine responses. Mice develop disease, demonstrated by weight loss, by 2 days post infection and usually die by 7-10 days post infection. Disease is shorter in duration than human SARS, but it is lethal. The pathologic changes in MHV-1 infected A/J mice displayed multiple features observed in SARS-CoV infected patients including interstitial pulmonary infiltrates, hyaline membrane formation, multinucleated syncytia, congestion, hemorrhage in the lung, pulmonary edema and the presence of virus in the liver.

Khanolkar et al compared the T-cell CD4 and CD8 responses in C3H/HeJ mice susceptible to lethal infection with the responses in B6 mice that survive MHV-1 infection (Khanolkar *et al.*, 2009, 2010). Susceptible C3H/HeJ mice generated a stronger CD4 T-cell response that mapped primarily to epitopes contained in 2 regions in S protein, 2 regions in N protein, and 1 region in M protein. Resistant B6 mice had a stronger CD8 T-cell response that mapped mostly to S, with none of the CD4 or CD8 responses mapping to the N protein. CD8 T-cell response in B6 mice was ~11 fold greater than the response in C3H/H3J mice, but CD 4 response was ~4 fold higher in C3H/HeJ. MHV-1 infection induces a more robust and broader CD4 T-cell response in susceptible mice, whereas resistant mice mount a “broad and vigorous” CD8 T-cell response. Because B6 mice lack the I-E^b allele and are I-A^b restricted and are unable to bind certain peptide sequences. It is uncertain as to the role of this restriction in pathogenesis.

Similar to SARS-CoV infected patients there is a marked elevation of IL-6 and IP-10 during MHV-1 infection (Dufour *et al.*, 2002; Kebaabetswe *et al.*, 2013; Khanolkar *et al.*, 2009). It has been reported in MHV-1 susceptible mice that IFN-γ and TNF-α coproduction by CD8 T-cells is reduced in the lung compared to levels in B6 mice that do not develop lethal disease, but

not in the spleen or lymphoid tissues and that CD4 coproduction of IFN-γ and TNF-α is increased in all tissues compared to B6 resistant mice (Khanolkar *et al.*, 2010). C3H/HeJ mice also had a higher fraction of IFN-γ and IL-2 coproduction in spleen and draining lymph nodes, but not in the lung, whereas B6 resistant mice produced more IL-2 in the lung than in the spleen.

The MHV-1 model has several advantages as a model for studying the pathogenesis of coronavirus induced severe respiratory diseases. MHV-1 requires no BSL3 facilities, is a lower risk pathogen than SARS-CoV, it naturally infects the lungs of mice, and creates a lethal SARS-CoV like disease in a specific mouse strain (A/J) while still causing non-lethal lung disease in other strains. Because MHV-1 produces a non-lethal pulmonary infection in most strains, various mouse strains can be used to evaluate gain of function or effect of genes in mutated or recombinant MHV-1 viruses and to interrogate the role of specific host genes. However, the MHV-1 model also has admitted limitations. The absence of exact copies of SARS-CoV specific genes makes it difficult to evaluate those genes' role in pathogenesis. To date no complete reverse genetic system is available for MHV-1, however there is a targeted recombination system that could be used to introduce some of the specific SARS-CoV genes into MHV-1and study their effect on pathogenesis in this model (Leibowitz *et al.*, 2010). Another issue is the different receptors utilized by cell entry by the two viruses. SARS-CoV utilizes ACE2 and thus impacts a major signaling cascade that is not affected in the MHV-1 model.

CONCLUSIONS

Animal models will likely not be able to completely recapitulate disease and pathology that occurs during infection of humans with SARS-CoV. Models should be able to accurately represent what occurs in human and should be able to do so in a manner that is safe for researchers and that is not overly expensive. While primate models of disease are, generally,

considered to accurately mimic human disease they are expensive and difficult to handle. Smaller mammals are safer and less expensive to work with and house, but usually require host-adapted viruses to recapitulate human disease. These models still require BSL3 containment to work with them safely. Related coronaviruses that are non-infectious to humans that naturally infect a small mammal are ideal in terms of cost and safety. However, a recent publication has called into question the relevance of much of the mouse data regarding human inflammatory diseases (Seok *et al.*, 2013). Thus, differences between humans and mice can make understanding the pathogenesis of SARS-CoV difficult. However, we have demonstrated that the models of SARS-CoV do, in part, mimic the disease course that is seen in humans not only in terms of cytokine/chemokine response, but also in histology and cellular pathology.

ACKNOWLEGEMENTS

The authors gratefully acknowledge support from US National Institutes of Health Grant AI078148.

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894 **Table 1. List of Cytokines/Chemokines elicited during a SARS-CoV infection of humans, cells, and animals**

Cytokine/ Chemokine	Function[#]	Increase or Decrease			References
		Human	Cell line	Animal Model	
IFN-β	Antiviral properties	No change	No change	↑early	(Nagata *et al.*, 2010; Versteeg *et al.*, 2007)
TNF-α	mainly secreted by macrophages, involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation	↑/no change conflicting	↑	↑	(Rockx *et al.*, 2009; Zhang *et al.*, 2004)
TGF-β	Multifunctional protein that controls proliferation, differentiation and other functions in many cell types	↓↑ conflicting	↑	nf	(Zhang *et al.*, 2004; Zhao *et al.*, 2008)
IFN-γ	produced by lymphocytes, potent activator of macrophages	↑	↓	↓↑	(Day *et al.*, 2009; Huang *et al.*, 2005; de Lang *et al.*, 2007; Yoshikawa *et al.*, 2010)
IL-18/ IGIF	cytokine that augments natural killer cell activity in spleen cells, and stimulates interferon gamma production in T-helper type I cells	↑	↑	↓	(Clay *et al.*, 2014; Huang *et al.*, 2005)
IL-6	functions in inflammation and the maturation of B cells, primarily produced at sites of inflammation	↑end	↑	↑	(Rockx *et al.*, 2009; Smits *et al.*, 2010; Yoshikawa *et al.*, 2010; Zhang *et al.*, 2004)
IL-8	chemotactic factor that attracts neutrophils, basophils, and T-cells, but not monocytes; involved in neutrophil activation	↓ ↑progressive and end	↑	↑	(Rockx *et al.*, 2009; Smits *et al.*, 2010; Yoshikawa *et al.*, 2010; Zhang *et al.*, 2004)
STAT	signal transducer and transcription activator that mediates cellular responses to interferons, cytokines, and growth factors	↑activation ↓nuclear transport	↑activation	↑activation	(Smits *et al.*, 2010)
CCL-20	chemotactic factor that attracts lymphocytes and neutrophils, but not monocytes; involved in mucosal lymphoid tissues by attracting lymphocytes and dendritic cells towards epithelial cells.	↑early	↑early	nf	(Clay *et al.*, 2014; Yoshikawa *et al.*, 2010)
CXCL-10/ IP-10	stimulation of monocytes, natural killer and T-cell migration, and modulation of adhesion molecule expression	↑	↑	↑	(Glass *et al.*, 2004b; de Lang *et al.*, 2007; Rockx *et al.*, 2009; Yoshikawa *et al.*, 2010)

CCL-2/ MCP-1	chemotactic activity for monocytes and basophils but not for neutrophils or eosinophils. It has been implicated in the pathogenesis of diseases characterized by monocytic infiltrates.	↑	↑	↑	(Day *et al.*, 2009; Glass *et al.*, 2004b; Huang *et al.*, 2005; Rockx *et al.*, 2009; Yoshikawa *et al.*, 2010)
CCL-5/ RANTES	functions as a chemoattractant for blood monocytes, memory T helper cells and eosinophils; causes the release of histamine from basophils and activates eosinophils.	↑	↑	↑	(Day *et al.*, 2009; Glass *et al.*, 2004b; Law *et al.*, 2007)
CXCL9/ MIG	thought to be involved in T cell trafficking as a chemoattractant	↑	↑	nf	(Glass *et al.*, 2004b; Yoshikawa *et al.*, 2010)
CCL-3	involved in the recruitment and activation of polymorphonuclear leukocytes	↑	↑	↑	(Chen *et al.*, 2010; Clay *et al.*, 2014; Glass *et al.*, 2004a)
IL-10	produced primarily by monocytes and to a lesser extent by lymphocytes; down-regulates the expression of Th1cytokines, MHC class II Ags, and costimulatory molecules on macrophages; enhances B cell survival, proliferation, and antibody production.	↓infected ↑convalescent	nf	NC or ↓	(Day *et al.*, 2009; Huang *et al.*, 2005; Jones *et al.*, 2004; Li *et al.*, 2010; Nagata *et al.*, 2008; Yoshikawa *et al.*, 2009)
IL-12	Acts as a growth factor for activated T and NK cells, enhance the lytic activity of NK/lymphokine-activated Killer cells, and stimulate the production of IFN-gamma by resting PBMC	↓	nf	↑ ↓aged	(Clay *et al.*, 2014; Day *et al.*, 2009)

[#] **information adapted from www.genecards.org**

NC- No Change reported

nf- data not found in literature at time of search

904 **Table 2. Comparison of animal model with available virus for study**

Model Animal	Virus	Advantages	Disadvantages
Inbred mouse strain	Mouse adapted SARS-CoV	Less host-related variability, inexpensive	Must use aged animals which are harder to acquire, required BL-3 containment
Inbred mouse strain	MHV-1	Inexpensive, SARS-CoV like pathology, no BL3 containment required	Different strains have different pathologies
Rat	Rat adapted SARS-CoV	Previous use in Acute Respiratory Distress Syndrome studies, infection produced similar lesions to SARS-CoV infected patients, inexpensive	Lack of mortality, require adult animals
Golden Syrian hamsters	SARS-CoV	Support viral replication, modest lung disease, virus present in other organs, inexpensive	Lack of mortality, no clinical disease, resolving lung pathology, requires immunosupression for disease model
Civet Cats	SARS-CoV	become lethargic, develop fever, leucopenia, and interstitial pneumonitis	Expensive to obtain and house
Ferrets	SARS-CoV	able to transmit virus by aerosol, animals become lethargic, lung lesions present	Expensive to purchase and house
Domestic Cats	SARS-CoV	able to transmit virus by aerosol, lung lesions present, lesions in Peyer's Patches	No lethargy or difficulty breathing, expensive to house
Marmosets	SARS-CoV	SARS-CoV like lung disease	Not Susceptible to lethal SARS-CoV disease, expensive to purchase and house
Macaques	SARS-CoV	Produce mild SARS-CoV infection illness in young (rhesus and cynomolgus, Conflicting data), aged animals produce severe SARS-CoV disease (cynomolgus)	Not Susceptible to lethal SARS-CoV disease, data is conflicting, expensive to purchase and house

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