Short Communication

The ADP-ribose-1″-monophosphatase domains of severe acute respiratory syndrome coronavirus and human coronavirus 229E mediate resistance to antiviral interferon responses

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Several plus-strand RNA viruses encode proteins containing macrodomains. These domains possess ADP-ribose-1″-phosphatase (ADRP) activity and/or bind poly(ADP-ribose), poly(A) or poly(G). The relevance of these activities in the viral life cycle has not yet been resolved. Here, we report that genetically engineered mutants of severe acute respiratory syndrome coronavirus (SARS-CoV) and human coronavirus 229E (HCoV-229E) expressing ADRP-deficient macrodomains displayed an increased sensitivity to the antiviral effect of alpha interferon compared with their wild-type counterparts. The data suggest that macrodomain-associated ADRP activities may have a role in viral escape from the innate immune responses of the host.

Coronaviruses are mainly associated with respiratory and enteric disease in livestock, companion animals and humans. The group includes severe acute respiratory syndrome coronavirus (SARS-CoV), which was responsible for a worldwide outbreak in 2003 (Peiris *et al.*, 2004), and several other established or recently identified human pathogens (Pyrc *et al.*, 2007). With the exception of SARS-CoV, human coronaviruses (HcoV), such as for example HCoV-229E, are only mildly pathogenic. The family *Coronaviridae* has been divided into three genera (Gorbalenya *et al.*, 2004), with the alpha genus including HCoV-229E, the beta genus containing SARS-CoV and the gamma genus representing avian coronaviruses (ICTV, 2009; Wevers & van der Hoek, 2009).

The large positive-stranded RNA genome of coronaviruses (27–32 kb) is polycistronic, with the two 5′-proximal ORFs (1a and 1b) forming a huge 'replicase/transcriptase' gene. Translation of this gene results in the synthesis of two polyproteins, which are processed by viral proteases to generate 15 or 16 mature non-structural proteins (nsps). The nsps are driving genome replication and mRNA transcription (Gorbalenya *et al.*, 2006; Ziebuhr, 2008), but there is increasing evidence that some of them have additional functions (Bechill *et al.*, 2008; Devaraj *et al.*, 2007; Eriksson *et al.*, 2008; Narayanan *et al.*, 2008; Snijder *et al.*, 2003; Wathelet *et al.*, 2007; Züst *et al.*, 2007).

The largest nsp, nsp3, is a multi-functional protein that contains one or several macrodomains (Neuman *et al.*, 2008; Snijder *et al.*, 2003; Tan *et al.*, 2009; Ziebuhr *et al.*, 2001). Macrodomains have a globular structure and are related to the non-histone domain of human histone macroH2A (Till & Ladurner, 2009). They are present in eukaryotes, bacteria and members of the family *Coronaviridae* and several other positive-strand RNA viruses (Draker *et al.*, 2006; Gorbalenya *et al.*, 1991; Schütze *et al.*, 2006; Ziebuhr *et al.*, 2001). Several

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coronavirus macrodomains were shown to have ADP-ribose-1″-monophosphatase (ADRP) activities (Egloff et al., 2006; Putics et al., 2005, 2006; Saikatendu et al., 2005). Recent evidence suggests that some viral macrodomains also bind poly(ADP-ribose), poly(A) or poly(G) (Egloff et al., 2006; Neuvonen & Ahola, 2009; Piotrowski et al., 2009; Tan et al., 2009). Despite the conservation of macrodomains across the family Coronaviridae, an ADRP-deficient mutant of HCoV-229E, termed HCoV_1ab-N1305A, exhibited no serious growth defects in cell culture (Putics et al., 2005). Similarly, an ADRP-deficient mutant of the mouse hepatitis coronavirus (MHV) replicated normally in cell culture (Eriksson et al., 2008). However, in contrast to the wild-type (wt) virus, this MHV mutant did not induce liver pathology in the mouse. Most likely this difference in MHV pathogenesis was due to the inability of the ADRP mutant to trigger a pro-inflammatory cytokine response (Eriksson et al., 2008). In line with this, macrodomain mutants of Sindbis virus (SINV) replicate normally in BHK cells but are attenuated in neurons and have reduced virulence in mice (Park & Griffin, 2009).

To investigate the role of the ADRP macrodomain of SARS-CoV, we used a reverse genetics system for the isolate HKU-39849 (Zeng et al., 2003) (K. K. Eriksson and others, unpublished data). A recombinant vaccinia virus containing a full-length cDNA of the SARS-CoV genome was constructed (Fig. 1a). Genomic DNA isolated from this virus was used as a template for in vitro transcription of SARS-CoV genomic RNA using previously described protocols (Thiel et al., 2001). Furthermore, we generated an ADRP mutant, SARS-CoV_1ab-N1040A, in which the pp1a/pp1ab Asn-1040 codon was replaced with an Ala codon (Fig. 1b). Asn-1040 is part of the SARS-CoV ADRP active site and corresponds to Asn-1305 in HCoV-229E pp1a/pp1ab (Putics et al., 2005) and Asn-1348 in MHV (Eriksson et al., 2008). This substitution was previously shown to abolish ADRP activity in vitro (Egloff et al., 2006). To rescue recombinant wt and mutant SARS-CoV, respectively, BHK-SARS-CoV N cells were electroporated with in vitro transcribed virus RNA and subsequently mixed with Vero E6 cells in a 1:1 ratio. At 72 h post-transfection, supernatants were harvested and used to grow virus stocks. Genotypes were confirmed by sequencing RT-PCR products of the ADRP domain (data not shown). In infected Vero E6 cells, the recombinant wt SARS-CoV (strain HKU-39849) exhibited a clear-plaque phenotype that was similar to the Frankfurt-1 isolate used previously in our lab (Fig. 1c, left panel and data not shown). By contrast, the ADRP mutant SARS-CoV_1ab-N1040A formed turbid plaques (Fig. 1c, right panel). Moreover, a time-course analysis revealed that the N1040A mutant grows slower than wt virus, but reaches similar end titres (Fig. 1d).

The data show that ADRP activity is not essential for SARS-CoV replication in cell culture. We went on to address the question of whether the enzyme may have a role in modulating host responses. Antiviral cytokines such as type I interferons (IFN-α/β) and chemokines are key determinants of viral pathogenesis (Hosking & Lane, 2010; Randall & Goodbourn, 2008; Weber & Haller, 2007). SARS-CoV is known to efficiently suppress IFN and cytokine synthesis, thus promoting rapid spread of the virus in the host organism (reviewed by Thiel & Weber, 2008). To investigate potential effects of ADRP activity on cellular cytokine profiles during virus infection, 293lp cells were infected for 16 h and expression of cellular cytokines and IFN-stimulated genes (ISGs) was determined by real-time RT-PCR analysis as described previously (Overby et al., 2010). Fig. 2(a) shows that the recombinant SARS-CoV (isolate HKU-39849) did not significantly induce IFN-β expression, which is in agreement with our previous data obtained for the strain Frankfurt-1 (Spiegel et al., 2005). The SARS-CoV_1ab-N1040A mutant slightly upregulated IFN-β gene expression. However, the IFN-dependent genes ISG56 and OAS1 were not influenced by any of the two recombinant SARS-CoV strains, confirming our previous observations that 10-fold induction of IFN-β is below the threshold for IFN secretion (Kuri et al., 2009; Overby et al., 2010). Similar to IFN-β, the mRNAs for the chemokines IP-10 and RANTES are induced 10- to 30-fold by the N1040A mutant, but not by the wt strain. However, compared to Rift Valley fever virus mutant Clone 13 (Billecocq et al., 2004), which we used as a positive control, the N1040A mutant of SARS-CoV proved to be a weak cytokine inducer (Fig. 2b). Whereas SARS-CoV_1ab-N1040A upregulated transcripts for IFN-β, IP-10 and RANTES by one order of magnitude, Clone 13 infection stimulated expression of these cytokines by four to five orders of magnitude, suggesting that ADRP activity has a minor role in modulating cytokine induction by SARS-CoV.

We also assessed the sensitivity of the ADRP-deficient mutant to the antiviral action of IFNs. To this end, parallel wells of Vero E6 cells were treated with increasing amounts of IFN-α, infected 16 h later with an m.o.i. of 0.001, and virus titres in cell culture supernatants were determined after another 72 h of incubation. Fig. 2(c) (left panel) shows that, in the presence of 100 U IFN-α ml^{-1}, SARS-CoV titres were reduced by one order of magnitude while virus reproduction was strongly suppressed in the presence of 1000 U IFN-α ml^{-1} (four orders of magnitude). Strikingly, SARS-CoV_1ab-N1040A was much more strongerly affected by IFN-α. Application of as little as 10 U ml^{-1} reduced titres by one order of magnitude, and 100 U ml^{-1} was enough to completely abolish the production of infectious virus. A similar, but less pronounced effect was observed for IFN-γ (Fig. 2d). Here, wt SARS-CoV was almost insensitive to even 1000 U IFN-γ ml^{-1}, whereas the N1040A mutant was reduced by more than two orders of magnitude in the presence of 100 U ml^{-1}, and reduced by five orders of magnitude if 1000 U ml^{-1} was used. The data indicate that the macrodomain-associated ADRP activity contributes to the resistance of SARS-CoV to the antiviral action of IFNs.
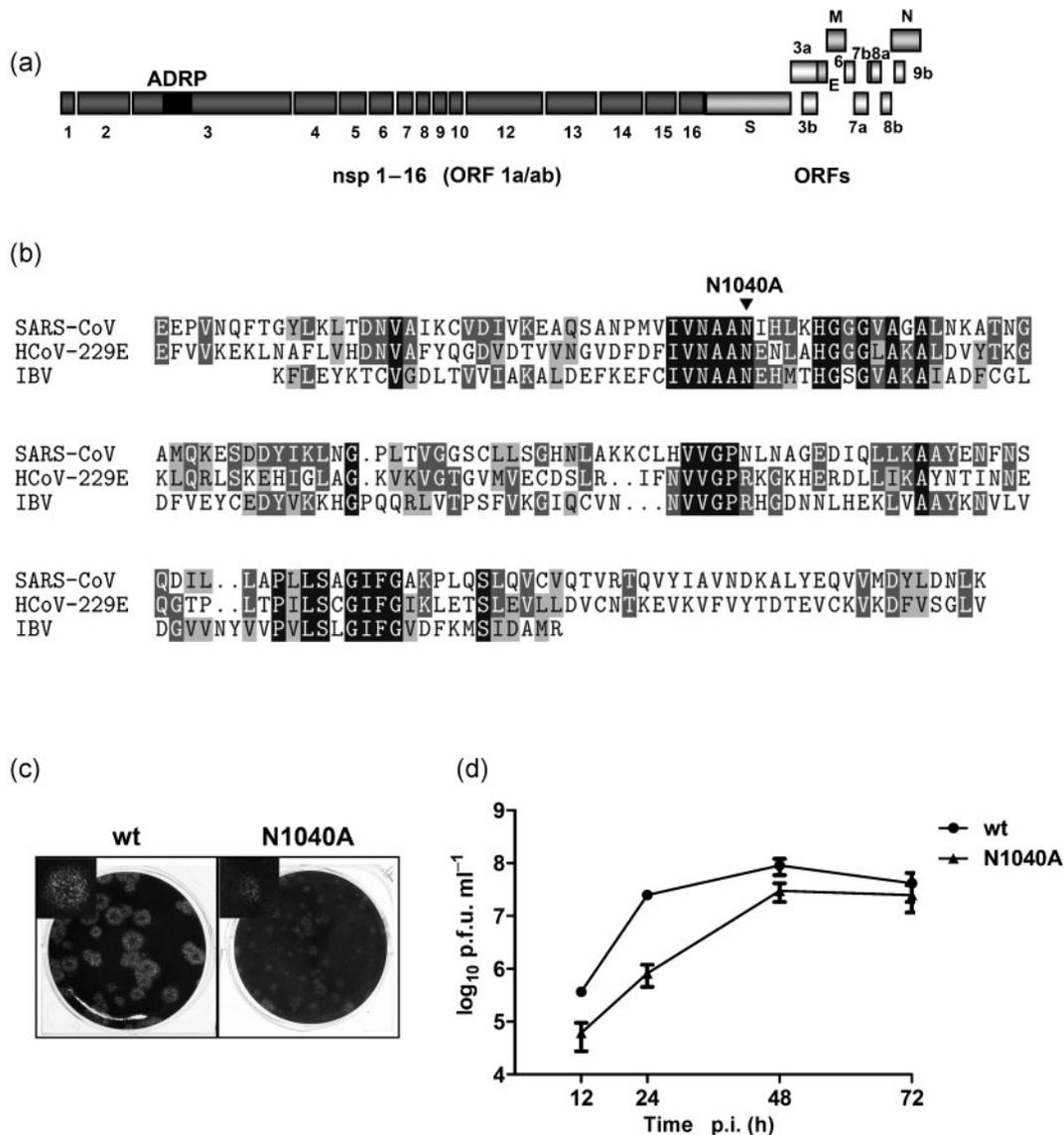
Fig. 1. Recombinant wt and ADRP-inactivated SARS-CoV strain HKU-39849. (a) Schematic representation of the proteins produced from the various ORFs present in the SARS-CoV genome. Nsp1−16 (shown in grey) are encoded by ORF 1a and 1b. The ADRP domain located in nsp3 is indicated in black. (b) Alignment of the macrodomain sequences of SARS-CoV (beta genus), HCoV-229E (alpha genus) and infectious bronchitis virus Beaudette (gamma genus). CLUSTAL W (Higgins *et al.*, 1992; Thompson *et al.*, 1994) was used for sequence alignment, and TeXshade (Beitz, 2000) for visualization. Sequences are derived from GenBank entries AY291315 (SARS-CoV), AF304460 (HCoV-229E) and NC_001451 (IBV). (c) Plaque phenotype of recombinant SARS-CoV (strain HKU-39849) and SARS-CoV_1ab-N1040A growing on Vero E6 cells. (d) Time-course analysis. Vero E6 cells were infected with an m.o.i. of 0.001 and virus titres in the supernatants were determined by plaque assay at specific time points (post-infection, p.i.). Mean values and SD from three independent experiments are shown. For these and for all subsequent experiments, RT-PCR-confirmed SARS-CoV ADRP mutants were used, which were derived from the first passage after rescue. Later passages were omitted since they contained some clear-plaque clones, indicating emergence of revertants.

We wondered whether the ADRP-deficient mutant of HCoV-229E may have a similar phenotype. To measure viral cytokine induction, we infected MRC-5 cells with wt HCoV-229E and the HCoV_1ab-N1305A mutant for 14 h, and performed real-time RT-PCR to measure innate immunity-related transcripts as described above. However, neither virus upregulated mRNAs for IFN-β, ISG56, OAS1, IP-10 or RANTES (Fig. 3a). This indicates that the ADRP of HCoV-229E has no role in modulating cellular cytokine profiles and, at the same time, identifies HCoV-229E as a poor inducer of antiviral IFNs and chemokines. However, some caution in interpreting these data is suggested since the
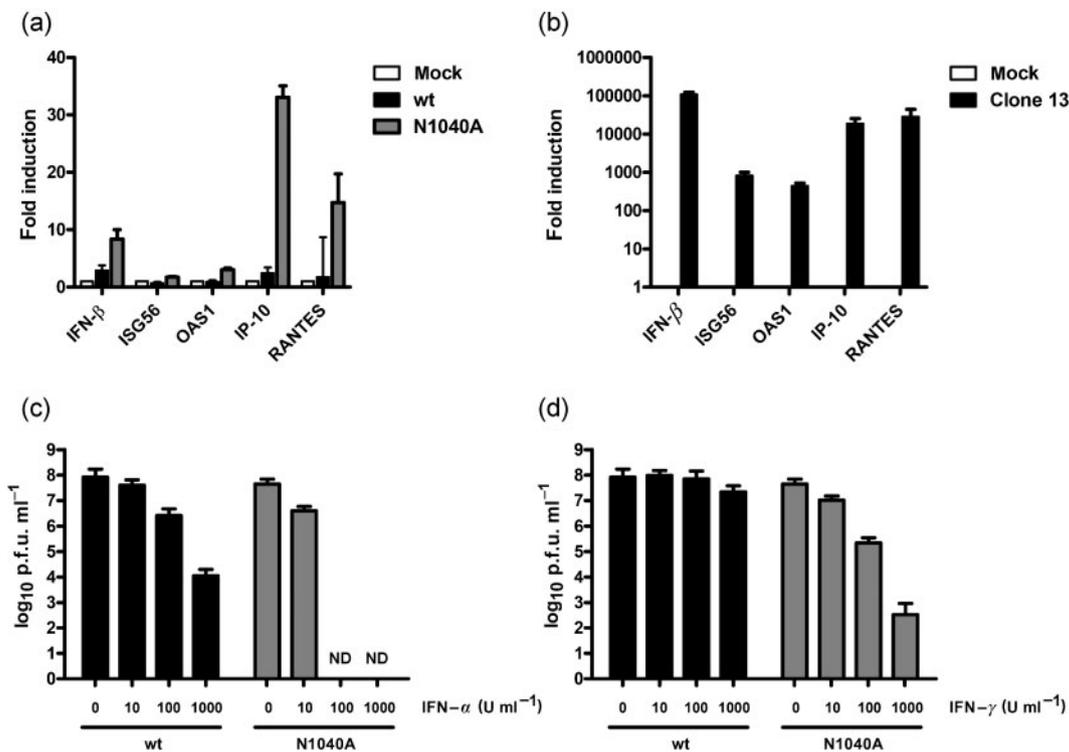
Fig. 2. Interaction of recombinant wt SARS-CoV and SARS-CoV_1ab-N1040A with the innate immune response. (a) Induction of an innate immune response. Human 293lp cells were infected with wt SARS-CoV or SARS-CoV_1ab-N1040A at an m.o.i. of 1, or left uninfected (mock) and total RNA was extracted at 16 h p.i.. Levels of mRNAs for IFN-β, the IFN-stimulated genes (ISGs) ISG56 and OAS1, and the chemokines IP-10 and RANTES were measured by real-time RT-PCR analysis, normalized to the cellular γ-actin mRNA, and set in relation to mRNA levels of mock-infected cells. (b) Host response to the positive control virus Clone 13, using the same experimental conditions and analysis methods as in (a). (c) Sensitivity to type I IFN. Vero E6 cells were pretreated with 0, 10, 100 or 1000 U IFN-α ml^{-1} [recombinant human IFN-α B/D (Horisberger & de Staritzky, 1987)] and infected 16 h later with wt SARS-CoV or SARS-CoV_1ab-N1040A at an m.o.i. of 0.001 p.f.u. per cell. At 72 h p.i., viral titres in the supernatants were determined by plaque assay as described previously (Spiegel et al., 2004). ND, Not detectable (detection limit 100 p.f.u. ml^{-1}). (d) Sensitivity to type II IFN. Cells were pretreated with 0, 10, 100 or 1000 U IFN-γ (Sigma) ml^{-1} and then infected and assayed for virus multiplication as indicated for (c). Mean values and SD from three independent experiments are shown.

recombinant 'wt' HCoV-229E is based on a laboratory-adapted strain known to express a truncated ORF4 (Dijkman et al., 2006).

We also measured sensitivity to IFN-α, the IFN which was most efficient against the ADRP mutant of SARS-CoV (see Fig. 2c). IFN sensitivity was measured under multistep growth conditions known to be a sensitive indicator of innate immunity-related replication defects. With decreasing m.o.i., growth defects of the N1305A mutant became increasingly apparent (Fig. 3b). At an input m.o.i. of 0.001 TCID$_{50}$ per cell, the N1305A mutant did not multiply at all. We therefore used an input m.o.i. of 0.3 TCID$_{50}$ per cell to measure IFN sensitivity of HCoV-229E. These conditions allow comparable growth properties without IFN, but still require multistep multiplication. MRC-5 cells were pretreated with 1000 U IFN-α ml^{-1}, infected with wt and the N1305A mutant, and virus titres were determined at 24, 32 and 40 h post-infection (p.i.). Fig. 3(c) shows that IFN

inhibits wt HCoV-229E by approximately 1.5 orders of magnitude, whereas that of HCoV_1ab-N1305A was reduced by almost 2.5 orders of magnitude. The N1305A mutant has thus a 10-fold increased IFN sensitivity compared with wt virus. We concluded that, consistent with the data obtained for SARS-CoV, the HCoV-229E macrodomain-associated ADRP activity counteracts antiviral activities of type I IFNs.

Coronaviruses share macrodomains with alphaviruses, e.g. Chikungunya virus and SINV, and several other positive-strand RNA viruses such as hepatitis E virus and rubella virus (Gorbalenya et al., 1991). This high level of conservation suggests a critical function in the viral life cycle. For both the coronavirus MHV as well as for the alphavirus SINV it was reported that macrodomain mutants can grow normally in cell culture, but are severely virulence-attenuated in vivo (Eriksson et al., 2008; Park & Griffin, 2009). Moreover, the ADRP mutant of MHV,
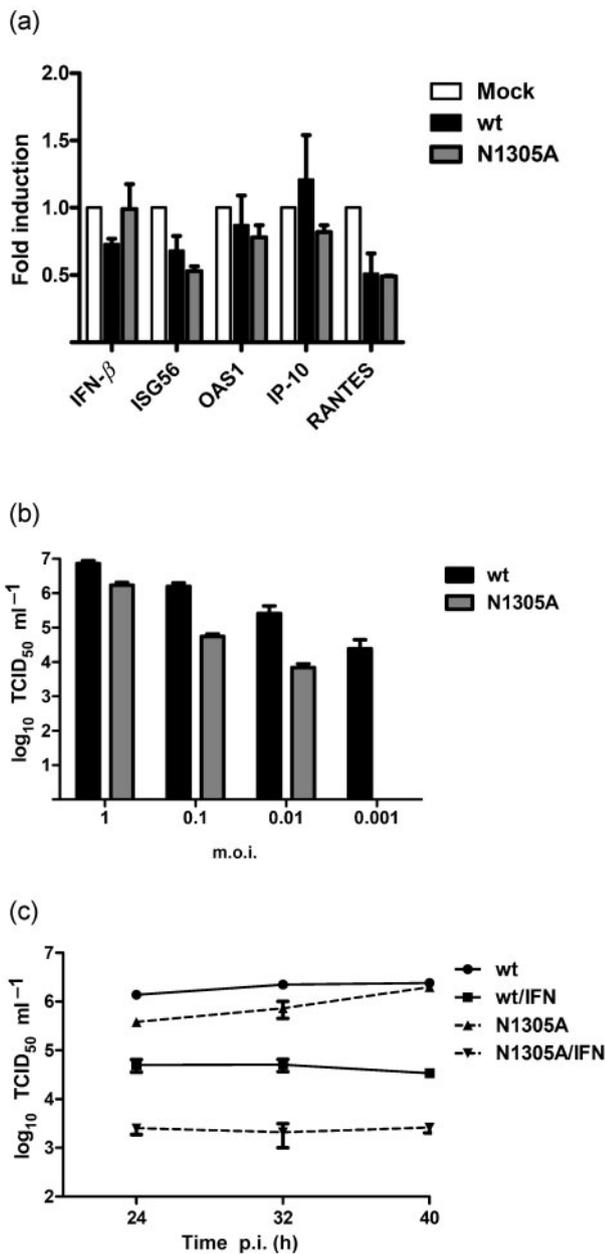
Fig. 3. Interaction of wt HCoV-229E and HCoV_1ab-N1305A with the innate immune response. (a) Induction of cytokines and chemokines. MRC-5 cells were infected for 14 h at an m.o.i. of 1 $TCID_{50}$ per cell of wt and ADRP-inactivated HCoV-229E, respectively. Levels of IFN-β, ISG56, OAS1, IP-10 and RANTES mRNAs were measured by real-time RT-PCR as indicated for Fig. 2(a). (b) Growth of recombinant HCoV-229E viruses. Human MRC-5 cells were infected with wt and ADRP-inactivated virus strains at an input m.o.i. of 1, 0.1, 0.01 or 0.001 $TCID_{50}$ per cell, and virus titres were determined at 48 h p.i. as described previously (Putics et al., 2005). (c) Sensitivity to type I IFN. Cells were either left untreated or pretreated for 16 h with 1000 U IFN-α ml^{-1}, and then infected with an m.o.i. of 0.3. Virus titres in the supernatants were determined at 24, 32 and 40 h p.i. as indicated for (b). Mean values and SD from three independent experiments are shown. The ADRP mutation in HCoV-229E was previously found to remain stable during cell culture passages (Putics et al., 2005).

which replicated *in vivo* to similar levels as the wt virus, displayed a significantly reduced induction of proinflammatory cytokines (Eriksson et al., 2008). This indicates that the macrodomain is a driver of MHV pathogenesis. The ADRP activities of SARS-CoV and HCoV-229E, by contrast, do not appear to modulate cytokine profiles, but make the virus less sensitive to IFN. Different observations have been made for MHV, where wt and ADRP mutant exhibit similar IFN sensitivities (Eriksson et al., 2008). A better understanding of the molecular mechanisms involving ADRP activities will be required to explain this discrepancy. Also, the characterization of the specific role(s) of two other macrodomains identified recently in the SARS-CoV unique domain (SUD) in nsp3 may help explain differences between different coronaviruses in dealing with host cell response (Tan et al., 2009).

Taken together, our data demonstrate that (i) human coronavirus HCoV-229E appears to be a poor inducer of the innate immune system, and (ii) macrodomain-associated ADRP activities of HCoV-229E and SARS-CoV contribute to resistance to the innate immune system. Although the molecular details remain to be studied, it seems likely that this ADRP function involves binding and/or processing of poly(ADP-ribose), ADP-ribose-derived metabolites or host mRNAs bearing particular sequence motifs (Neuvonen & Ahola, 2009; Tan et al., 2009). Our current efforts are aimed at elucidating the so-far unknown innate immune mechanisms counteracted by coronavirus macrodomains. Our study supports the idea that genetically engineered coronaviruses that lack ADRP activity may be promising candidates for attenuated life vaccines. These mutants can be grown to high titres in cell culture, but display an increased sensitivity to antiviral innate immunity responses, thus limiting their pathogenicity in the natural host.

Acknowledgements

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