Dampened NLRP3-mediated inflammation in bats and implications for a special viral reservoir host

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Bats are special in their ability to host emerging viruses. As the only flying mammal, bats endure high metabolic rates yet exhibit elongated lifespans. It is currently unclear whether these unique features are interlinked. The important inflammatory sensor, NLR family pyrin domain containing 3 (NLRP3), has been linked to both viral-induced and age-related inflammation. Here, we report significantly dampened activation of the NLRP3 inflammasome in bat primary immune cells compared to human or mouse counterparts. Lower induction of apoptosis-associated speck-like protein containing a CARD (ASC) speck formation and secretion of interleukin-1β in response to both ‘sterile’ stimuli and infection with multiple zoonotic viruses including influenza A virus (− single-stranded (ss) RNA), Melaka virus (PRV3M, double-stranded RNA) and Middle East respiratory syndrome coronavirus (+ssRNA) was observed. Importantly, this reduction of inflammation had no impact on the overall viral loads. We identified dampened transcriptional priming, a novel splice variant and an altered leucine-rich repeat domain of bat NLRP3 as the cause. Our results elucidate an important mechanism through which bats dampen inflammation with implications for longevity and unique viral reservoir status.

Over-activation of the NLRP3 inflammasome has been linked to a hyper-inflammatory state and immunopathology in viral infection with minimal effect on the viral load12−15. However, nothing is known about NLRP3-mediated inflammation in bats. On the basis of the observations of increased longevity and viral asymptomaticity in bats, we hypothesize that the NLRP3 inflammasome, as a central player in viral infection and aging, differs between bats and other mammals.

Here, we report a mechanism by which bats dampen host inflammation in response to both ‘sterile’ danger signals and infections with three different zoonotic RNA viruses, without affecting the viral loads in primary immune cells. This involves dampened transcriptional priming and a lower functional capacity of bat NLRP3. We also discover that bat-borne Pteropine orthoreovirus 3 (PRV3M, previously known as Melaka virus) and MERS coronavirus (MERS-CoV) induce NLRP3-mediated inflammation in mouse or human. Bats have naturally dampened stress-related and virus-induced host inflammatory responses, with implications for longevity and asymptomatic viral reservoir status.

Results

Activation of NLRP3 inflammasome is dampened in bat primary immune cells. Following priming (signal 1) and activation (signal 2), NLRP3 triggers assembly of the diffuse cytosolic apoptosis-associated speck-like protein containing a CARD (ASC) protein to form ASC specks35. These then recruit and activate caspase-1 to promote inflammatory cell death via pyroptosis and cleavage/secretion of the potent pro-inflammatory cytokine interleukin-1β (IL-1β) (Fig. 1a).

To assess the activation of the NLRP3-inflammasome in primary
Fig. 1 | Activation of the NLRP3 inflammasome is dampened in bat PBMCs, BMDMs and BMDCs. **a**, Schematic model for NLRP3 inflammasome activation. **b**, Representative single-cell images of gated monocytes of human (left) and bat (right) PBMCs, primed with LPS for 3 h, with or without stimulation by ATP or nigericin for 30 min. Cells were acquired using ImageStream. DAPI, blue; ASC, red; arrowheads, ASC specks. Scale bars, 10 µm. **c**, Quantification of ASC specks in gated monocytes of PBMCs (left), BMDMs (middle) or BMDCs (right) by ImageStream, unprimed (Un.) or primed with LPS for 3 h, with or without stimulation with ATP or nigericin for 30 min. **d**, LDH release in cell-free supernatants of PBMCs (left), BMDMs (middle) or BMDCs (right), primed with LPS for 3 h, with or without stimulation by ATP or nigericin for 1 h. **e,f**, Detection of human (e) and bat (f) C-terminal pro-IL-1β peptides in cell lysates (left) or supernatants (right) of PBMCs as in d, using PRM-based targeted mass spectrometry with heavy isotope-labelled internal standards. K indicates heavy labelled lysine (13C6, 15N2); NS, not detected; ND, not significant. **g**, Secretion of IL-1β by ELISA in the supernatant of BMDMs (left) or BMDCs (right) as stimulated in d. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; two-tailed unpaired t-test. Exact P values are provided in Supplementary Table 5. Data are representative of three biological replicates (n = 3) in b, or mean ± s.d. of three biological replicates (n = 3) in c–g.

immune cells, peripheral blood mononuclear cells (PBMCs) of bat (Pteropus alecto) and human, or bone-marrow-derived macrophages (BMDMs) or dendritic cells (BMDCs) of bat and mouse, were tested. Cells were primed with lipopolysaccharide (LPS) and then stimulated by potent NLRP3 activators, ATP or nigericin, representing a host- or pathogen-derived danger signal, respectively27. ASC specks in bat cells were visualized with a monoclonal antibody specifically raised against P. alecto ASC protein (Supplementary Fig. 1a–c). Quantification of ASC specks in human and bat PBMCs was first validated by confocal microscopy (Supplementary Fig. 1d) and ImageStream imaging flow cytometry (Fig. 1b). Both approaches similarly detected ASC specks formation on treatment of human
PBMCs with ATP or nigericin (Fig. 1c and Supplementary Fig. 1e). To quantify the specks in high throughput, we used ImageStream for the remainder of the experiments. We detected low-level ASC specks in LPS-primed bat cells, with negligible induction in ATP- or nigericin-treated cells, in contrast to treated human PBMCs, mouse BMDMs or BMDCs (Fig. 1c). A key feature of pyroptotic cell death is destabilization of the cell membrane and release of cytosolic contents\(^\text{11}\). We measured pyroptosis by the release of lactate dehydrogenase (LDH) and observed less LDH release in treated bat immune cells (Fig. 1d). To examine the downstream secretion of IL-1β in limited amounts of bat PBMCs, we used highly sensitive and specific parallel reaction monitoring (PRM)-based targeted mass spectrometry with heavy isotope-labelled peptide standards\(^\text{12}\). Levels of human N- or C-terminal pro-IL-1β peptides decreased in cell lysates from treated PBMCs, correlating with significantly increased levels in supernatants (Fig. 1e and Supplementary Fig. 2a,c). In contrast, from treated PBMCs, correlating with significantly increased levels of peptides decreased in cell lysates.

Secretion of IL-1β was also lower in bat BMDMs or BMDCs as β was generated with cross-reactive antibodies (Supplementary Fig. 3). In contrast, the levels of pro-IL-1β peptides in bat cells did not decrease with treatment, correlating with a lack of detection of endogenous peptides in supernatants, despite the detection of spiked internal standards (Fig. 1f and Supplementary Fig. 2b,d). In addition, an in-house sandwich enzyme-linked immunosorbent assay (ELISA) for bat IL-1β was generated with cross-reactive antibodies (Supplementary Fig. 3). Secretion of IL-1β was also lower in bat BMDMs or BMDCs as measured by ELISA of IL-1β (Fig. 1g). Altogether, activation of the NLRP3 inflammasome is dampened in primary bat immune cells.

**Transcriptional priming of bat NLRP3 is impaired, independent of TLRs.** Lack of ASC speck formation and downstream activation can be attributed to either the adaptors ASC or the sensor NLRP3, or both. NLRP3 expression is rate-limiting and transcriptional upregulation by NF-κB through Toll-like receptors (TLRs) is an essential first step required for the inflammasome activation\(^\text{13,14}\). We thus examined the transcriptional priming of both NLRP3 and IL-1β, along with classical NF-κB-induced genes IL-6 and TNF-α. BMDMs, BMDCs or PBMCs of bat and mouse or human were treated with various doses of four different NF-κB-activating TLR agonists: LPS (bacterial lipopolysaccharide) for TLR4, Pam3CSK4 (bacterial triacylated lipopeptide) for TLR1/2, CL264 (adenine analogue) for TLR7 and poly(I:C) (viral dsRNA analogue) for TLR3. The majority of the TLR stimulations induced IL-1β, IL-6 and TNF-α, up to 115-, 67- and 57-fold, respectively, in bat BMDMs (Fig. 2a), 40-, 38- and 39-fold in bat BMDCs (Fig. 2c) and 40-, 552- and 95-fold in bat PBMCs (Fig. 2e), suggesting robust NF-κB activation in these bat immune cells. However, NLRP3 was not induced with most of the TLR stimuli, except at higher doses when it was up to only threefold in bat BMDMs, fourfold in bat BMDCs and ninefold in bat PBMC (Fig. 2a,c,e). In contrast, NLRP3 was significantly induced with higher amplitude for most stimuli in mouse BMDMs or BMDCs and human PBMCs (Fig. 2b,d,f). Despite the differences in the amplitudes of IL-1β, IL-6 or TNF-α responses to various stimulations between species, a more robust induction of NLRP3 in mouse or human cells compared to bat cells was observed with the TLR stimuli of similar potency for IL-1β, IL-6 or TNF-α between species, such as CL264 in bat and mouse BMDMs (Fig. 2a,b), CL264 and poly(I:C) for bat and mouse BMDCs, respectively (Fig. 2c,d), and CL264 and LPS for bat and human PBMCs (Fig. 2e,f). We excluded the possibility of a difference in NLRP3 basal expression level by comparing mRNA levels across bat and mouse tissues (Supplementary Fig. 4). Comparable levels of NLRP3 transcripts relative to housekeeping genes between bat and mouse tissues were observed. Basal NLRP3 expression across bat tissues was 100 to 1,000 times lower than constitutively expressed inflammasome genes such as ASC and caspase-1, which is also consistent with the pattern seen in mouse tissues (Supplementary Fig. 4).

Collectively, these data indicate that bat NLRP3 has dampened transcriptional priming independent of TLR signalling. The function of all four bat NLRP3 isoforms, but not ASC, is dampened. To characterize NLRP3 and ASC functions, we examined their major isoforms in bat immune cells. Transcriptome analysis revealed only one ASC isoform, yet a NLRP3 splice-variant was identified. The alignment of full-length human and *P. alecto* NLRP3 proteins revealed 79% identity with two potential start codons in bat NLRP3 (Fig. 3a). De novo assembly of mapped RNA-sequ reads from *P. alecto* splenocyte subsets revealed an exon 7-skipping splice variant in bat NLRP3, not previously described in other mammalian orders (Fig. 3b). The exon 7 region was located within the leucine-rich repeat (LRR) domain of NLRP3. Both exon 7-positive and exon 7-negative variants were validated by PCR and sequencing from spleen and thymus cDNA. This exon 7-negative isoform constituted more than 60% of NLRP3 transcripts in different *P. alecto* tissues (Fig. 3c). The four isoforms with different start codons and/or alternative splicing are designated 1 to 4 (Fig. 3d).

Human embryonic kidney (HEK293T) reporter cells stably expressing human/bat ASC–mPlum fusions and transiently expressing human/bat NLRP3–mCitrine fusions were used for ImageStream and immunoblot analysis. For ImageStream analysis, double-positive cells of similar fluorescent intensity for ASC–mPlum and NLRP3–mCitrine were gated. This revealed that formation of bat ASC specks and their downstream cleavage of IL-1β was not different from human ASC, in response to all NLRP3 variants (Fig. 3c,e,i). In addition, the microscopic structures of specks including size, density and shape were comparable between human and bat ASC (Supplementary Fig. 5). In contrast, ASC speck and IL-1β cleavage by bat NLRP3 isoforms was lower than that of human NLRP3 (Fig. 3e–i). Notably, exon 7-negative isoforms 2 and 4 were less functional than exon 7-positive isoforms 1 and 3. The alternative translational start resulted in no difference in activity. Together, these data show that the function of all four NLRP3 isoforms but not ASC is dampened compared to the human NLRP3 and that alternative splicing of exon 7 contributes to further dampening of bat NLRP3 activity.

**NLRP3 isoform activity in bat cells from both major bat lineages is reduced.** Chiroptera is a diverse order consisting of more than a thousand bat species. To further examine whether our findings for NLRP3 in *P. alecto* from the Yinpterochiroptera suborder are also true in evolutionarily distant bats, we extended our observations to *Myottis davidi* in the other suborder, Yangochiroptera. Only one start codon matching the second start of the *P. alecto* protein was identified in *M. davidi* NLRP3 (Fig. 4a). Similarly, both exon 7-positive (isoform 1) and exon 7-negative (isoform 2) isoforms were identified by PCR and sequencing from spleen and kidney cDNA. To assess NLRP3 function in native bat cells of different species origin, we established *P. alecto* kidney (PaKi) and *M. davidi* kidney (MdKi) cells stably expressing the homologous ASC–mPlum reporter construct. Overexpression of human NLRP3–mCitrine in both bat cell types led to redistribution of diffuse bat ASC into specks (Fig. 4b–d). In comparison, the activity of all bat NLRP3 isoforms was significantly lower than that of the human protein (Fig. 4b–d). Importantly, both forms of *M. davidi* NLRP3 also displayed reduced activation. Consistent with previous findings in HEK293T cells, lack of exon 7 resulted in additional dampening of NLRP3 function. To map the region responsible for the reduced function of exon 7-positive isoforms, we generated chimaeras between human NLRP3 and *P. alecto* NLRP3 isoform 1 by substituting the human PYRIN, NACHT and LRR domains, respectively (Fig. 4e). While chimaeras 1 and 2 showed increased activity, only chimaera 3 exhibited lower activity than the wild-type human protein, suggesting that the LRR domain is responsible for the reduced function. Evolutionary analysis using 10 bat and 17 non-bat mammalian NLRP3 sequences confirmed NLRP3 was under higher selection pressure in the bat ancestor lineage compared to other mammals and the LRR domain was the only domain under significant positive selection.

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selection (Supplementary Tables 1–3), consistent with our chimeraic functional analysis. Overall, these data show that a bat-specific splice variant and/or an altered LRR domain are responsible for the dampened activity of all NLRP3 isoforms.

NLRP3-mediated inflammation is dampened in bat immune cells in response to three different types of RNA virus. To confirm our results in a more physiological setting of immune cells, we infected LPS-primed mouse or bat BMDMs or BMDCs with IAV at different multiplicities of infection (MOI = 1, 2, 5 or 10). MOI-dependent induction of ASC speck and IL-1β secretion was observed in infected mouse BMDMs or BMDCs (Fig. 5d,e and Supplementary Fig. 7a,b). Although the IAV titre was similar between mouse and bat cells, ASC speck formation and IL-1β secretion were minimally or not induced in infected bat BMDMs or BMDCs (Fig. 5d–f and Supplementary Figs. 7a–c and 8a).

To confirm that the observation is not IAV-specific, PRV3M45, a bat-borne zoonotic reovirus of double-stranded RNA (dsRNA), (IAV), strain A/NWS/33. It is a negative-sense single-stranded RNA (–ssRNA) virus, known to activate the NLRP3 inflammasome37. All bat NLRP3 isoforms were significantly less responsive to IAV infection, while viral titres from supernatants were not affected (Fig. 5a–c). To validate this finding in freshly derived myeloid cells, we infected LPS-primed mouse or bat BMDMs or BMDCs with IAV at different multiplicities of infection (MOI = 1, 2, 5 or 10). MOI-dependent induction of ASC speck and IL-1β secretion was observed in infected mouse BMDMs or BMDCs (Fig. 5d,e and Supplementary Fig. 7a,b). Although the IAV titre was similar between mouse and bat cells, ASC speck formation and IL-1β secretion were minimally or not induced in infected bat BMDMs or BMDCs (Fig. 5d–f and Supplementary Figs. 7a–c and 8a).

To confirm that the observation is not IAV-specific, PRV3M45, a bat-borne zoonotic reovirus of double-stranded RNA (dsRNA),
The function of all four bat NLRP3 isoforms, but not ASC, is reduced. a, Amino acid sequence alignment of human and bat (P. alecto) NLRP3, with identical residues highlighted in dark blue and mismatches in light blue. Black boxes, alternative translation start sites. Red box, boundaries of exon 7. b, Read-mapping analysis of two splice variants (exon 7-positive/negative) by RNA-seq read counts of the P. alecto splenocytes subsets. Arrows, exon-exon junctions. c, Percentage of exon 7-positive or exon 7-negative isoform in P. alecto tissues measured by qPCR. d, Schematic representation of four bat NLRP3 isoforms.

NLRP3 #1

NLRP3 #2

Human

Bat

ASC

Control

SN

CL

β-Actin

Pro-IL-1β (p31)

IL-1β (p17)

NLRP3

ASC

β-Actin

Fig. 3 | The function of all four bat NLRP3 isoforms, but not ASC, is reduced. a, Amino acid sequence alignment of human and bat (P. alecto) NLRP3, with identical residues highlighted in dark blue and mismatches in light blue. Black boxes, alternative translation start sites. Red box, boundaries of exon 7. b, Read-mapping analysis of two splice variants (exon 7-positive/negative) by RNA-seq read counts of the P. alecto splenocytes subsets. Arrows, exon-exon junctions. c, Percentage of exon 7-positive or exon 7-negative isoform in P. alecto tissues measured by qPCR. d, Schematic representation of four bat NLRP3 isoforms.

was used to infect reconstituted imACs. Viruses from the dsRNA group have not been demonstrated to activate the NLRP3 inflammasome. Our data show that PRV3M activated the inflammasome in an NLRP3-dependant manner as measured by ASC speck and IL-1β secretion, with decreased activation from bat NLRP3 compared to the human counterpart (Fig. 5g,h and Supplementary Fig. 8d). Additionally, there was a decrease in viral titres with NLRP3 but no obvious difference between human and bat isoforms (Fig. 5i). PRV3M infection (MOI = 1, 2, 5 or 10) in bat BMDMs or BMDCs also triggered less inflammasome activation without
the MOI-dependent increase in ASC speck formation and IL-1β secretion seen in infected mouse cells, despite the similar viral titres between mouse and bat cells (Fig. 5j–l and Supplementary Fig. 8c).

To expand this investigation to a third type of RNA virus, we interrogated inflammasome activation in response to MERS coronavirus (MERS-CoV) infection, a positive-sense (+)ssRNA zoonotic virus from a recent epidemic with potential bat ancestry66. As mouse cells are not permissive to MERS-CoV67, human and bat PBMCs were infected. MERS-CoV infection (MOI = 0.25, 0.5 or 1) activated ASC speck and IL-1β secretion in a MOI-dependent manner in human PBMCs (Fig. 5m,n). This activation, abolished by pharmacological inhibition of NLRP3 with a small-molecule inhibitor MCC95048, suggests NLRP3-dependent activation of the inflammasome by MERS. In contrast, MERS-CoV infection failed to trigger an inflammasome response in bat PBMCs, although viral titres only differed minimally between human and bat PBMCs (Fig. 5m–o and Supplementary Fig. 8c). Taken together, our findings demonstrate dampened activity of NLRP3 in immune cells and reduced activation of the NLRP3 inflammasome in response to infection with three very different viruses of public health concern, without affecting viral load.

Discussion

In summary, our results demonstrate an overall dampening of NLRP3 inflammasome activation in bat primary immune cells (Supplementary Fig. 9). Dampened transcriptional priming, a bat-specific splice variant and an evolutionarily adapted LRR domain of NLRP3 are responsible for the reduction of inflammation from ‘sterile’ danger signals (ATP) and infection of different zoonotic RNA viruses. Additionally, we discovered that both PRV3M and MERS-CoV activate the NLRP3 inflammasome in mouse and human, which also expands our current knowledge of the role of the NLRP3 inflammasome in the dsRNA virus group and family Coronavirusidae. A clear demonstration of dampened host inflammatory responses without affecting viral load is consistent with the unique asymptomatic viral reservoir status of bats.

We report multiple mechanisms, at both the mRNA and protein level, underlying the dampened NLRP3-mediated inflammation. Our data demonstrate dampened transcriptional priming, specific for NLRP3, following different TLR stimulations in P. alecto kidney (PaKi) cells stably expressing bat ASC, transfected with mCitrine (control), human or bat NLRP3–mCitrine. A Quantification of ASC specks by ImageStream in M. davidii kidney cells stably expressing M. davidii ASC, transfected with mCitrine (control), human or M. davidii NLRP3–mCitrine. Representative ImageStream images of P. alecto or M. davidii cells stably expressing P. alecto or M. davidii ASC, respectively, transfected with empty vector, human or bat NLRP3–mCitrine. NLRP3–mCitrine, green; ASC–mPlum, red. Scale bars, 10 μm. Left, Schematic representation for chimaeras between human NLRP3 and P. alecto NLRP3 isoform 1. Right, Quantification of ASC specks by ImageStream in PaKi cells stably expression P. alecto ASC, transfected with mCitrine (control), human or bat NLRP3 or chimaera–mCitrine. *P < 0.05, ***P < 0.001, ****P < 0.0001 by one-way ANOVA with Bonferroni’s multiple comparisons test. Exact P values are provided in Supplementary Table 5. Data are presented as mean ± s.d. of three independent experiments or representative of three independent experiments.
**Fig. 5** | Bat NLRP3-mediated inflammation in immune cells is dampened in response to IAV, PRV3M and MERS-CoV infection. **a-c, g-i**, Quantification of ASC specks by ImageStream (a.g), immunoblot analysis (b.h) and virus titration (c.i) of mouse NLRP3+/− iMACs reconstituted with mCitrine alone (control), human or bat (P. alecto) NLRP3–mCitrine, primed with LPS for 3 h, followed by infection with IAV (MOI = 0.5, a-c) or PRV3M (MOI = 2, g-i) for 24 h. NLRP3–mCitrine, primed with LPS for 3 h, followed by infection with IAV (MOI = 0.5, 1) for 24 h. MCC950 inhibitor was added 1 h before infection for human PBMCs. Titres in the supernatant are expressed as median tissue culture infectious dose (TCID50) ml−1. **d-f, j-l** Quantification of ASC specks by ImageStream (m), IL-1β secretion by ELISA (n) and virus titration (o) of human or bat PBMCs, primed with LPS for 3 h, followed by infection with MERS-CoV (MOI = 0.25, 0.5, 1) for 24 h. MCC950 inhibitor was added 1 h before infection for human PBMCs. Titres in the supernatant are expressed as TCID50 ml−1. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, NS, not significant, by one-way ANOVA (a.c.g.i) or two-way ANOVA (d-f, j-o) with Bonferroni’s multiple comparisons test. Exact P values are provided in Supplementary Table 5. Data are presented as mean ± s.d. of three biological replicates (d-f and j-o) or three independent experiments (a.c.g.i), or representative of three independent experiments (b.h).
model, however, failed to identify a positively selected residue of NLRP3 in the bat ancestral lineage. This suggests that an altered LRR domain is probably a conserved feature in bats and possibly involves accumulated changes in different residues in different lineages of bats. At the steady state, the LRR domain plays a role in auto-inhibition by preventing oligomerization of NLRP3. Auto-inhibition of the LRR domain in bat NLRP3 could possibly be enhanced, hence making it harder for NLRP3 to trigger downstream activation. Interestingly, the same priming step for transcriptional upregulation also licenses NLRP3 activation by inducing post-translational modifications including deubiquitination in the LRR domain. No key residues have been identified for auto-inhibition or ubiquitin modifications in the human/mouse LRR domain so far, and their roles are yet to be determined in the altered bat LRR.

The NLRP3 inflammasome is increasingly being recognized to play a critical role in the immune response to viruses. In this study, we have discovered NLRP3-dependent activation of inflammation for a bat-related dsRNA virus (PRV3M) and a coronavirus (MERS-CoV) with significant human spillover events. NLRP3 has no effect on viral titres for IAV and MERS-CoV infection in reconstituted iMAGs and PBMCs, respectively. Interestingly, both human and bat NLRP3 mediated a similar decrease in viral titres from reconstituted iMAGs infected by PRV3M, suggesting an NLRP3-dependent and inflammasome-independent mechanism. NLRP3 inflammasome activation by dsRNA virus has only been inferred from transfecting isolated dsRNA from another reovirus. No unified mechanisms for activating the NLRP3 inflammasome have been identified for viruses. While genomic or intermediate dsRNA of PRV3M and viroporin encoded by MERS-CoV E protein are possible candidate NLRP3 activators, further studies are needed to determine the activation mechanisms.

Observations in both wild-caught and experimentally infected bats suggest the ability of bats to tolerate viral disease, even during a transient phase of high viral load. For instance, in vivo virus challenge with high-dose Ebola virus and MERS-CoV caused no clinical disease and limited pathology, despite high viral titers detected in tissues or sera. The viral load can reach as high as 10^7 TCID50 equivalents per gram of lung tissues for MERS-CoV and 10^6.4 (fluorescent focus-forming units)/ml of sera for Ebola virus. While a ‘light as a fever’ theory and an ‘always on’ interferon (IFN) system might suggest elevated antiviral immunity in bats, a dampened STING-dependent IFN response to a DNA virus and an inhibitory state of natural killer cells inferred from genome analysis support enhanced immune tolerance. These hypotheses still largely lack functional confirmation, particularly with RNA virus infection. A recent study demonstrated a temperature-independent replication of Filoviruses on bat cells, as opposed to the ‘light as fever’ hypothesis. We demonstrate here a dampened NLRP3-mediated inflammatory response to three different types of RNA virus in bat immune cells, with no or minimal difference in viral titers. This supports an enhanced innate immune tolerance rather than an enhanced antiviral defence in bats. This may also contribute to our understanding of the role of the inflammasome in disease tolerance in bats as reservoir hosts, in contrast to severe pathogenesis in spillover hosts for many high-profile emerging zoonotic viruses. As NLRP3 is recognized to sense an increasing number of viruses, this finding may have broad application in a great variety of bat-borne viruses or viruses yet to be detected in bats. With the ongoing development of more experimental tools and reagents for bat immunology, a deeper characterization of immune responses in other cell types/tissues and in vivo will provide greater insight into the underlying mechanisms of viral disease tolerance in bats.

Methods

Reagents. Ultrapure LPS-B5, ATP, nigericin, Pam3CSK4, poly(I:C), CL264 and Hygromycin B Gold were obtained from InvivoGen. P. alecto ASC-specific monoclonal antibody (mouse IgG2b) was generated by GenScript’s monoclonal antibody service. Rabbit polyclonal anti-ASC (AL177) (human/mouse) was purchased from Abdigen. Goat polyclonal anti-dog IL-1β (ab193852) (cross-reactive with P. alecto), rabbit polyclonal anti-dog IL-1β (ab171928), rabbit polyclonal anti-mouse IL-1β (ab20516) and mouse polyclonal antibody (mAb) to IAV nucleoprotein (ab20343) were from Abcam. mAb to β-actin (A2228) was from Sigma-Aldrich and mAb to GAPDH and vimentin (including mCitrine) were from Roche (11814460001). Anti-dsRNA mAb J2 was purchased from SClCONS and anti-mouse/rabbit/goat/monkey horseradish peroxidase (HRP)-conjugated secondary antibodies were from Santa Cruz. mPlasmid antibody from OriGene was used to stain mPlasmid. Human mAb to MERS-CoV spike glycoprotein (m336) was provided by D. S. Dimitrov (NIH). Alexa 488/568/647 Zenon labelling kits (Invitrogen) were used for direct labelling of antibodies for confocal microscopy and ImageStream. Protein G agarose beads (Millipore) were used to purify P. alecto IL-1β-Fc cloned and expressed from HEK293T cells. Polyclonal macaque serum (#S244 D42, infected by PRV3M, produced in-house) was used for immunoblot analysis.

Viruses. Human H1N1 IAV strain A/NWS/33 (ATCC & VR-219) was propagated in Vero, clone E6 cells in DMEM, 0.3% BSA, 25 mM HEPES and 1 μg/ml TPCK-treated trypsin. Porcine reproductive and respiratory virus (PRV3M, Melaka virus) and MERS-CoV were propagated in Vero B4 cells in DMEM, 2% FBS. At a virus-induced cytopathic effect of 80–90%, viruses were harvested, clarified by centrifugation, and the virus-containing supernatant was stored at –80 °C. MERS-CoV was further purified using ultrafiltration and resuspended in DPBS. To titer IAV, A/NWS/33 Madin Darby canine kidney (MDCK, ATCC #CCCL-34) cells were infected with IAV for 1 h at 37 °C. After 1 h incubation, the medium was replaced with plaque medium (DMEM, 0.8% Avicol, 0.3% BSA, 25 mM HEPES and 1 μg/ml TPCK-treated trypsin). Plaques were fixed with 4% paraformaldehyde at 48 h post-infection and stained with 0.5% crystal violet, and titres were expressed in p.f.u. ml^-1. PRV3M and MERS-CoV titres were determined by limiting dilution. Immunofluorescence of serial diluted virus was added into a 96-well plate containing 1 × 10^4 Vero B4 cells per well. Cells were observed for cytopathic effect and the titres were expressed as TCID₅₀/ml. All work with live MERS-CoV was performed in BSL3 containment.

Plasmids. Expression constructs for ASC–mPlum, NLRP3–mCitrine, IL-1β-HA and empty vectors were generated in the retroviral backbone of pQCXIII (Clontech). NLRP3 chimaeras between human NLRP3 and the P. alecto NLRP3 isoform 1 were generated by overlap extension PCR. ASC–3xMyc and 3xMyc construct were generated in pDual GC (Agilent Technologies). P. alecto IL-1β-Fc construct containing the IL-2 signal sequence was generated from pFUSE-hlgG1-Fc plasmid (Inovigen). pSV-G envelope vector for retroviral packaging was obtained from Clontech. pCI-Caspase-1 construct encoding human pro-casparase-1 and pCMV-pro-IL-1β-Fc 1 construct encoding mouse pro-IL-1β were from Addgene. P. alecto and M. davidiid genes were cloned from Omniscript (Qiagen)-generated cDNA from bat tissues. All constructs were prepared with endotoxin-free plasmid maxi-prep kits (Omega Bio-tek).

Cells. All procedures in this study dealing with animal samples are in compliance with all relevant ethical regulations. Specifically, capturing and processing of bats (P. alecto) in Australia was approved by the Queensland Animal Science Precinct & University of Queensland Animal Ethics Committee (AEC#SVS/073/16/USMGS) and the South Australian Animal Health Laboratory (AAHL) Animal Ethics Committee (AEC#1389 and AEC#1557). Where possible, wild bats with irreparable physical damage (torn wings) already scheduled for euthanasia were utilized. Processing of bats has been described previously. Human blood was obtained from healthy donors with consent, approved by the National University of Singapore Institutional Review Board (NUS-IRB reference code H-18–029). PBMCs were isolated by Ficoll-Paque Plus (GE Healthcare) density gradient separation from P. alecto or human blood samples and stored in liquid nitrogen. Wild-type C57BL/6 mice were obtained with permission from the Singhealth IACUC committee. P. alecto and mouse splenocytes were isolated by grinding the spleen through a 100 μm cell strainer followed by red blood cell lysis. P. alecto bone marrow cells were differentiated with recombinant macrophage colony-stimulating factor (M-CSF) over 6 days by BMDMs or over 8 days with recombiant P. alecto granulocyte-macrophage colony-stimulating factor (GM-CSF) into BMDCs, as described previously. Mouse bone marrow cells were harvested and differentiated into BMDMs or BMDCs over 6 days or 8 days of cultures using 10 ng/ml mouse M-CSF or 20 ng/ml mouse GM-CSF recombinant proteins (R&D System). Immortalized P. alecto and M. davidiid kidney cell lines have been described previously and were provided by E. Latz (University of Bonn). Frozen PBMCs, splenocytes and bone marrow cells were thawed in 37 °C for 2 min, washed twice with warm maintenance medium and recovered at 37 °C for 2 h in RPMI 1640 medium containing 10% (vol/vol) FBS before experiments. PBMCs, splenocytes, BMDMs and reconstituted iMAGs were cultured in RPMI 1640 medium containing 10% FBS (Biological Industries). MDCK, Vero, GP2–293, HEK293T, PaK and MDKc cells were cultured in DMEM (Gibco) medium supplemented with 10% FBS. Sodium pyruvate and
NEAA (Life Technologies) were supplemented into the culture media of GP2−293 cells during retroviral packaging.

Generation of HEK293T, Pak4 and Mdk4 reporter cells. GP2−293 cells, stably expressing gag and pol proteins, were co-transfected with pSVS-G envelope vector and human or bat ASC-mPlum, or mPlum-only empty vector for 48 h. Retroviral supernatants were collected, centrifuged at 500g for 10 min and filtered through a 0.45 μm hydrophilic polycarbonate filter (Millipore). HEK293T, Pak4 or Mdk4 cells were transduced with a low MOI of retrovirus for 24 h to obtain a single viral copy per transduced cell, followed by Hygromycin B antibiotic selection 72 h post transduction for at least 1 month to ensure stable expression. HEK293T stable cells were further (fluorescence-activated cell sorting (FACS)-sorted for low mPlum-positive cells of similar fluorescent intensity to ensure no spontaneous activation of ASC−mPlum.

Reconstitution of NLRP3 in macrophages. Retroviral supernatants for human or bat NLRP3−mCitrine, or mCitrine-only, were similarly generated to transduce retrovirus NLRP3−knockout iMACs by first centrifuging cells with the retroviral supernatants at 300g for 1 h at 37°C, then incubating for 24 h. At 72 h after the transduction, cells were FACS-sorted for low mCitrine-positive cells of similar fluorescent intensity.

Inflammasome activation assays. PBMCs, BMDMs, BMDCs, splenocytes or reconstituted iMACs were seeded at 1 × 10^6 ml^−1 into 96-well, 24-well or 12-well plates. Cells were treated with LPS (10 μg ml^−1) and Pam3CSK4 (100 ng ml^−1) or IFN-α (2.5 IU ml^−1) or 100 ng ml^−1) and TMB stop solution (VWR) were used in the assay. Purified anti-human ASC antibody (Santa Cruz), 3,3′,5,5′-tetramethylbenzidine (TMB) (ab193852) as the capturing antibody and rabbit anti-mouse IL-1β (ab919852) as the detection antibody. ELISA was generated using the goat anti-dog IL-1β Sandwich ELISA for human/mouse IL-1β ELISAs for human/mouse IL-1β were performed on a 96-well plate. Cells were stimulated by LPS and washed and then incubated with a 1:1,000 dilution of primary antibodies overnight in a cold room in 5% BSA in TBS-T. Nuclei were stained with DAPI. Coverslips were subsequently mounted onto glass slides with Mowiol 4.88.

For IAV and PRV3M infections, BMDMs, BMDCs or mouse were seeded at 1 × 10^6 ml^−1 into 96-well, 24-well or 12-well plates. Cells were stimulated by LPS (10 μg ml^−1) and Pam3CSK4 (100 ng ml^−1) or IFN-α (2.5 IU ml^−1) and washed and then incubated with a 1:1,000 dilution of primary antibodies overnight in a cold room in 5% BSA in TBS-T. Nuclei were stained with DAPI. Coverslips were subsequently mounted onto glass slides with Mowiol 4.88.

LDH release assay. Cytosolic LDH is rapidly released into the supernatant medium following disruption of the plasma membrane. Cell culture media were collected and centrifuged at 500g for 5 min at 4°C. The release of LDH was measured in the cell-free supernatants using a Cytotoxicity Detection Kit PLUS (LDH) from Roche. The kit was used according to the manufacturer’s instructions. The percentage of LDH release was calculated as LDH release (% = (experiment value − low control)/(high control − low control)) × 100. Low control is the baseline LDH release from untreated cells, while high control is the maximum LDH release from cells treated with lysis solution.

Immunoblot analysis. Cells were lysed in lysis buffer containing Complete ULTRA protease inhibitor cocktail and PhosSTOP phosphatase inhibitors (Roche). Cell-free lysates were directly used or concentrated by methanol−chloroform precipitation and dissolved in SDS−sample buffers. Proteins were separated by 6−15% SDSPAGE gels, transferred onto 0.45 μm polyvinylidene difluoride membrane with a Trans-Blot Turbo transfer system (BioRad). Membranes were blocked with 5% BSA in TBS-T (10 μg ml^−1) and incubated with specific primary antibodies overnight in a cold room in 5% BSA in TBS-T. Membranes were washed and incubated with HRP-conjugated secondary antibody for 1 h. Membranes were developed using Amersham ECL Prime Western blotting detection reagent (GE Healthcare) and signals were detected with a myECL Imager (Thermo Scientific). Anti-GFP antibody was used to detect NLRP3−mCitrine immunoblots.

ELISAs. ELISAs for human/mouse IL-1β in cell-free supernatants were performed according to the manufacturer’s instructions (BD & Systems; DY201 and DY401). Sandwich ELISA for bat IL-1β was generated using the goat anti-dog IL-1β (ab199852) as the capturing antibody and rabbit anti-mouse IL-1β (ab92722) as the detection antibody. Bicarbonate/carbonate coating buffer (50 mM), OptEIA assay diluent (BD bioscience), donkey anti-rabbit HRP-conjugated secondary antibody (Santa Cruz), 3,3',5,5'-tetramethylbenzidine (TMB) chromogen solution (Invitrogen) and TMB stop solution (VWR) were used in the assay. Purified P. alecto IL-1β−Fc recombinant proteins were used as standards.

TLR stimulations and qPCR. BMDMs, BMDCs or PMDC of bat and mouse were seeded at 1×10^6 ml^−1 into a 96-well plate. Cells were stimulated by various concentrations of TLR ligands for 3 h: LPS-B5 (10, 100 and 1,000 ng ml^−1), PolyI:C (10 μg ml^−1), LPS+polyI:C (100 ng ml^−1 and 10 μg ml^−1), Pam3CSK4 (10, 100 and 1,000 ng ml^−1). Harvested tissues of mice or bats were homogenized using silicon-carbide sharp particles (BioSpec Products) in the FastPrep-24 5G Homogenizer (MP Biomedical). Tissue and cellular RNA was extracted using an RNeasy Mini kit and an RNeasy Plus Micro Kit (Qiagen), respectively. RNA was converted into cDNA using a QuantiTect reverse transcription kit (Qiagen). qPCR reactions were prepared using the SensiFAST SYBR No-ROX Kit (Bioline) and were run on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using the following following cycling condition: 95°C for 5 min, 40 cycles of 95°C for 10 s and 55°C for 30 s, with a melt curve cycle. Targeted genes (NLRP3, ASC, caspase-1 and IL-1β) were normalized to the geometric mean of three housekeeping genes (β2-Microglobulin, MHC-II, and 18S rRNA) and used for PCR efficiency by standard curves from sample serial dilution. Fold induction of cytokines (NLRP3, IL-1β, IL-6 and TNF-α) was normalized to housekeeping (GAPDH) and compared to mock treated. The relative abundance of exon 7−positive and exon 7−negative NLRP3 isoforms was determined by quantifying total NLRP3 and exon 7+ positive isoforms using specific primers targeting a common region and exon 7, respectively. Serially diluted plasmids of both isoforms were used to perform standard curve calculation. qPCR primers for target and reference genes are listed in Supplementary Table 4.

Isoform analysis. Illumina HiSeq RNA-seq sequencing was performed on both CD11b+/MHC-II+ (putative monocytes/macrophages) and CD11b+MHC-II− (putative DCs) of P. alecto splenocytes (n = 5) as described previously. We used TopHat to map the pooled reads from all data sets to the genomic locus of P. alecto NLRP3 and used Cufflinks and Velvet to perform de novo assembly. TopHat was then used to map the pooled reads to the two splice variants identified. For PCR validation of isoforms, RNA extraction from the spleen and thymus of an Amnis ImageStream® MK II imaging flow cytometer using x40 magnification. At least 10,000 single cells were acquired per sample and analysed with the inbuilt IDEAS software. First, cells in focus were gated using the bright-field gradient r.s.m.v. Single cells with an intermediate bright fluorescence ratio were selected. For PBMCs, the monocyte population was gated and separated from the lymphocyte population using bright-field area and side scatter. Based on the DAPI intensity, sub-G1/G0 cells were excluded. Double-positive cells of similar fluorescent intensity for ASC−mPlum and NLRP3−mCitrine or virus-infected cells were also gated. ASC speck formation was then analysed by gating on a small area and a high maximum pixel value of ASC signal. Mean pixel intensity, size and circularity score (average distance of the object boundary from its centre divided by the variation of this distance) were measured for detected ASC specks.

Images were acquired using a 40× objective (Nikon) and a high power objective (Nikon) and images were overlaid using ImageJ (NIH). The specificity of the binding was confirmed by using the following isotype controls: mouse I-Ak, mouse I-Ek, mouse IgG1, mouse IgG2a, mouse IgG2b, mouse IgG3 and mouse IgM (clone 96-243-125).
P. alecto or the spleen and kidney from M. davidii was performed using the RNeasy mini kit (Qiagen) and cDNA was produced using an Omniscript (Qiagen) reverse transcription kit. PCR amplification was performed using Q5 polymerase (NEB).

**Targeted mass spectrometry analysis.** Cells and concentrated cell-free supernatants (methylchloroform precipitation) were lyzed and denatured in 8 M urea/50 mM Tris-HCl buffer pH 8.0. Proteins were reduced with 25 mM TCEP for 20 min at 25 °C and alkylated with 55 mM 2-chloroacetamide (CAA) for 30 min, in the dark, at room temperature. Before digestion, samples were diluted with 100 mM triethylammonium bicarbonate buffer. Protease digestion was carried out with LysC enzyme (Wako) for 4 h, followed by trypsin (Promega) treatment for 18 h at 25 °C (1:100, enzyme:protein ratio). On the following day, samples were acidified with 1% trifluoroacetic acid and peptides were purified by Sep-Pak C18 cartridges (Waters). Elution of peptides was performed with 0.5% acetic acid, 80% acetonitrile followed by peptide concentration using a vacuum concentrator system. Vacuum dried peptides were subsequently analysed on an EASY-nLC 1000 (Thermo) chromatography system coupled with Orbitrap Fusion mass spectrometer (Thermo). Each sample was separated on 70 min gradient (0.1% formic acid in water and 99.9% acetonitrile with 0.1% formic acid) using a 50 cm x 75 μm inner diameter EASY-Spray Reverse Phase Column (C-18, 2 μm particles, Thermo).

Data were acquired in targeted PRM mode together with IL-1β peptide internal standards and peptide mass inclusion list. For acquisition, an Orbitrap analyser with ion targets and resolution (OT-MS 45 eV ions 120; PRM TMS/MS 35 eV ions 15k) was used. The total area MS1 of each peptide was analysed and exported using Skyline software (version 3.7.0). Values for endogenous peptides were normalized by those for the heavy isotope labelled internal standards.

**Evolutionary analysis of mammalian NLRP3.** The coding sequences for NLRP3 were collected from GenBank and Ensembl for 27 species representing the major phylogeny, using a positive selection and statistically different to the rest of the phylogeny, using a likelihood ratio test.

**Statistical analysis.** Data are presented as mean and s.d. or s.e.m. of multiple biological replicates or independent experiments (as indicated). Statistical analysis was performed using GraphPad Prism software. Results were tested for significance using unpaired two-tailed Student’s t-tests when two conditions were compared. One-way or two-way ANOVA involving one or two independent variables and Bonferroni’s multiple comparisons test were performed if multiple samples or conditions were compared. Data were considered significant if \( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \), ****\( P < 0.0001 \). Exact \( P \) values are provided in Supplementary Table 5.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The data supporting the findings of this study are available from the corresponding authors upon request. RNA-seq data used in this study have been deposited in the NCBI Sequence Read Archive (SRRR8382151). The bat NLRP3 sequences generated in this study have been deposited in GenBank under accession numbers MK355440–MK355443. Supplementary figures and tables are provided in the Supplementary Information.
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Author contributions
M.A., A.T.I. and L.-F.W. conceived the study. J.H.J.N., Z.-L.S. and L.-F.W. provided assistance in the Duke-NUS ABSL3 Facility. The authors thank the following for help with bat sampling: Crameri Research Consulting, J. Meers, H. Field and Duke-NUS team members (for a detailed listing see Supplementary Information). They acknowledge assistance in the Duke-NUS ABSL3 Facility.

Competing interests
The authors declare no competing interests.

Additional information
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- **☐**: Clearly defined error bars
- **☐**: State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

- **Data collection**: NA
- **Data analysis**: GraphPad Prism 6, Microsoft Excel 2011, TopHat 2.0.14, Cufflinks 2.2.1, Velvet 1.2.10, Unipro UGENE v1.13.3, IDEAS 6.0, Imaris 9.2.0, ImageJ 2.0.0

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The data supporting the findings of this study are available from the corresponding authors upon request. RNA-seq data used in this study have been deposited in
NCBI Sequence Read Archive (SRA: SRR8382151). Bat NLRP3 sequences generated in this study have been deposited in the GenBank under the accession number MK355440-MK355443. Supplementary figures and tables are available in the Supplementary Information file.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Biological triplicates (n=3) were performed for primary tissues or cells. |
|-------------|-------------------------------------------------------------------------|
| Data exclusions | No data exclusions. |
| Replication | Three independent experiments were performed with technical duplicates or triplicates. |
| Randomization | Bat materials were randomized by the bat caring group. Human blood materials were randomized by the blood collector. Mouse materials were randomized by the animal facility staff. |
| Blinding | Standard measurements (such as absorbance, chemiluminescence, ImageStream acquisition) and same templates (such as ImageStream analysis and FACS gating) were applied to all samples. |

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☑️ | Unique biological materials |
| ☑️ | Antibodies |
| ☑️ | Eukaryotic cell lines |
| ☑️ | Palaeontology |
| ☑️ | Animals and other organisms |
| ☑️ | Human research participants |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑️ | ChIP-seq |
| ☑️ | Flow cytometry |
| ☑️ | MRI-based neuroimaging |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

Bat materials can be requested from the corresponding authors, but subject to the availability.

Antibodies

Antibodies used

- Pteropus alecto ASC-specific monoclonal antibody (mouse IgG2b) was generated by GenScript’s monoclonal antibody service.
- Rabbit polyclonal anti-ASC (Clone: AL177; Cat#: AG-25B-0006) (human/mouse) was purchased from Adipogen.
- Goat polyclonal anti-dog IL-1β (ab193852) (cross-reactive to P. alecto), rabbit polyclonal anti-mouse IL-1β (ab9722), monoclonal antibody (mAb) to influenza A virus (IAV) nucleoprotein (Clone: AASH; Cat#: ab20343) were from Abcam.
- mAb to β-actin (Clone: AC-74; Cat#: A2228) was from Sigma-Aldrich.
- mAb to GFP and variants (including mCitrine) was from ROCHE (Clone: 7.1 and 13.1; Cat#:11814460001).
- Anti-dsRNA mAb (Clone:J2; Cat#:10010500) was purchased from SCICONS.
- Anti-mouse/rabbit/goat/donkey HRP-conjugated secondary antibody were from Santa Cruz (Cat#: sc-2005/2004/2458).
Goat polyclonal antibody to mPlum from Origene (Cat#: TA150098) was used to stain mPlum.

Human mAb against MERS-CoV spike glycoprotein (Clone: m336) was kindly provided by Dimiter S. Dimitrov (NIH, USA).

Polyclonal macaque serum (#5244 D42, infected by PRV3M) was produced in-house.

**Validation**

Pteropus alecto ASC-specific monoclonal antibody (mouse IgG2b) was validated with immunoblot and ImageStream analysis (Date provided in SI).

Goat polyclonal anti-dog IL-1β (ab193852) and rabbit polyclonal anti-mouse IL-1β (ab9722) were validated to be cross-reactive to P. alecto IL-1β by immunoblot and ELISA analysis (Date provided in SI).

Polyclonal macaque serum (#5244 D42, infected by PRV3M) was produced in-house and validated by immunoblot and ImageStream analysis (Date provided in SI).

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### Eukaryotic cell lines

Policy information about **cell lines**

**Cell line source(s)**

Human embryonic kidney fibroblast cells (HEK293T, ATCC#CRL-3216) were obtained from ATCC.

Immortalized P. alecto and M. davidii kidney cells have been generated and described previously (Cramer, G. et al and Li, Y. et al.).

GP2-293 retroviral packaging cells were obtained from Clontech.

The NLRP3-knockout immortalized mouse macrophages (iMACs) have been described previously and were kindly provided by Eicke Latz (University of Bonn, Germany).

**Authentication**

HEK293T cells were from ATCC with authentication. Bat cells made by ourselves were authenticated by species-specific qPCR and NGS.

**Mycoplasma contamination**

We confirm that all cells were tested as mycoplasma negative.

**Commonly misidentified lines**

None of the cell lines used are listed in the ICLAC database.

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### Animals and other organisms

Policy information about **studies involving animals; ARRIVE guidelines** recommended for reporting animal research

**Laboratory animals**

CS7BL/6 mice from 9-14 weeks (male or females) were obtained with permission from the Singhealth IACUC committee.

**Wild animals**

Capturing and processing of bats (P. alecto) in Australia was approved by the Queensland Animal Science Precinct & University of Queensland Animal Ethics Committee (AEC# SVS/073/16/USGMS) and the Australian Animal Health Laboratory (AAHL) Animal Ethics Committee (AECR 1389 and AEC# 1557). Where possible, wild bats (juvenile or adult bats; males or females) with irreparable physical damage (torn wings) already scheduled for euthanasia were utilized. Processing of bats has been described previously. Processed materials were frozen and transported by air shipping in dry ice.

**Field-collected samples**

Study did not involve samples collected from the field.

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### Human research participants

Policy information about **studies involving human research participants**

**Population characteristics**

Age of 21-60 years old, weight of at least 50kg, generally in good health without any symptoms of infection for at least one week.

**Recruitment**

Human blood was obtained from healthy donors with consent, approved by the National University of Singapore Institutional Review Board (NUS-IRB Reference Code: H-18-029). Healthy volunteers meeting the above criteria were recruited by poster advertisements.