Complementary regulation of caspase-1 and IL-1β reveals additional mechanisms of dampened inflammation in bats

Geraldine Goh[1]*, Matae Ahn[1]*, Feng Zhu[1], Lim Beng Lee[1], Dahai Luo[2,3], Aaron T. Irving[1,4]†, Lin-Fa Wang[1,5]†

[1]*Duke-NUS Medical School, Programme in Emerging Infectious Diseases*
[2]*Lee Kong Chian School of Medicine, Nanyang Technological University, Experimental Medicine Building 03-07, 59 Nanyang Drive, Singapore 636921.*
[3]*NTU Institute of Structural Biology, Nanyang Technological University, Experimental Medicine Building 06-01, 59 Nanyang Drive, Singapore 636921.*
[4]*Zhejiang University-University of Edinburgh Institute, Zhejiang University School of Medicine, Zhejiang University International Campus, China*
[5]*Singhealth Duke-NUS Global Health Institute, Singapore*

* These authors contributed equally to this work.
† Corresponding authors: Aaron T. Irving, Lin-Fa Wang

Email: aaron.irving@u.nus.edu, linfa.wang@duke-nus.edu.sg
Address: 8 college road, Singapore 169857. Tel: +65 6516 8397.

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Author Contributions
G.G., M.A., A.T.I. and L.-F.W. conceived the study. M.A., A.T.I., and L.-F.W. provided resources and materials; G.G., B.L., and M.A. performed experiments. G.G., M.A., F.Z., and D.L. analyzed the data, and G.G. and L.-F.W. wrote the manuscript with input from all authors. Correspondence and requests for materials should be addressed to A.T.I. and L.-F.W.

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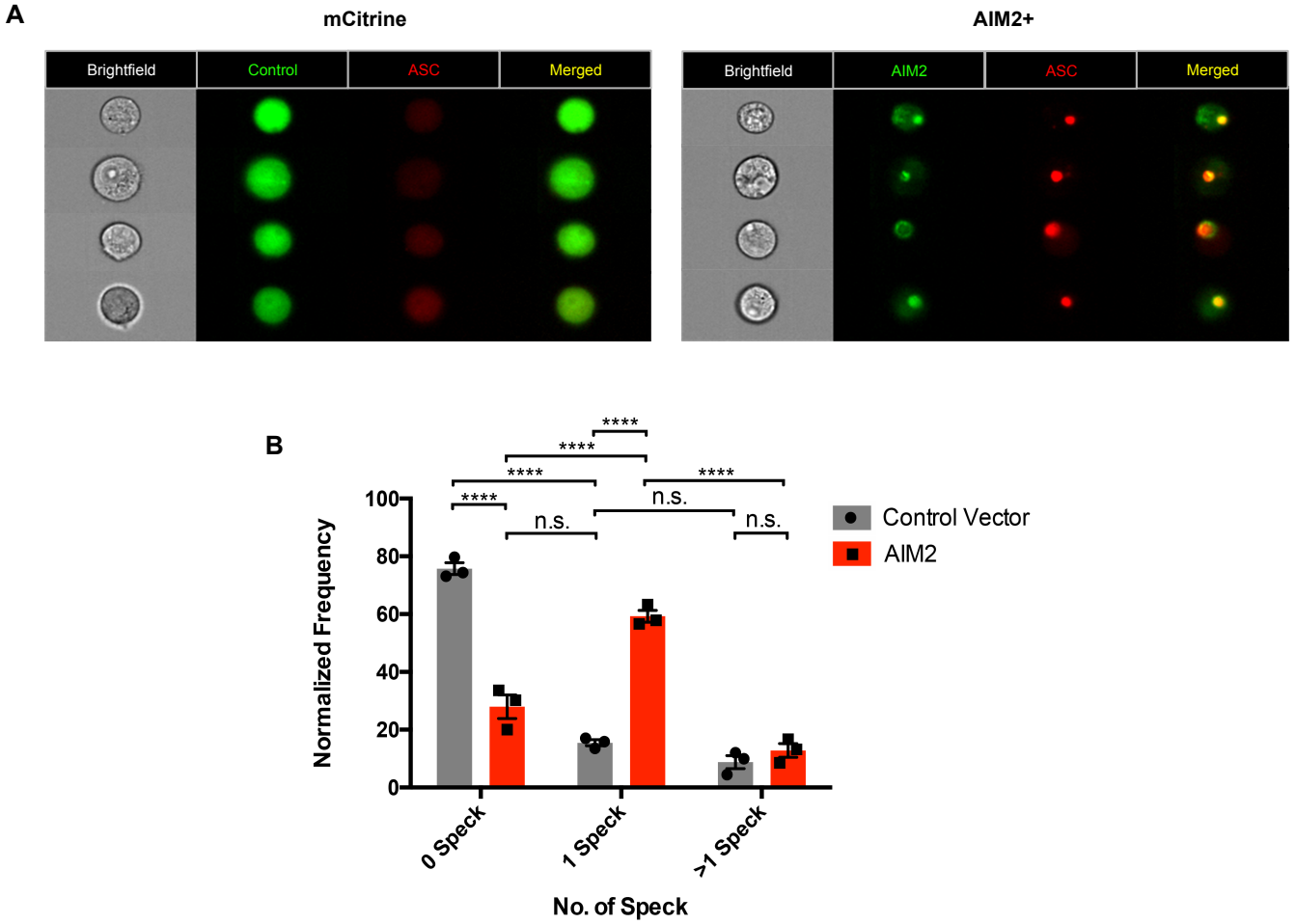
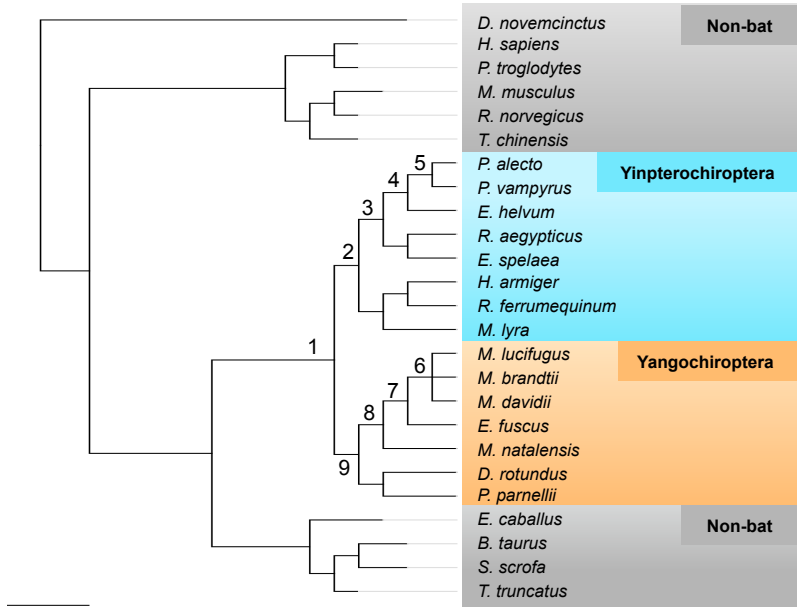


Fig S1. *In vitro* AIM2 reconstitution restores ASC-speck in bat cells.

P. alecto kidney cells (PakiS) stably expressing ASC-mPlum were reconstituted with either mCitrine-only control vector or AIM2-mCitrine by transient transfection. After 48 h incubation, cells were harvested and live cells run on Imagestream. Fluorescent signal was detected, and flow cytometry data was analyzed on IDEAS software. Single live double positive cells were gated and ASC speck quantified by parameters mPlum mean fluorescence intensity and max pixel intensity. Shown here diffuse and aggregated ASC speck (red), and diffuse and oligomerized AIM2 (green), and merged. (B) Quantification of ASC speck count in control or AIM2 transient expression in PakiS cells, ranging from 0, 1, and >1 ASC speck was performed using the Spot count wizard on the IDEAS software. Statistical analysis conducted using two-way ANOVA with Bonferroni's multiple comparisons test, ****$P <$ 0.0001, n.s. = non-significant. Figures are representative of three independent experiments (A-B).

A



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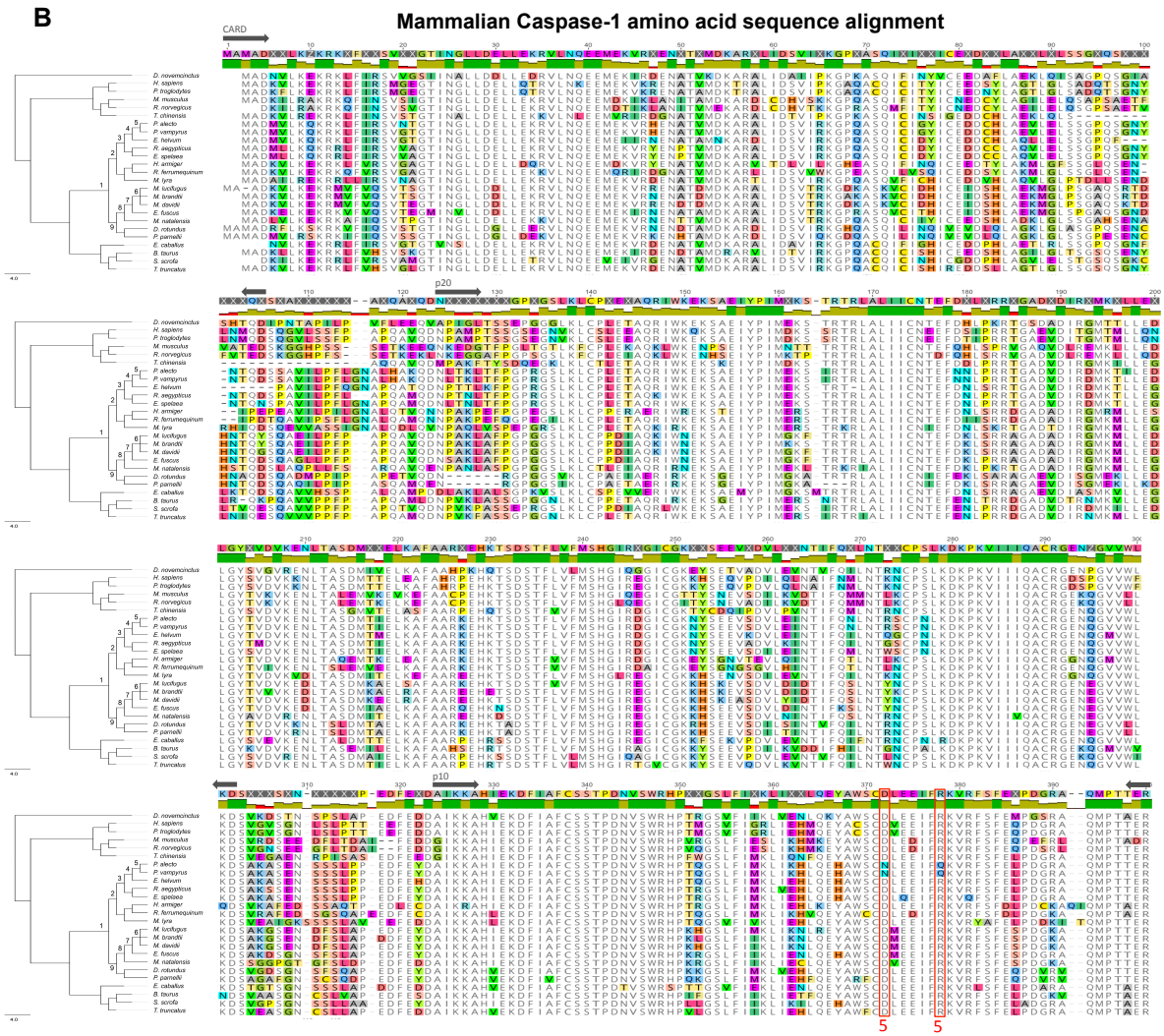


Branch	dN/dS	Sites of high positive selection pressures (p)
1	0.3354	
2	1.57607	
3	0.4106	
4	999	
5	0.39675	328 (***365***) D 0.670 334 (***371***) R 0.610

Fig S2. Phylogenetic tree and positive selection analysis of mammalian caspase-1 amino acid sequence.

(A) Caspase-1 CDS sequences of 15 bats and ten non-bat species were retrieved using discontinuous Megablast from NCBI or manually using cloning of bat cDNA. Sequences were aligned using MAFFT and phylogeny of bats referenced from existing literature. Euarchontoglires and Laurasiatheria species were represented, including primates (*H. sapiens and P. troglodytes*) and ungulates from land and sea (*S. scrofa, B. taurus, E. caballus and T. truncatus*) respectively. In total, 10 non-bat species were included, and 8 bats from Yinpterochiroptera suborder and 7 from Yangochiroptera suborders were represented. Branches among the bat phylogenetic tree are numbered from 1 (bat ancestral species) to 9 in order of speciation. Tree was rooting using the nine-banded armadillo (*D. novemcinctus*). (B) Protein sequence alignment of caspase-1 as shown using both bats and non-bat species as described in Methods. Positive selection was performed using CodeML in PAML to investigate for either lineages, or sites, which have potentially undergone evolutionary selection. Shown in the table by branch is the calculated dN/dS substitution rate, along with sites (p) identified by branch-site test showing codons which may be subject to higher positive selection pressure. Highlighted (red boxes) are the two residues identified by branch-site testing in the pteropid lineage and investigated in this study.

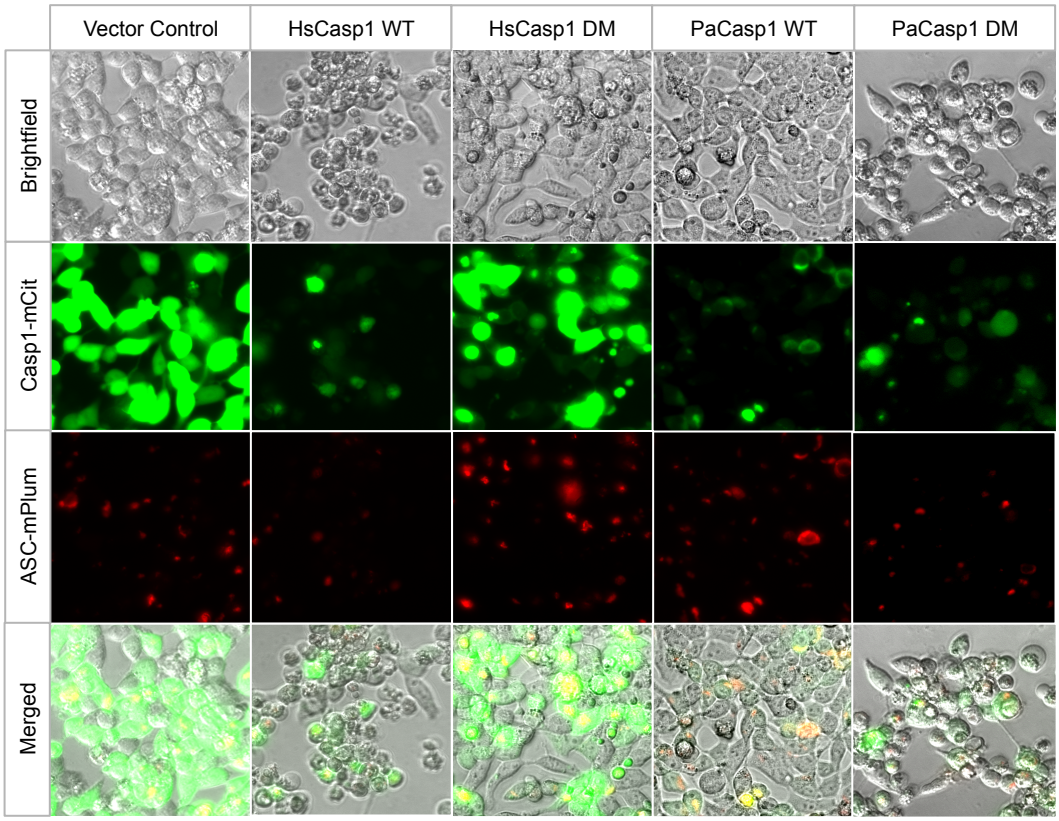


Fig S3. Cell death is induced in wild-type HsCasp1 or double mutant PaCasp1 cells. HEK293T cells were reconstituted with AIM2-3xFlag (or empty vector control), ASC-mPlum and Casp1-mCitrine (Human WT, DM or *P. alecto* WT, DM) and incubated for 48 h. Brightfield and fluorescent images were collected and analyzed for cellular stress, morphological changes, caspase-1 and ASC distribution. Figures are representative of three independent experiments.

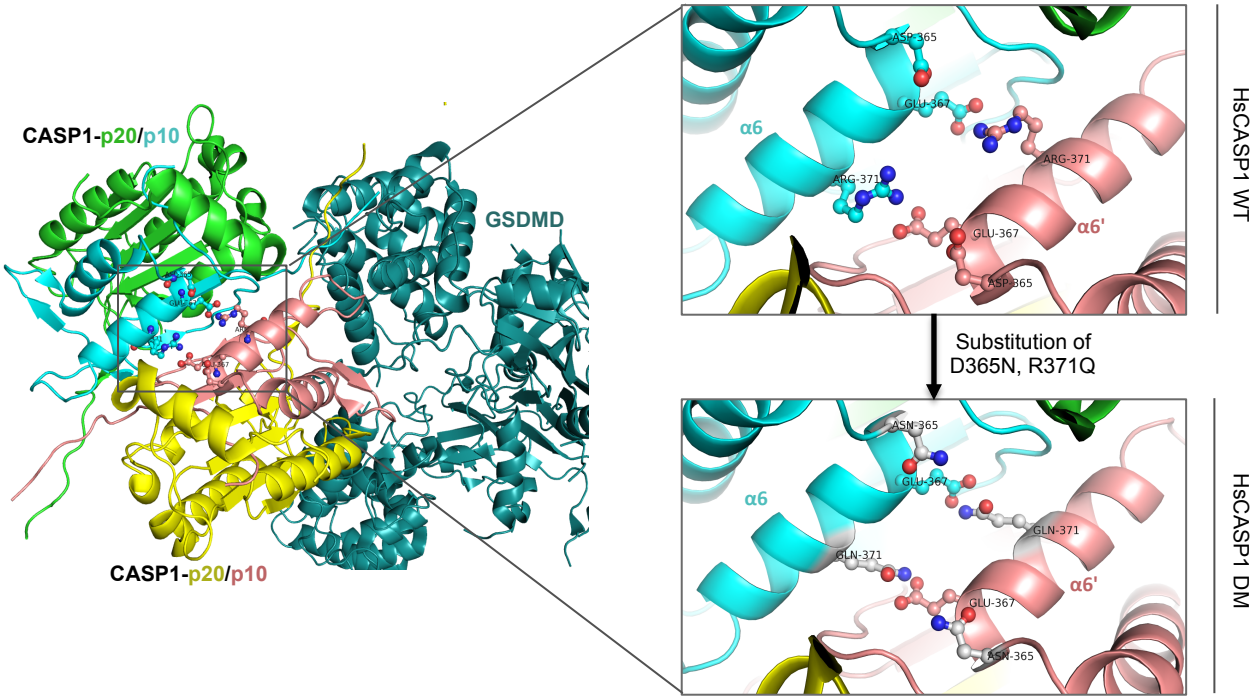
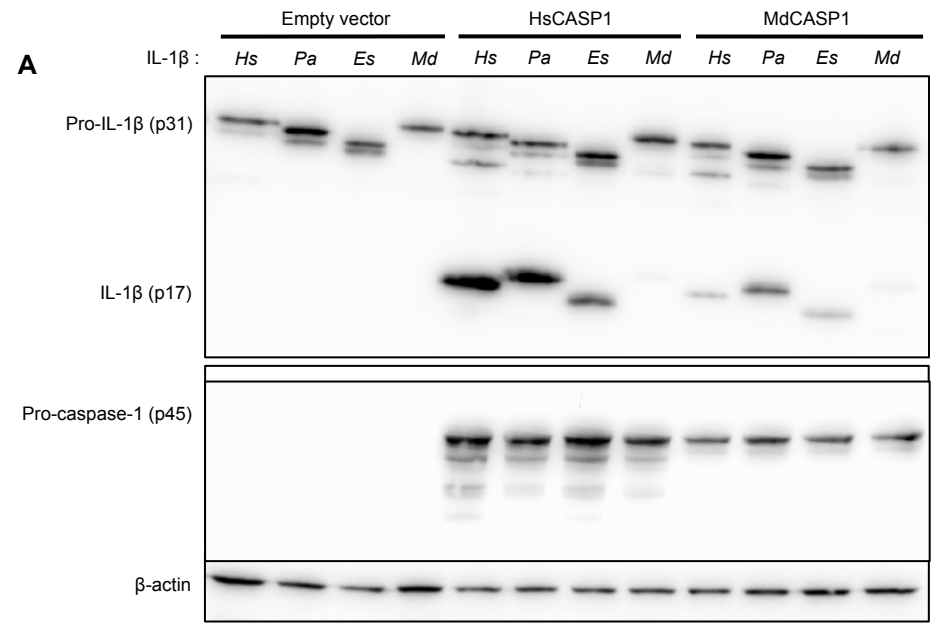
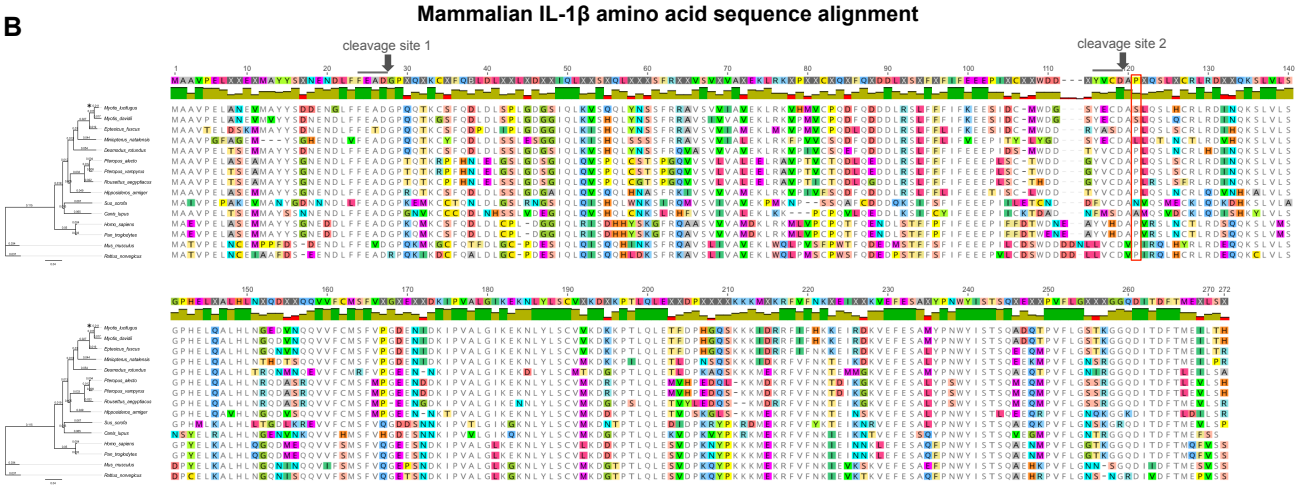


Fig S4. D365N and R371Q leads to loss of electrostatic attraction between the p10-p10 interface of the caspase-1 tetramer

Overall structure of HsCASP1 WT bound to substrate GSDMD (in teal) (left), R371 and D365 interaction across the p10-p10 interface in the post-substrate bound activated state (right, top panel), compared with substitution of D365N, R371Q in the HsCASP1 DM mutant (right, bottom panel) with altered electrostatic interaction at the dimer interface. We refer to the recently published structure of human Caspase 1 in complex with GSDMD to investigate the Casp1 interface (pdb code: 6VIE). The figure was prepared using PyMOL (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC. URL https://pymol.org/).





Branch Site Model (BSM) (Myotis branch vs. other species)
Sites of high positive selection pressures (p)
9 N 0.557 11 V 0.518 101 (**110**) G 0.823 108 (**117**) S 0.531 113 H 0.557 204 H 0.862 230 D 0.839

Fig S5. Impaired MdIL-1β cleavage despite intact MdCasp1 upstream functionality.

(A) Variable co-expression of human, *P. alecto*, *E. spelaea* or *M. davidii* IL-1β in HEK283T reconstituted with either empty vector, HsCASP1 or MdCasp1, in a NLRP3 and ASC upstream system. 48 h post-transfection, cell lysate was harvested and cleavage of IL-1β

measured by immunoblotting with anti-HA, pro-caspase-1 (p45) stained with anti-FLAG, and blots were normalized by β-actin. Western blot shown is representative of three independent experimental replicates. (B) Alignment of IL-1β of 15 mammalian species, including 9 bat and 6 non-bat species. Positive selection analysis was performed using phylogenetic analysis by maximum likelihood (PAML) in branch mode (BM), branch-site mode (BSM) and site model (SM) employed for detection of significantly selected residues. Shown in the table are sites (p) identified by the Bayes empirical Bayes (BEB) method which may be subject to higher selection pressure.

Gating Strategy for Casp1-FLICA-660 Assay:

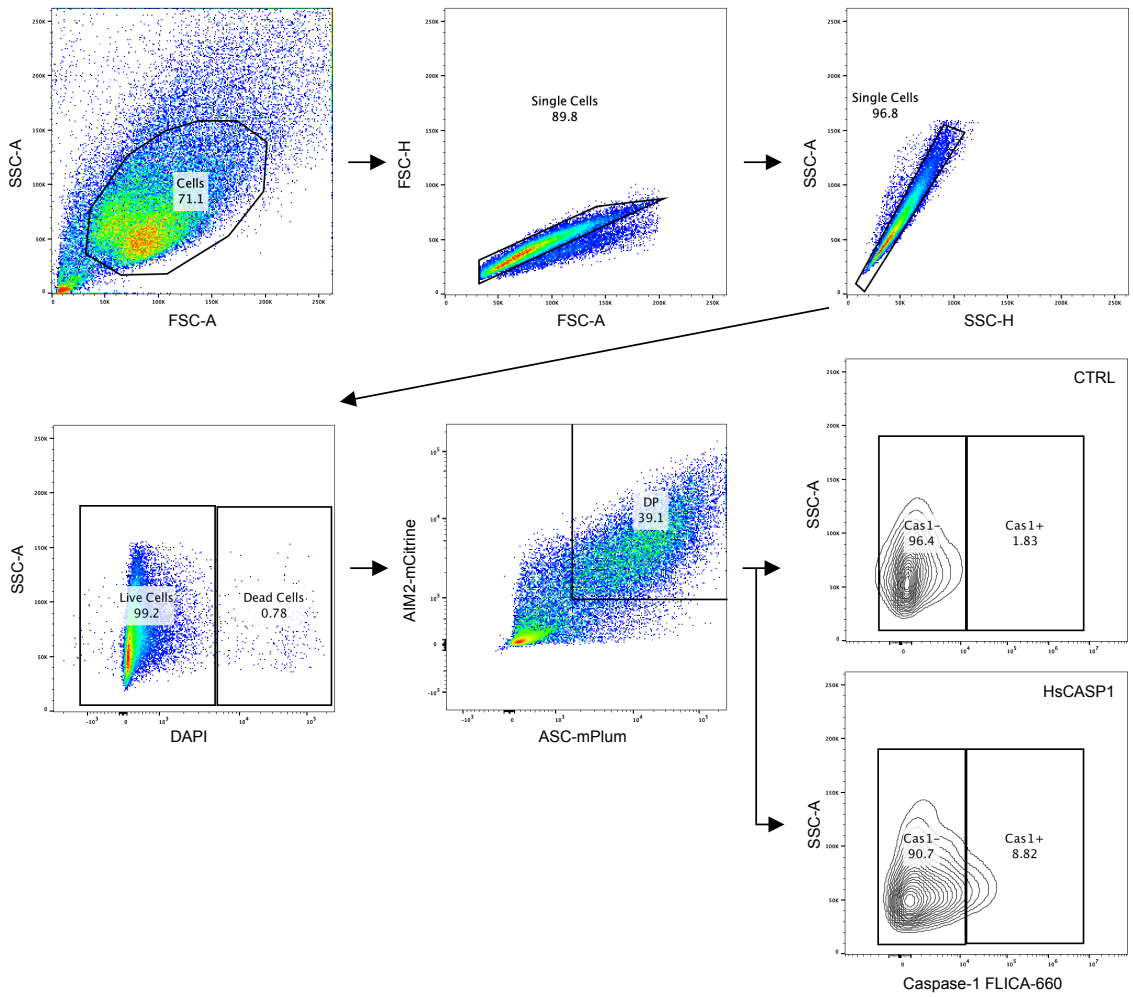


Fig S6. Caspase-1 FLICA assay with AIM2 and ASC expression gating.

The AIM2 inflammasome axis was reconstituted in HEK293T cells as described in methods. Cells were incubated for 48 h and harvested, incubated with 660-YVAD-fmk caspase-1 FLICA substrate for 1 h, and run on flow cytometry. Analysis was done on FlowJo with single color and fluorescence minus one (FMO) controls included. Cells were gated for forward scatter (FSC-A) and side scatter (SSC-A), then with FSC-A and FSC-H to exclude duplets or larger events, and again with SSC-H and SSC-A to achieve a single-cell population. Live cells were gated by exclusion of DAPI, and subsequently for double positive AIM2-mCitrine and ASC-mPlum expression. Shown here is a negative control with no FLICA substrate staining, with minimal (1.83%) fluorescent signal detected. Gating was batch-applied across all samples and conditions.

Supplementary Table S1

Flanking primers used in AIM2, caspase-1 and IL-1β cloning	
HsAIM2_F	ATGGAGAGTAAATACAAGGAGATA
HsAIM2_R	TGTTTTTTTTTTGGCCTTAATAAC
HsCASP1_F	ATGGCCGACAAGGTCCTGAAG
HsCASP1_R	ATGTCCTGGGAAGAGGTAGAA
PaCASP1_F	ATGGCTGACATGGTGCTGAAG
PaCASP1_R	CATATCCTGGAAAGAGGTAGAA
PaCASP1 N365D, Q371R DM (overlap PCR) FWD	TGGTCCTGTGATCTGGAGGAAATTTTCCGCAAGGTTCGA
PaCASP1 N365D, Q371R DM (overlap PCR) REV	TCGAACCTTGCGGAAAATTTCCTCCAGATCACAGGACCA
PaCASP1 N365D-only (overlap PCR) FWD	TGGTCCTGTGATCTGGAGGAA
PaCASP1 N365D-only (overlap PCR) REV	TTCCTCCAGATCACAGGACCA
PaCASP1 Q371R-only (overlap PCR) FWD	GAAATTTTCCGCAAGGTTCGA
PaCASP1 Q371R-only (overlap PCR) REV	TCGAACCTTGCGGAAAATTTC
HsCASP1 D365N, R371Q DM (overlap PCR) FWD	TGTTCCTGTAATGTGGAGGAAATTTTCCAAAAGGTTCGA
HsCASP1 D365N, R371Q DM (overlap PCR) REV	TCGAACCTTTTGGAAAATTTCCTCCACATTACAGGAACA
HsCASP1 D365N-only (overlap PCR) FWD	TGTTCCTGTAATGTGGAGGAA
HsCASP1 D365N-only (overlap PCR) REV	TTCCTCCACATTACAGGAACA
HsCASP1 R371Q-only (overlap PCR) FWD	GAAATTTTCCAAAAGGTTCGA
HsCASP1 R371Q-only (overlap PCR) REV	TCGAACCTTTTGGAAAATTTC
MdIL-1β_F	ATGGCAGCAGTGCCTGAAC
MdIL-1β_R	GTGAGTCAGGATTTCCATGGTGA
HsIL-1β_F	ATGGCAGAAGTACCTGAGCT
HsIL-1β_R	GGAAGACACAAATTGCATGG
PaIL-1β_F	ATGGCAGCAGTGCCTG
PaIL-1β_R	GTGAGAAAGGATTCCAGGG
EsIL-1β_F	ATGGCAGCAGTGCCTGAACT
EsIL-1β_R	GTGAGAAAGGACTTCCAGGG
MdCASP1_F	ATGGCAGACAAAGTCCTGAA
MdCASP1_R	ATTGCCAGGAAAGAGGTAGAAAC
EsCASP1_F	ATGGCTGACATGCTGCTGAA
EsCASP1_R	ATATCCTGGAAAGAGGTAGAAACAT

Table S1. Flanking primers used in AIM2, caspase-1 and IL-1β cloning.

Primers were designed against human, *P. alecto*, *E. spelaea* and *M. davidii* caspase-1 and IL-1β genes for PCR cloning and expression in plasmid vectors. Overlap PCR primers were generated for site-directed mutagenesis of human and *P. alecto* caspase-1 cloning, as listed.

Supplementary Table S2

Flanking primers used in IL-1β and GSDMD cloning	
PalL-1β DG110/111GS (overlap PCR) F	TGCACGTGGGACGGCAGTTACGTGTGCGAC
PalL-1β DG110/111GS (overlap PCR) R	GTCGCACACGTAACTGCCGTCCCACGTGCA
PalL-1β V113E (overlap PCR) F	GACGACGGGTACGAGTGCGACGCGCCCCTG
PalL-1β V113E (overlap PCR) R	CAGGGGCGCGTCGCACTCGTACCCGTCGTC
PalL-1β P117S (overlap PCR) F	GTGTGCGACGCGTCCCTGCAGTCGCTGAGC
PalL-1β P117S (overlap PCR) R	GTGTGCGACGCGTCCCTGCAGTCGCTGAGC
PalL-1β S122Q (overlap PCR) F	CTGCAGTCGCTGCAGTGCAGGCTCCGG
PalL-1β S122Q (overlap PCR) R	CCGGAGCCTGCACTGCAGCGACTGCAG
PalL-1β Pa→Md 110-122 (overlap PCR) F	ACGTGGGACGGCAGTTACGAGTGCGACGCGTCCCTGCAGTCGCT GCAGTGCAGGCTCCGG
PalL-1β Pa→Md 110-122 (overlap PCR) R	CCGGAGCCTGCACTGCAGCGACTGCAGGGACGCGTCGCTCACGT AACTGCCGTCCCACGT
MdIL-1β GS110/111DG (overlap PCR) F	TGCATGTGGGATGACGGGTACGAGTGTGAT
MdIL-1β GS110/111DG (overlap PCR) R	ATCACACTCGTACCCGTCATCCCACATGCA
MdIL-1β E113V (overlap PCR) F	GATGGCAGTTACGTGTGTGATGCATCCCTA
MdIL-1β E113V (overlap PCR) R	TAGGGATGCATCACACACGTAACTGCCATC
MdIL-1β S117P (overlap PCR) F	TGTGATGCA CCT CTACAATCG
MdIL-1β S117P (overlap PCR) R	CGATTGTAG AGG TGCATCACA
MdIL-1β Q122S (overlap PCR) F	TCCCTACAATCGCTGAGCTGCAGGCTCCGG
MdIL-1β Q122S (overlap PCR) R	CCGGAGCCTGCAGCTCAGCGATTGTAGGGA
MdIL-1β Md→Pa 110-122 (overlap PCR) F	ATGTGGGATGACGGGTACGTGTGTGATGCACCCCTACAATCGCTGA GCTGCAGGCTC
MdIL-1β Md→Pa 110-122 (overlap PCR) R	GAGCCTGCAGCTCAGCGATTGTAGGGGTGCATCACACACGTACCC GTCATCCCACAT
HsGSDMD F	ATGGGGTCGGCCTTTGAGCGGGTAGTCCGGAGAGTG
HsGSDMD R	GTGGGGCTCCTGGCTCAGTCCTGATAGCAGTGC
PaGSDMD F	ATGGCGTCGGCCTTCGAGGGGGTGGTCAGGAGCGTGGTC
PaGSDMD R	GCGCAGCTGGCTCAGCTTCAGCAGTAGGGCCAG
2xMYC F	ATGGAACAAAAGTTGATTTCTGAAGAAGATTTGAACGGTGAACAAAA GCTAATCTCCGAGGAAGACTTG

Table S2. Flanking primers used in IL-1β and GSDMD cloning.

Overlap PCR primers were generated for site-directed mutagenesis of *P. alecto* and *M. davidii* **IL-1β** cloning, as listed. Flanking primers were designed and used for human GSDMD and PaGSDMD PCR cloning, and overlap extension to create a N-terminal 2xMyc tag.

Supplementary Table S3

Non-bat mammalian caspase-1 gene sequences

No.	Species	NCBI Ref.
1	*Dasypus novemcinctus* (nine banded armadillo)	XP_0234443082.1, caspase 1 [Dasypus novemcinctus]
2	*Homo sapiens* (human)	NP_150634.1, caspase 1 [Homo sapiens]
3	*Pan troglodytes* (chimpanzee)	XP_024203161.1, caspase 1 [Pan troglodytes (chimpanzee)]
4	*Mus musculus* (house mouse)	NP_033937.2, caspase 1 [Mus musculus]
5	*Rattus norvegicus* (Norway rat)	NP_036894.2, caspase 1 [Rattus norvegicus]
6	*Tupaia chinensis* (Chinese tree shrew)	XP_027628645.1, LOC102468460 caspase-1-like [Tupaia chinensis]
7	*Equus caballus* (horse)	NP_001075311.1, caspase 1 [Equus caballus]
8	*Bos taurus* (cattle)	XP_024831465.1, caspase 1 [Bos taurus]
9	*Sus scrofa* (pig)	NP_999327.1, caspase 1, apoptosis-related cysteine peptidase [Sus scrofa]
10	*Tursiops truncatus* (bottlenose dolphin)	XP_004319499.1, caspase 1 [Tursiops truncatus]

Bat caspase-1 gene sequences

No.	Species	NCBI Ref.
1	*Pteropus alecto* (black flying fox)	PCR cloned gene sequence
2	*Pteropus vampyrus* (large flying fox)	XP_011366415.1, caspase 1 [Pteropus vampyrus]
3	*Eidolon helvum* (straw-colored fruit bat)	Contig KE817771.1
4	*Rousettus aegyptiacus* (Egyptian fruit bat)	XP_015988899.1, caspase 1 [Rousettus aegyptiacus]
5	*Eonycteris spelaea* (lesser dawn bat)	Contig PUFA01000168.1
6	*Hipposideros armiger* (great roundleaf bat)	XP_019489681.1, caspase 1 [Hipposideros armiger]
7	*Rhinolophus ferrumequinum* (greater horseshoe bat)	Contig KI177206.1
8	*Megaderma lyra* (greater false vampire bat)	Contigs AWHB01513333.1, AWHB01308376.1
9	*Myotis lucifugus* (little brown bat)	XP_023620676.1, LOC102430672 caspase-1-like [Myotis lucifugus]
10	*Myotis brandtii* (Brandt's bat)	XP_014395312.1, caspase 1 [Myotis brandtii]
11	*Myotis davidii* (David's myotis)	XP_015419332.1 (404), LOC102769699 caspase-1 [Myotis davidii]
12	*Eptesicus fuscus* (big brown bat)	XP_008154318.1, caspase 1 [Eptesicus fuscus]
13	*Miniopterus natalensis* (Natal long-fingered bat)	XP_016062165.1, LOC107532340 caspase-1-like [Miniopterus natalensis]
14	*Desmodus rotundus* (common vampire bat)	XP_024426637.1, caspase 1 [Desmodus rotundus]
15	*Pteronotus parnellii* (Parnell's mustached bat)	Contigs KE915949.1 KE877022.1

Table S3. Non-bat mammalian caspase-1 gene sequences

Caspase-1 coding sequences (CDS) were retrieved from NCBI for one armadillo (*D. novemcinctus*) and many Boreoeutheria species including Euarchontoglires and Laurasiatheria. Euarchontoglires species include two primates (Human and P. *troglodytes*), two rodents (rat and mouse) and one tree shrew (Chinese tree shrew, *T. chinensis*). Homologs of bat caspase-1 from 15 species are shown as identified by discontinuous MegaBLAST or PCR sequencing from cloned caspase-1 genes.

Supplementary Table S4

Non-bat mammalian IL-1β gene sequences

No.	Species	NCBI Ref.
1	*Sus scrofa* (pig)	NP_999220.1, interleukin-1 beta [Sus scrofa]
2	*Homo sapiens* (human)	XP_016859477.1, interleukin-1 beta isoform X1 [Homo sapiens]
3	*Canis lupus familiaris* (dog)	NP_001033060.1, interleukin-1 beta [Canis lupus familiaris]
4	*Pan troglodytes* (chimpanzee)	XP_001147075.1, interleukin-1 beta [Pan troglodytes]
5	*Mus musculus* (house mouse)	XP_006498858.1, interleukin-1 beta isoform X1 [Mus musculus]
6	*Rattus norvegicus* (Norway rat)	NP_113700.2, interleukin-1 beta [Rattus norvegicus]

Bat IL-1β gene sequences

No.	Species	NCBI Ref.
1	*Myotis davidii* (David's myotis)	PCR cloned gene sequence
2	*Myotis lucifugus* (little brown bat)	XP_006103069.1, interleukin-1 beta [Myotis lucifugus]
3	*Eptesicus fuscus* (big brown bat)	XP_008158481.1, interleukin-1 beta [Eptesicus fuscus]
4	*Miniopterus natalensis* (Natal longfingered bat)	XP_016057251.1, PREDICTED: interleukin-1 beta [Miniopterus natalensis]
5	*Desmodus rotundus* (common vampire bat)	XP_024414191.1, interleukin-1 beta [Desmodus rotundus]
6	*Pteropus alecto* (black flying fox)	PCR cloned gene sequence
7	*Pteropus vampyrus* (large flying fox)	XP_011376173.1, interleukin-1 beta [Pteropus vampyrus]
8	*Hipposideros armiger* (great roundleaf bat)	XP_019518405.1, PREDICTED. interleukin-1 beta isoform X1 [Hipposideros armiger]
9	*Eonycteris spelaea* (lesser dawn bat)	PCR cloned gene sequence
10	*Rousettus aegyptiacus* (Egyptian fruit bat)	XP_016008605.1, interleukin-1 beta isoform X2 [Rousettus aegyptiacus]

Table S4. Non-bat mammalian IL-1β gene sequences

IL-1β coding sequences (CDS) were retrieved from NCBI for 6 Euarchontoglires species including two primates (Human and P. *troglodytes*), two rodents (rat and mouse) and one dog and one pig (*C. lupus familiaris, S. scrofa*). Homologs of bat IL-1β from 10 species are shown as identified by discontinuous MegaBLAST or PCR sequencing from cloned IL-1β genes.