Supplementary Information

Title: Robust Dengue virus infection and limited innate immune responses in bat cells coupled with positive serology from bats in IndoMalaya and Australasia

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Supplementary Methods:
Primer Sequences for
IFIT1: For CCTCCACCCATCTTAGGTTTATAG, Rev CATCACTGGGTACTCTCATGTC, BST2: For GCAGAGCAGAAGTGTCGAAATA, Rev AGAGTCTCCACAGTCTGGTT,
CCL4 For GACAGCCTGCTGCTTCTCTT, Rev CTGGGGTTGGCACAGACTTG,
TNF: For, CTGGCCTCCCACTAATCAAA, Rev ACTGGAGCTGTCCCTCGGCT,
IFNβ: For CTCTAGCACTGGCTGGAATGAA, Rev TGCCCACCGAGTGTCTCA,
IFNα3: For CATTTCATGGCAACCCCTTCC, Rev ACCTGCCTAGCAGGATCTCA,
IL1β: For CTCCGGGACATAAACCAGAAG, Rev CTGGGATCTTGTCATCGTTCT C,
MX1: For TGGTGGTGCCCAGTAACGT, GCTTCGTCAGGATTCCTATGGT,
SNRPD3: For AGGTATACATCCGTGGCAGC, Rev CCACTTGGGCCTTCAGAATA,
DenV NS2a: For ACCTGGGAAGAGTGATGGTTATGG, Rev ATGGTCTCTGGTATGGTGCTCTGG.

Proteomics pipeline:
Samples were enriched for proteins using an acid precipitation-based protocol (2D-Clean up, GE Healthcare). Protein was resuspended in 7 M urea, 2 M thiourea, 4% CHAPS, 2% SDS and quantified (2D Quant kit, GE Healthcare); 40 µg of protein was reduced by the addition of 10 mM DTT and alkylated with 25 mM iodoacetamide and the protein precipitated by addition of 9 volumes -20ºC methanol overnight at -20ºC. The resulting pellet was washed with a further 1 mL of 90% methanol and digested using a LysC (1:100) trypsin (1:50) digest as per Glatter *et al.*(2012). Digests were acidified with 3 µl 10% TFA, stored at -80ºC.
Acidified digests were subjected to NanoHPLC-MS/MS analysis using a nanoAcquity nanoHPLC system (Waters, Milford, MA, USA) interfaced with a linear ion-trap (LTQ)-Orbitrap Velos Pro hybrid mass spectrometer (Thermo Fischer Scientific, Bremen, Germany). Digests were loaded onto a 5 µm Symmetry 180 µm × 20 mm

C18 trap column (Waters) at 5 µL/min in 99.5% solvent A (0.1% (v/v) aqueous formic acid) and 0.5% solvent B (0.1% (v/v) formic acid in 100% acetonitrile) for 3 min at 22 ◦C then switched in-line with a pre-equilibrated analytical column (BEH130 C18 1.7 µm, 75 µm × 200 mm, Waters) at a flow rate of 0.3 µlL/min and 98% solvent A, 1% solvent B. Peptides were separated at 35 ◦C using a sequence of linear gradients: starting from 5% B over 5 min, to 27% B over 235 min, to 40% B over 20 min, and finally, to 80% B over 5 min, before holding the column at 80% B for a further 5 min. Eluates from the analytical column were introduced into the LTQ-Orbitrap Velos Pro throughout the entire run via a Nanospray Flex Ion Source (Thermo Fisher Scientific) using a 10µm P200P coated silica emitter (New Objective). Typical spray voltage was 2 kV with no sheath, sweep or auxiliary gases; heated capillary temperature was set to 275 ◦C. The LTQ-Orbitrap Velos Pro was controlled using Xcalibur 2.2 SP1.48 software, Tune 2.7.1103 SP1 (Thermo Fisher Scientific) and operated in a data-dependent acquisition mode to automatically switch between Orbitrap-MS and ion trap-MS/MS. The survey full scan mass spectra (from m/z 350–1700) were acquired in the Orbitrap with a resolving power of 60,000 after accumulating ions to an automatic gain control (AGC) target value of 1.0×10^6 charges in the LTQ (maximum injection time 200 ms); lock mass of 445.120024. MS/MS spectra were concurrently acquired in the LTQ on the 20 most intense ions from the survey scan (370-1400 mz), using an AGC target value of 5000 (maximum injection time 70 ms). Charge state filtering, where unassigned precursors and singly charged ions were not selected for fragmentation, and dynamic exclusion were used (repeat count 1, repeat duration 30 s, exclusion list size 500, exclusion duration 90 s). Fragmentation conditions in the LTQ were: 35% normalized collision energy, activation q of 0.25, 10 ms activation time, and minimum ion selection intensity of 500 counts.
Raw data was searched and LFQ quantitation performed using MaxQuant (ref. 2) (version 1.5.2.8) using the Uniprot reference proteome for Pteropus alecto (UP000010552, 28 April 2015, 19520 entries) and Dengue virus type 2 (strain Thailand/16681/1984) (DENV-2) (UP000002324) 4 May 2015, 1 entry), taxonomy:"Nelson Bay orthoreovirus [118027]" AND organism:"Nelson Bay orthoreovirus [118027]". Parameters used were: variable modifications oxidation methionine, deamidation glutamine asparagine, acetyl protein N-terminus; fixed modification carbamidomethyl cysteine; default orbitrap and ion-trap settings; FDR PSM and protein 0.01; match between runs and razor peptides used (full parameters in supplementary table X).
LFQ results were analysed in Perseus 1.5.16 (3) by the following steps: generic import including LFQ data; removal of identified by site/reverse categorical annotations; transform log2 (x); filter valid values 2 in one group; impute 1.9 SD 0.3 SD; two sample t-tests, both side, Benjamini-Hochberg correction 0.05 FDR.

VirScan:
Briefly, briefly a 200-mer oligonucleotide library of the human virome was synthesized and cloned into the T7 bacteriophage display vector [1]. The phage library was incubated with 20µl of 100-fold diluted bat serum and b[1]at serum antibodies bound to peptides displayed by phage were retained through affinity binding to Protein A- and Protein G-magnetic beads. Unbound phage was washed off and DNA from bound phage was subjected to polymerase chain reaction (PCR) followed by deep sequencing to quantify the enrichment of each peptide member due to serum antibody binding. Negative controls were included in the assay by having 6-8 reactions with no serum samples added. By quantifying the increase in

read counts of each peptide member compared to negative controls (which indicated baseline peptide stickiness to Protein A- and Protein G-magnetic beads), a peptide cumulative Z-score could be tabulated. A Z-score of more than 10 is considered to be a significantly enriched peptide.

NanoString

Nanostring was run according to the manufacturer's instructions from 200ng of RNA purified as per the qPCR protocol (previously) with the Omega Bio-Tek EZNA Total RNA Kit II. Hybridisation was performed overnight for 16-24 h for cell lines and 36h for BMDCs/BMDMs and run using the nCounter Sprint Profiler. The probe set was designed based on the 50 gene ISG panel detected by *Schoggins et al* previously[2] combined with an additional 73 inflammatory markers/cytokine genes designed against *R. aegyptiacus* but with conserved probes suitable for *P. alecto*. A full list of probes with accession numbers is available in Table S6. Access to the codeset PAISGIM1 is available upon request.

Online Supplement:

Table 1: Quantitative Proteomics of PakiT03 cells following polyIC, NBV or DENV treatment.
Majority protein IDs, LFQ counts, MS counts, Unique peptide numbers, peptide IDs and protein IDs (of proteins Identified in >3 samples) for Control, polyI:C and DENV2 NGC infected PakiT03 cells (48 h, MOI=0.1). All 3 biological replicates for each treatment is displayed.

Table 2: Repeat Quantitative Proteomics of PakiT03 cells with DENV2 ST treatment.
Significantly changed proteins, their LFQ counts, MS count, peptide ID, FASTA etc as per previous (Table 1) with an infection at 48 h of MOI=0.1 for Dengue Virus Type 2 ST 16681. Highlighted values indicate significantly changed, in all three samples and with a good peptide coverage across the protein with multiple unique peptides.

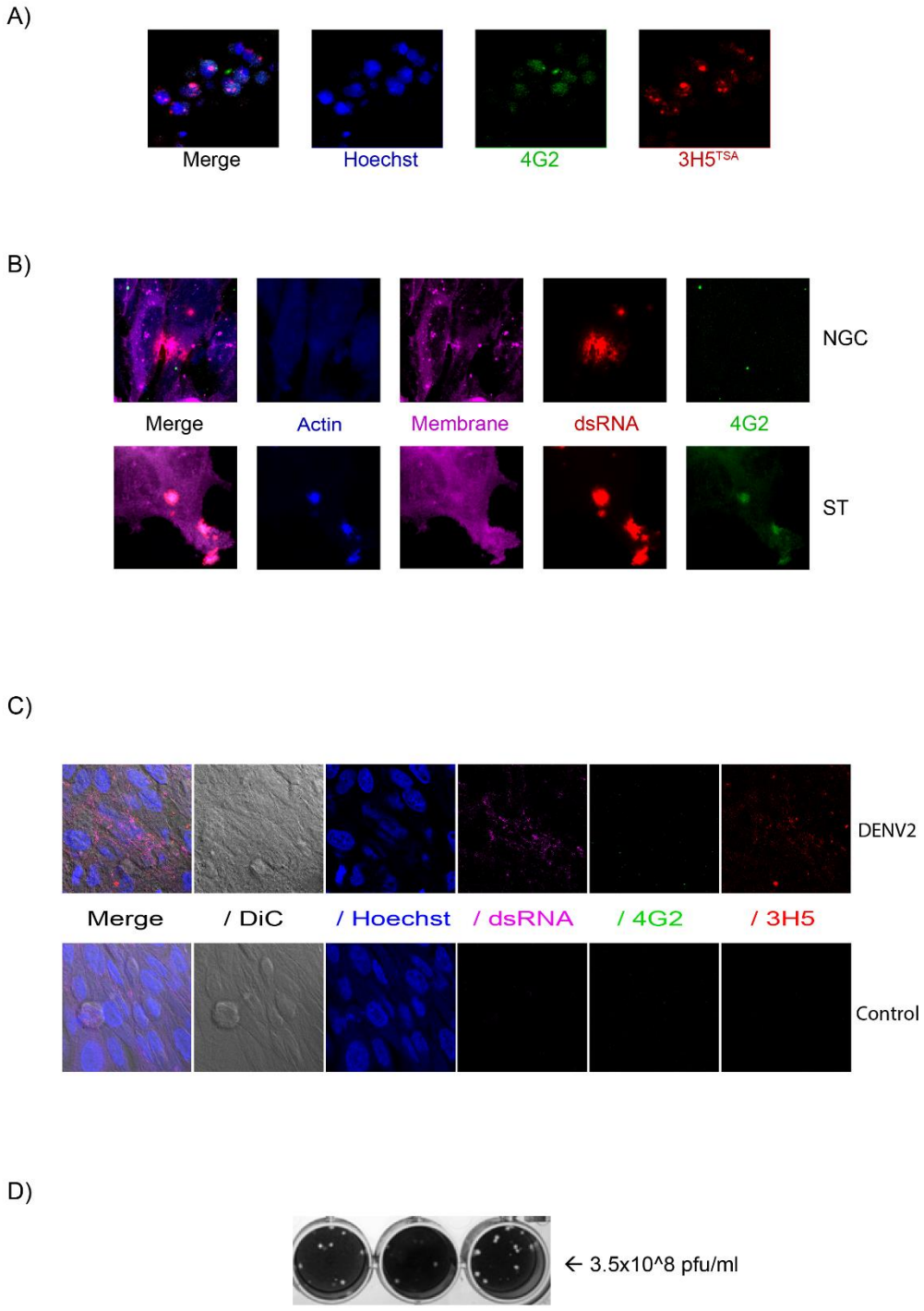
Table 3: Combined Serology data for LIPS, VirScan, DENV2 ELISA on IndoMalayan bats.
Serology results on sera from Eonycteris Spelaea and Cynopterus brachyotis for LIPS (DENV2 NS1), VirScan (DENV1,2,4), DENV2 IGG NS1 ELISA and a scoring if positive for multiple serology platforms.

Table 4: VirScan results for IndoMalayan bats of all flaviviruses.
VirScan unique peptide counts (number with a Z-Score >10), Z-Score mean, Z-Score Maximum and Protein ID listed by bat for all flaviviruses detected in the panel.

Table 5: VirScan results for Australian bats of all flaviviruses.
VirScan unique peptide counts (number with a Z-Score >10), Z-Score mean, Z-Score Maximum and Protein ID listed by bat for all flaviviruses detected in the panel.

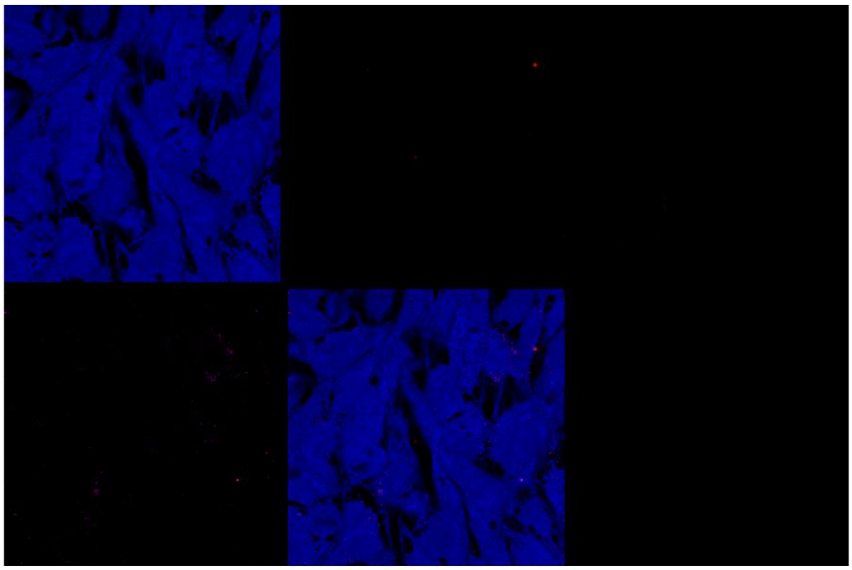
Table 6: Nanostring codeset details for PAISGIM1
Codeset probe ID, Gene names, sequence, start sites and cross-reactivity for all probes in the panel.

Supplemental Figures



Supplemental Figure 1: A) Dengue virus entry as per Fig 1A, single channel confocal images (as indicated). B) Single channel confocal images from Figure 1C, staining as indicated. C) Dengue virus replication in PaKiT03 cells after 96 h infection with an MOI of 0.1 with DiC-Differential Contrast Imaging (grey) J2-dsRNA staining (Magenta), Hoechst (Blue), 3H5-alexa594 (Red), 4G2 (Green) as previously. Uninfected control cells below. D) Full virus titration of supernatant from Fig. 1A showing $3.5x10^8$ pfu/ml produced from PakiT03 cells after an initial infection of 0.1 for 72 h. Starting MOI is the equivalent input of $4.98x10^5$pfu, ~3 log lower than the output.

A)

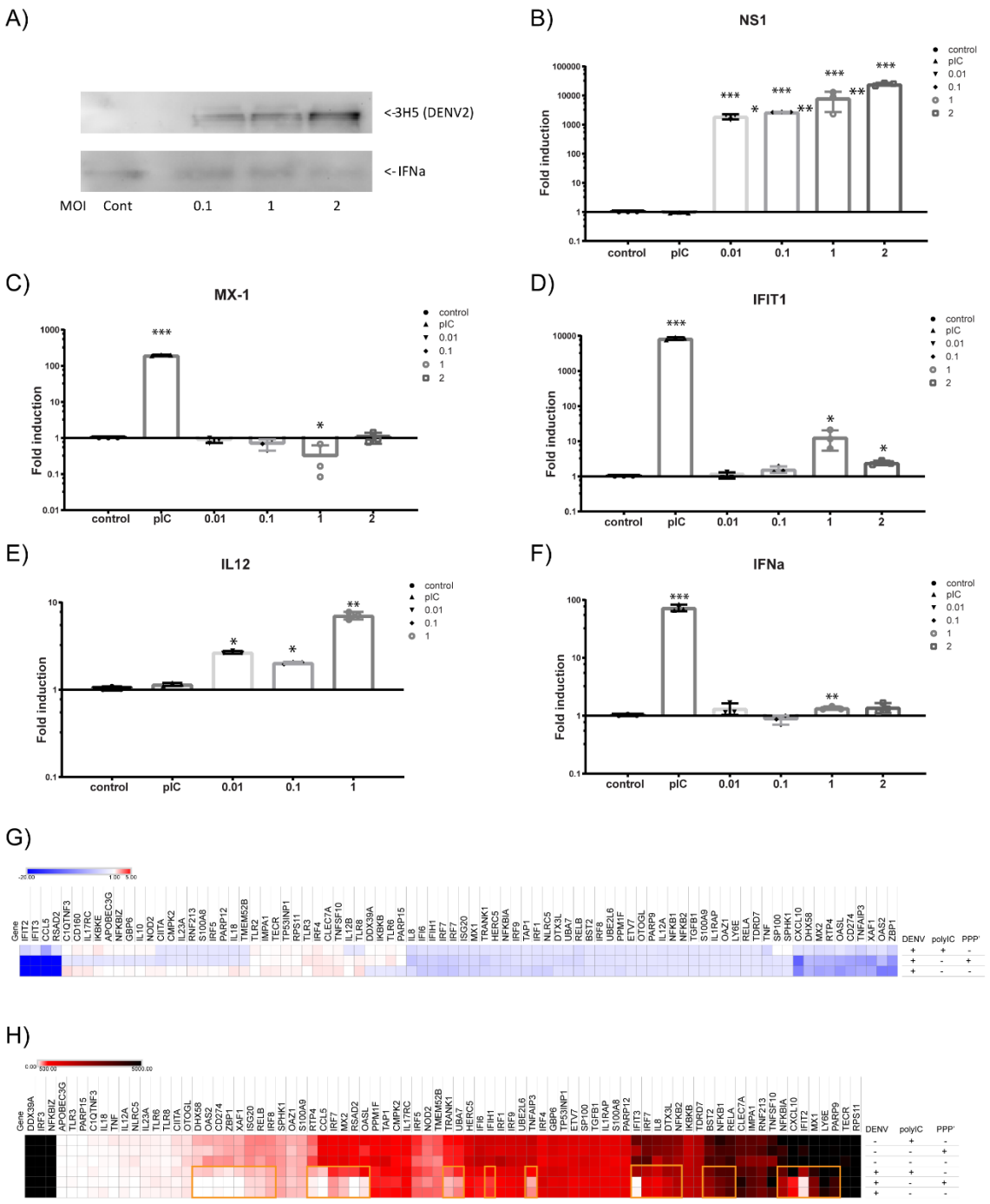


Actin / dsRNA / 3H5 / 4G2

B)

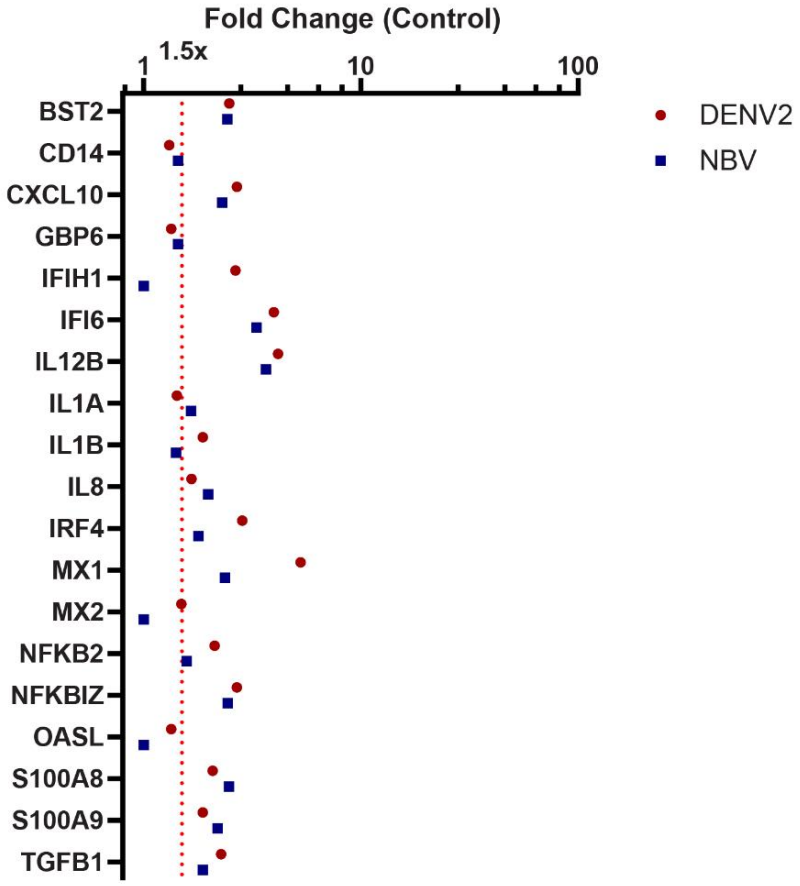
Actin / dsRNA / 3H5 / 4G2

Supplemental Figure 2: A) Uninfected PaLu cells by confocal, matched to Fig3A. Stained with Actin (Blue), dsRNA (Magenta) 3H5 (red), 4G2 (green) and a merged image. B) As per A for EsLu cells (uninfected).

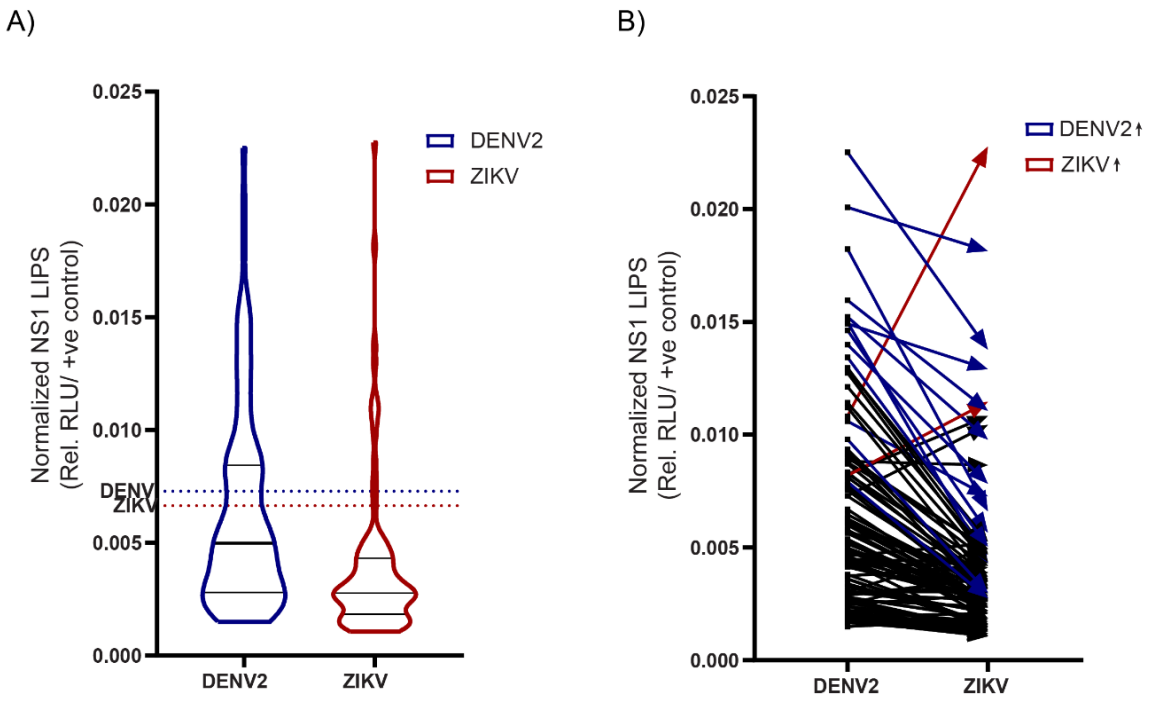


Supplemental Figure 3: A) IFN induction in the supernatant from paBMDCs (as per Figure 3C) showing no further induction of IFNα above baseline even up to an MOI of 2, despite increasing protein detected for DENV2 (3H5 antibody). B) qPCR, as previous, for DENV2 NS1 after various MOIs (0.1,1,2) or with a polyIC control for 24 h infection in PakiT03 cells. C) MX1, D) IFIT1, E) IL-12, F) IFNα3. G) NanoString (as per Figure 4) on RNA from PakiT03 cells with or without DENV infection for 24 h at an MOI of 0.1 followed by ligand treatment. Expressed as fold change (scale as indicated) relative to no virus control (e.g. DENV followed by no treatment vs no virus control and no treatment, DENV2 infection with poly IC treatment vs polyIC only). H) The same data as per G expressed as raw counts. Orange box indicates genes downregulated in the presence of DENV (MOI 0.1 for 24 h).

A)



Supplemental Figure 4: A) NanoString results as fold induction (Log_{10}) for genes induced post-infection (16hrs) with either DENV2 NGC or PRV1NB / NBV (Nelson Bay orthoreo-Virus) in *P. alecto* BMDC's (as published previously[3]) 1.5x cutoff is displayed (red line).



Supplemental Figure 5: A) Violin plot of LIPS-assay results for 102 *E. spelaea* bat sera run against DENV2 and ZIKA Virus (ZIKV) NS1 antigens (expressed as relative luciferase units, normalised against geometric mean of the respective four positive controls or Maximum signal). Cutoff thresholds displayed for DENV (blue line) and ZIKA (red line). B) Same results displayed as a Before-After plot for each bat with a directional arrow. Blue represents bats with higher scores for DENV2, red for ZIKV. Black represents values not greater than mean +4 SD.

Supplemental References:

1. Xu GJ, Kula T, Xu Q, Li MZ, Vernon SD, Ndung'u T, Ruxrungtham K, Sanchez J, Brander C, Chung RT, O'Connor KC, Walker B, Larman HB, Elledge SJ (2015) Viral immunology. Comprehensive serological profiling of human populations using a synthetic human virome. Science 348 (6239):aaa0698. doi:10.1126/science.aaa0698

2. De La Cruz-Rivera PC, Kanchwala M, Liang H, Kumar A, Wang LF, Xing C, Schoggins JW (2018) The IFN Response in Bats Displays Distinctive IFN-Stimulated Gene Expression Kinetics with Atypical RNASEL Induction. J Immunol 200 (1):209-217. doi:10.4049/jimmunol.1701214

3. Zhou P, Chionh YT, Irac SE, Ahn M, Jia Ng JH, Fossum E, Bogen B, Ginhoux F, Irving AT, Dutertre CA, Wang LF (2016) Unlocking bat immunology: establishment of Pteropus alecto bone marrow-derived dendritic cells and macrophages. Sci Rep 6:38597. doi:10.1038/srep38597