**Supplemental material**

**Title**

Tissue factor-coagulation factor VIIa PAR2-β-arrestin signaling in mice sustains organ failure in Coxsackievirus B3 infection

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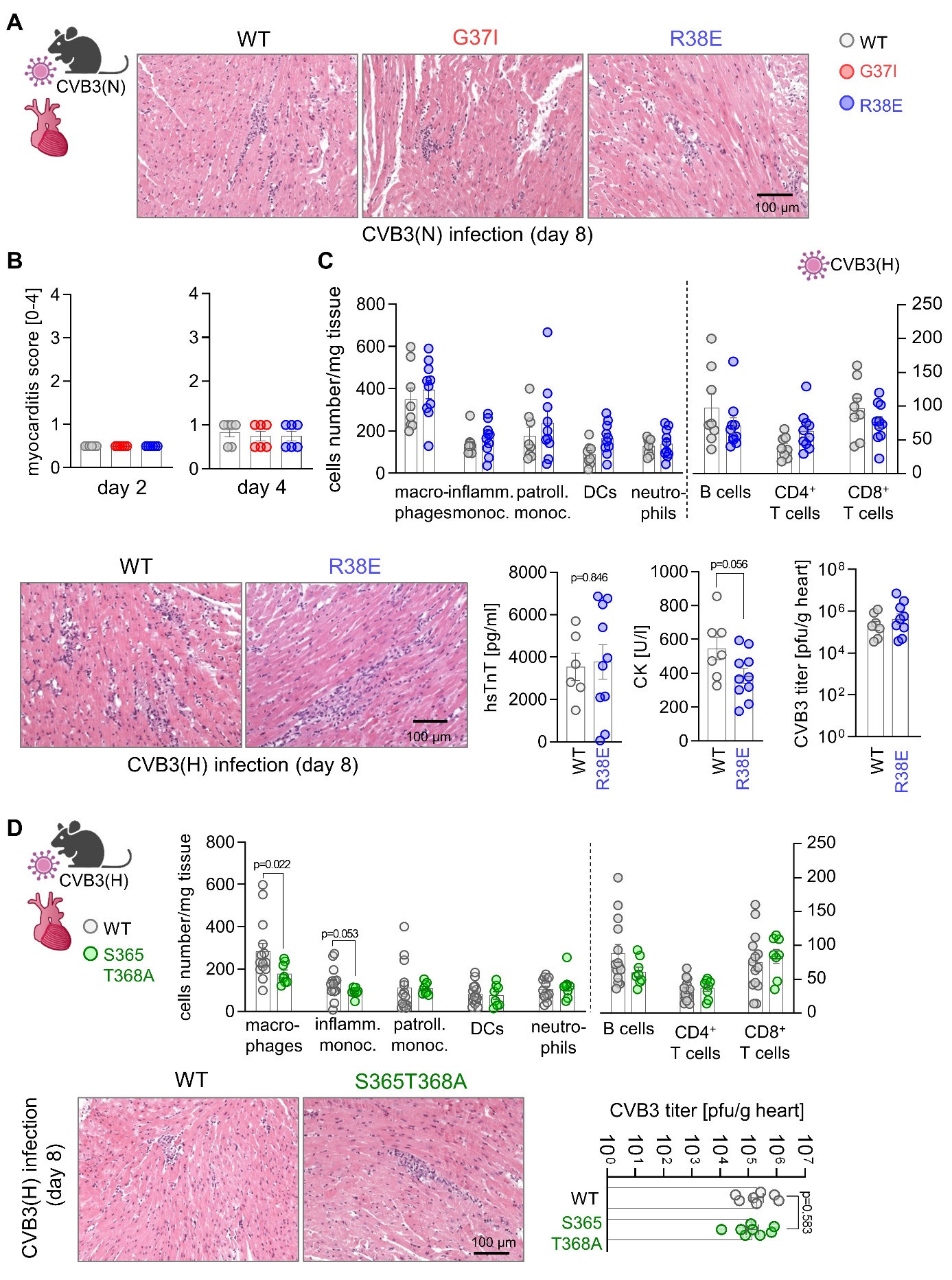
16 – Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Berlin, Germany

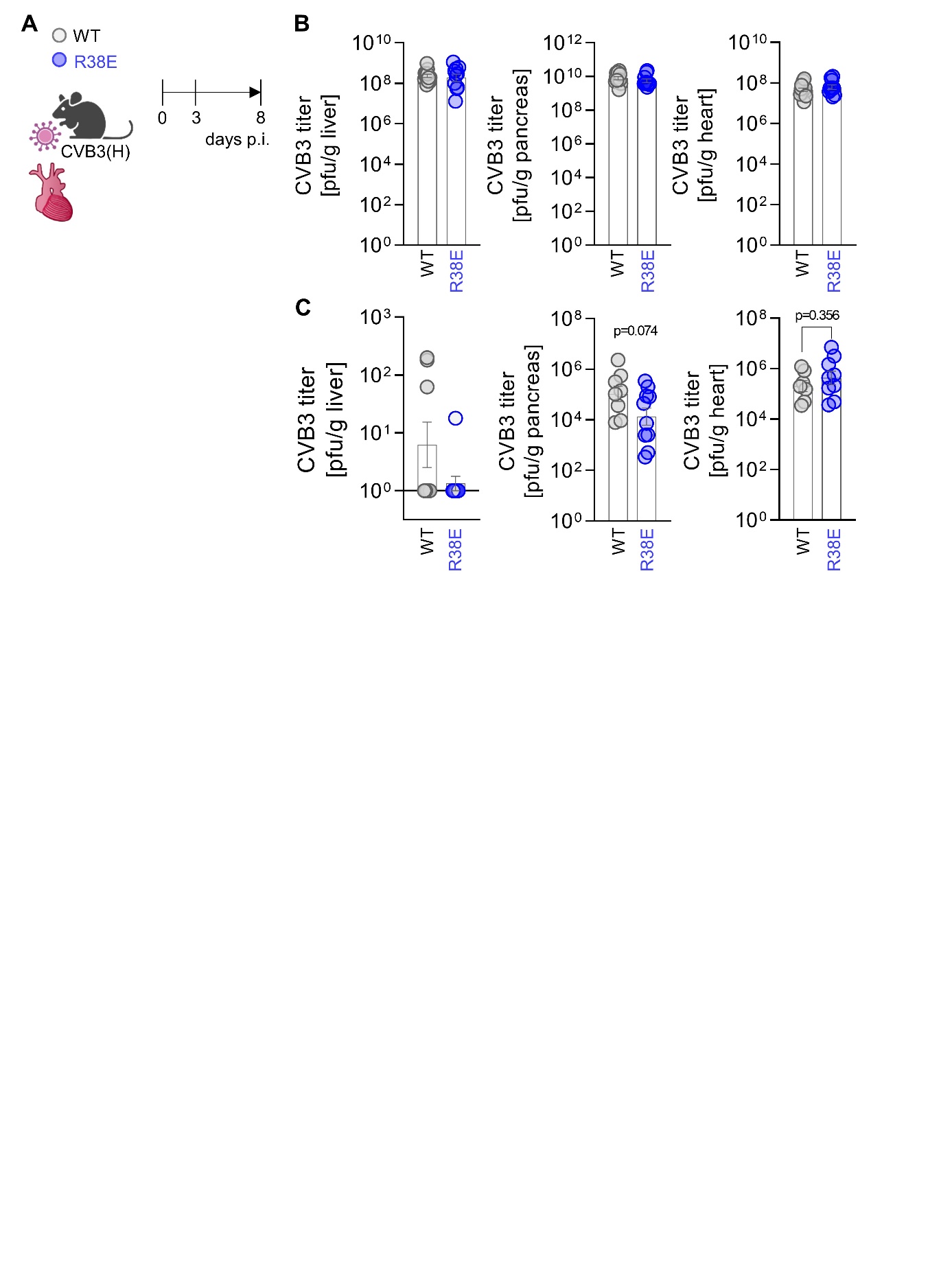
17 – University Medical Center Mainz, Institute for Pathology, Johannes-Gutenberg-Universität Mainz, Mainz, Germany

18 – University Medical Center Mainz, Research Center for Immunotherapy, Johannes-Gutenberg-Universität Mainz, Mainz, Germany

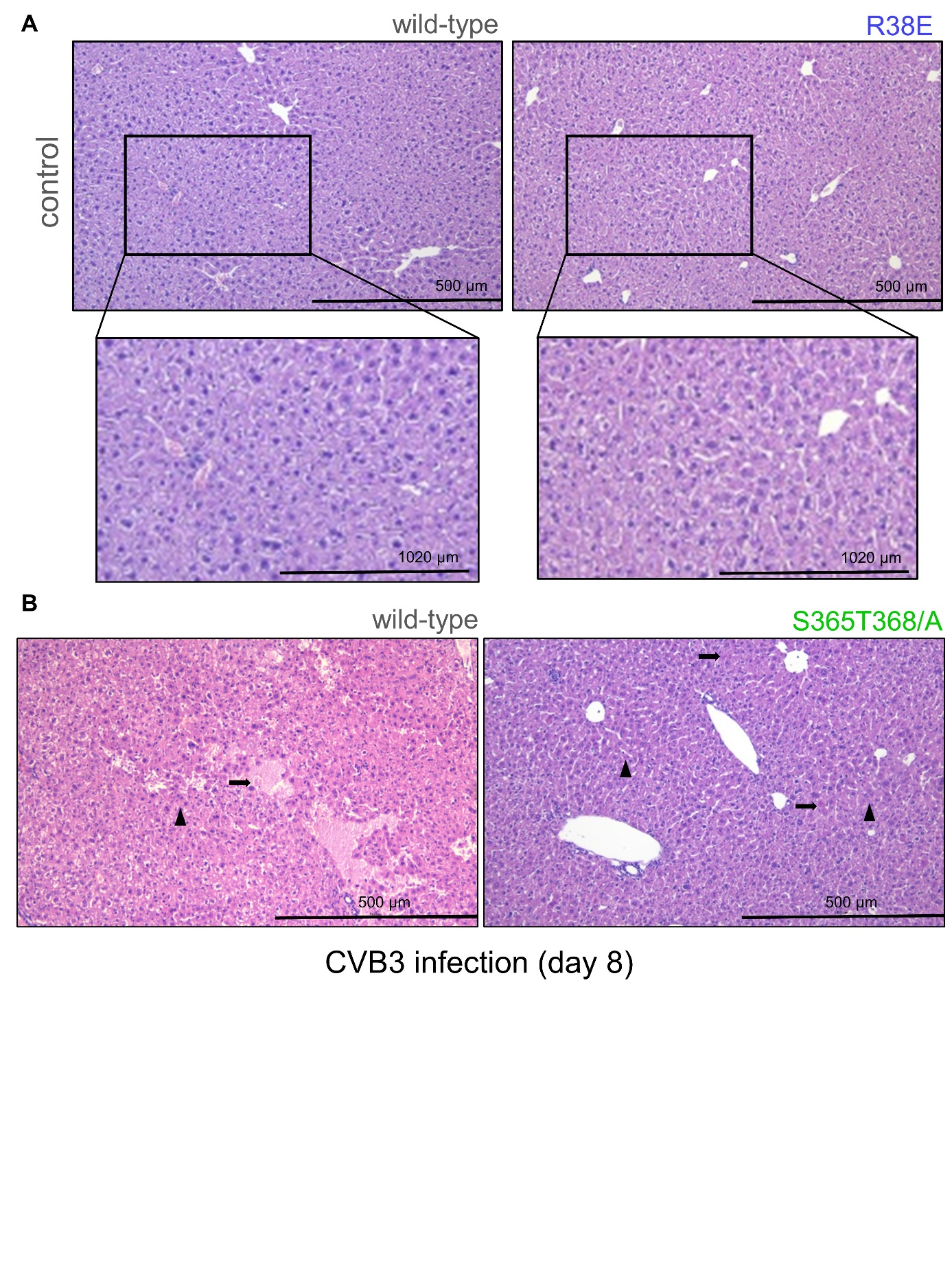
19 – Joint Unit Immunopathology, Institute of Pathology, University Medical Center, JGU-Mainz and TRON, Translational Oncology at the University Medical Center, Mainz, Germany

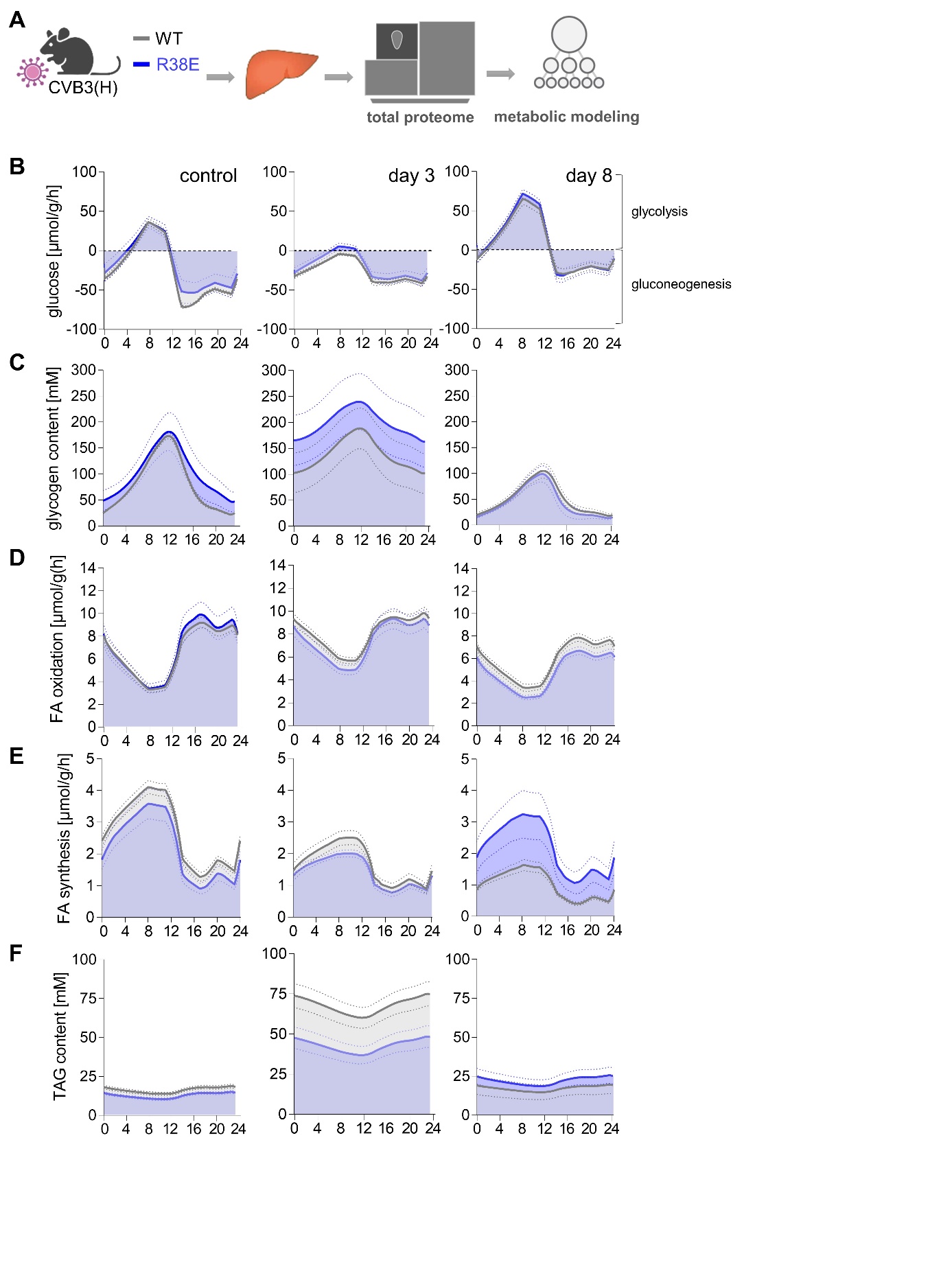
**Supplemental figures**

**Figure S1: The PAR2-β-arrestin signaling platform is without substantial relevance for virus-triggered inflammatory heart tissue injury. (A)** Wild-type (WT), PAR2-G37I (G37I) and PAR2-R38E (R38E) mice were infected with CVB3(N) and sacrificed after 8 days. Representative micrographs from heart tissue show mild myocarditis. **(B)** Wild-type (WT), PAR2-G37I (G37I) and PAR2-R38E (R38E) mice were infected with CVB3(N) and sacrificed after 2 and 4 days. Scoring of cardiac sections stained with hematoxylin/eosin. Each dot represents a different animal. In panel **(C)**, wild-type (WT) and PAR2-R38E (R38E) mice were infected with CVB3(H) and sacrificed after 8 days. Infiltrating myeloid and lymphoid immune cells in heart tissue quantified by flow cytometry (WT n=8, PAR2-R38E n=10). Representative micrographs (WT n=8, PAR2-R38E n=10) from heart tissue also show mild myocarditis. Serum high-sensitive troponin T (day 5) and creatinine kinase activity (day 8) (WT n=7, PAR2-R38E n=10). Infectious viral particles determined by plaque assay of heart tissue (WT n=7, PAR2-R38E n=9; day 8). **(D)** WT and PAR2-β-arrestin-coupling incompetent PAR2-S365T368/A mice were infected with CVB3(H) and sacrificed after 8 days. Representative micrographs from heart tissue showing mild myocarditis. Infiltrating myeloid and lymphoid immune cells in heart tissue quantified by flow cytometry after 8 days (WT n=14, PAR2-S365T368/A n=8). Infectious viral particles determined by plaque assay of heart tissue (WT n=7, PAR2-R38E n=8).

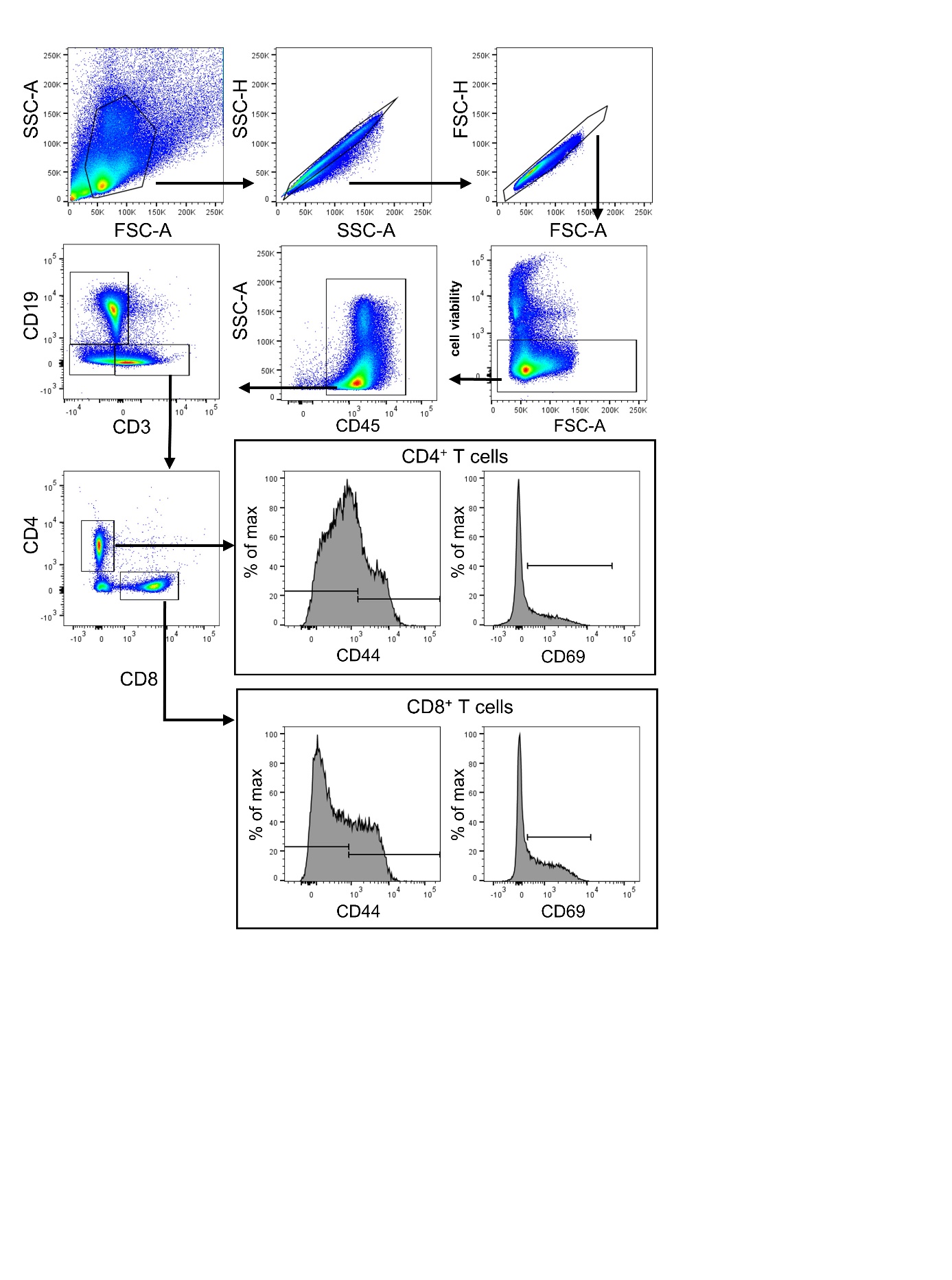
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**Figure S2:** **Virus concentrations are not affected by canonical PAR2 cleavage. (A)** Wild-type (WT) and PAR2-R38E (R38E) mice were infected with CVB3(H) and sacrificed after 3 (WT n=12, PAR2-R38E n=10) and 8 days (WT n=8, PAR2-R38E n=10). **(B/C)** Virus titers were determined using plaque assays. PAR2-R38E and WT mice did not statistically differ in terms of their CVB3 titers.

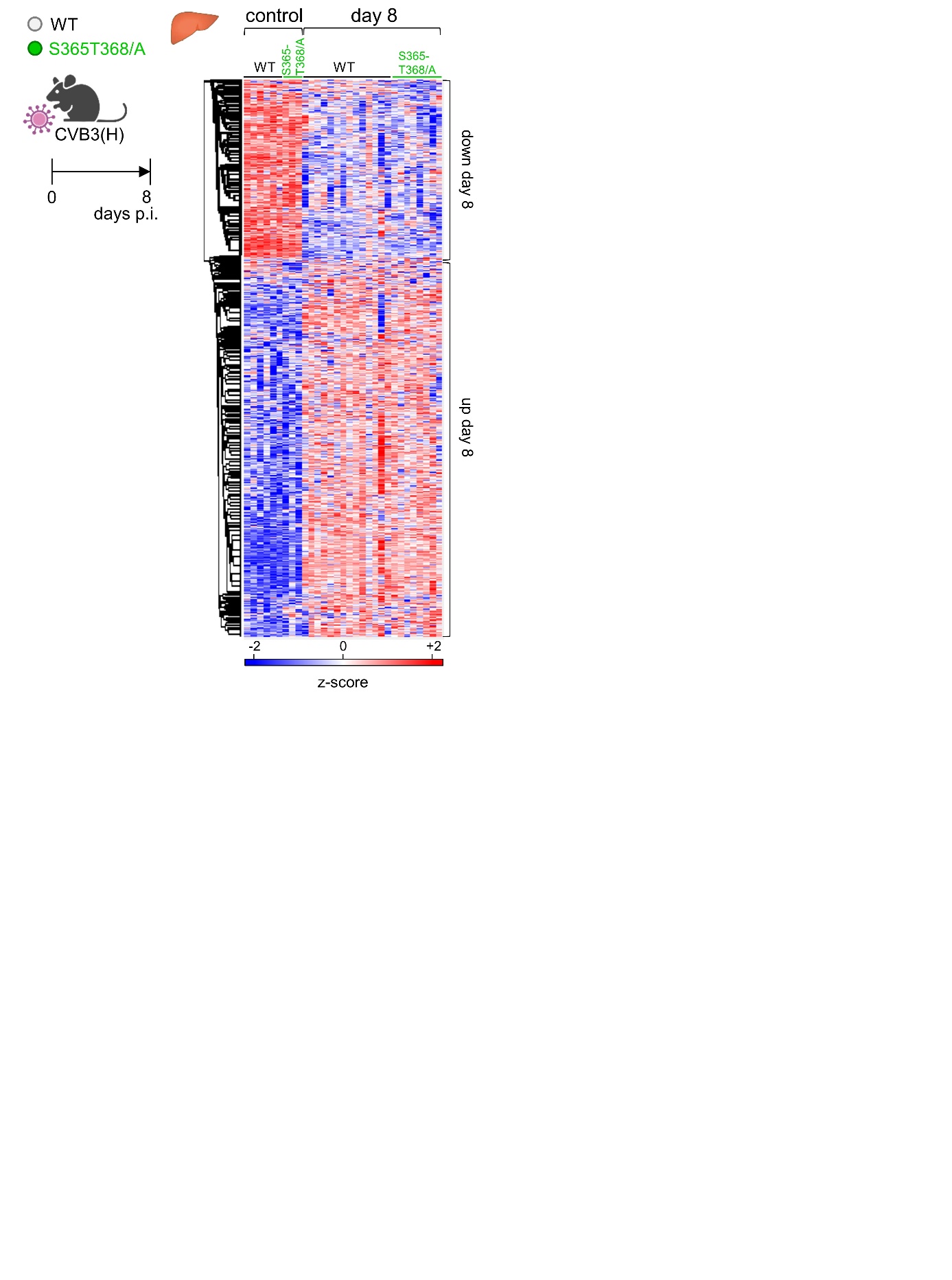
**Figure S3:** **Representative micrographs for liver histology. (A)** Uninfected wild-type (WT) and PAR2-R38E (R38E) mice were sacrificed and liver tissue samples were stained with hematoxylin and eosin. Liver pathology was categorized based on the severity of inflammation and necrosis using a scoring system adapted from Veteläinen *et al.*(Veteläinen et al, 2006). Based on this, all control mice were scored “0”. **(B)** WT and PAR2-β-arrestin-coupling incompetent PAR2-S365T368/A mice were infected with CVB3(H) and the mice were measured at day 8. Representative micrographs from liver tissue of WT mice and PAR2-S365T368/A mice (same image as shown in Figure 6H) stained with hematoxylin/eosin after eight days. Arrows indicate single cell necrosis and arrow heads mark intact hepatocytes.

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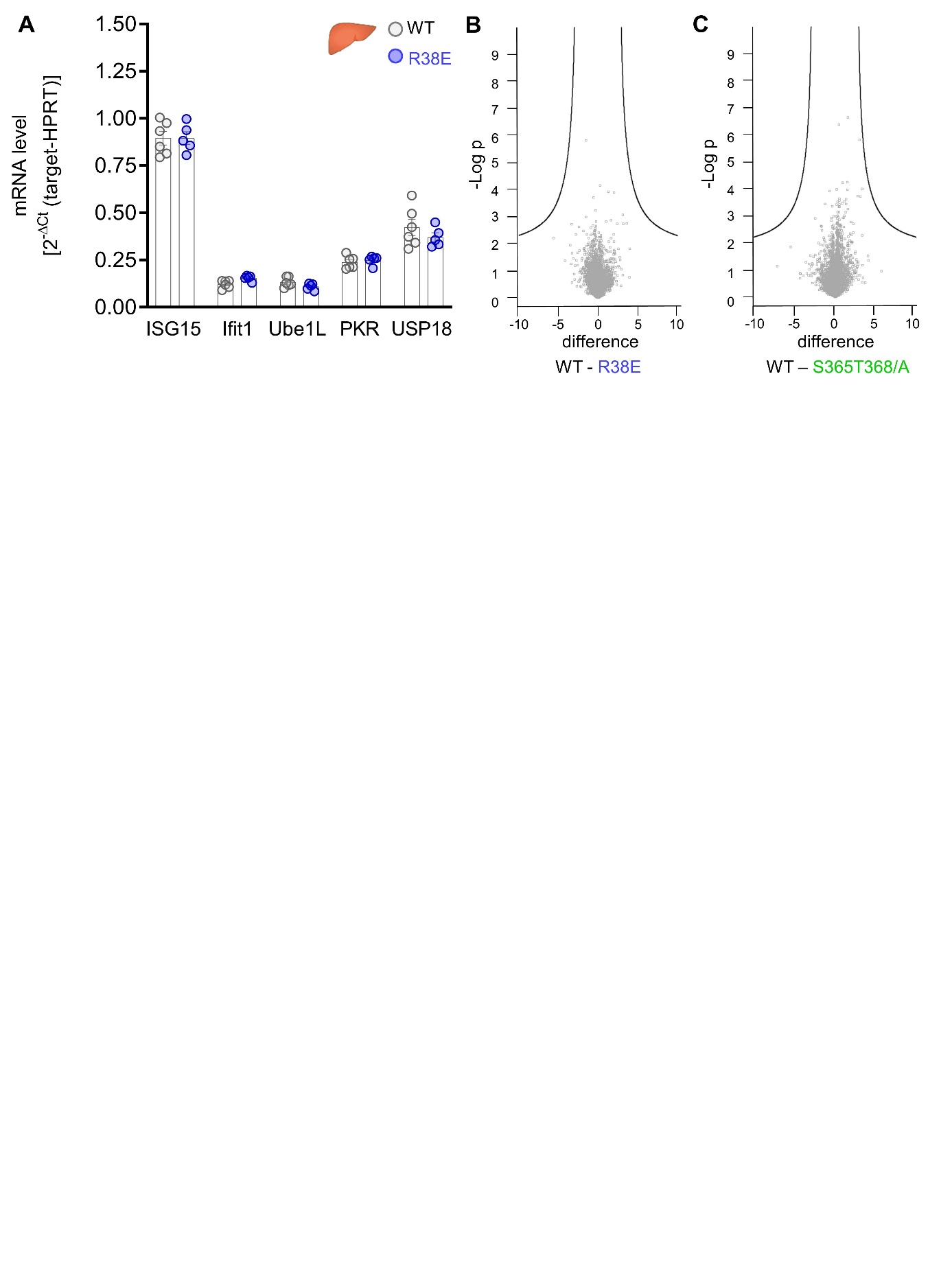
**Figure S4: Metabolic modelling of liver function. (A)** Liver proteome data integrated into the Hepatokin1 model to assess the metabolic alterations in liver tissue of WT and PAR2-R38E mice in CVB3(H) infection. Metabolic models for the different conditions were constructed by scaling the maximal activity for each enzyme using the LFQ intensities for each protein obtained from DIA-NN analysis at the respective point in time. For the indicated time points after infection, diurnal changes of selected metabolic functions/conditions (**B:** glucose consumption=glycolysis; glucose production=gluconeogenesis; **C:** glycogen content; **D:** fatty acid β-oxidation; **E:** fatty acid synthesis; **F:** triacylglycerol (TAG) content simulated for any time point of the 24-hour cycle using a standard diurnal metabolite plasma profile. Negative exchange fluxes, as partially shown for glucose, indicate net release from the liver to the plasma. Data are mean ± SEM (control: WT n=6, PAR2-R38E n=5; day 3: WT n=11, PAR2-R38E n=9; day 8: WT n=14, PAR2-R38E n=8).

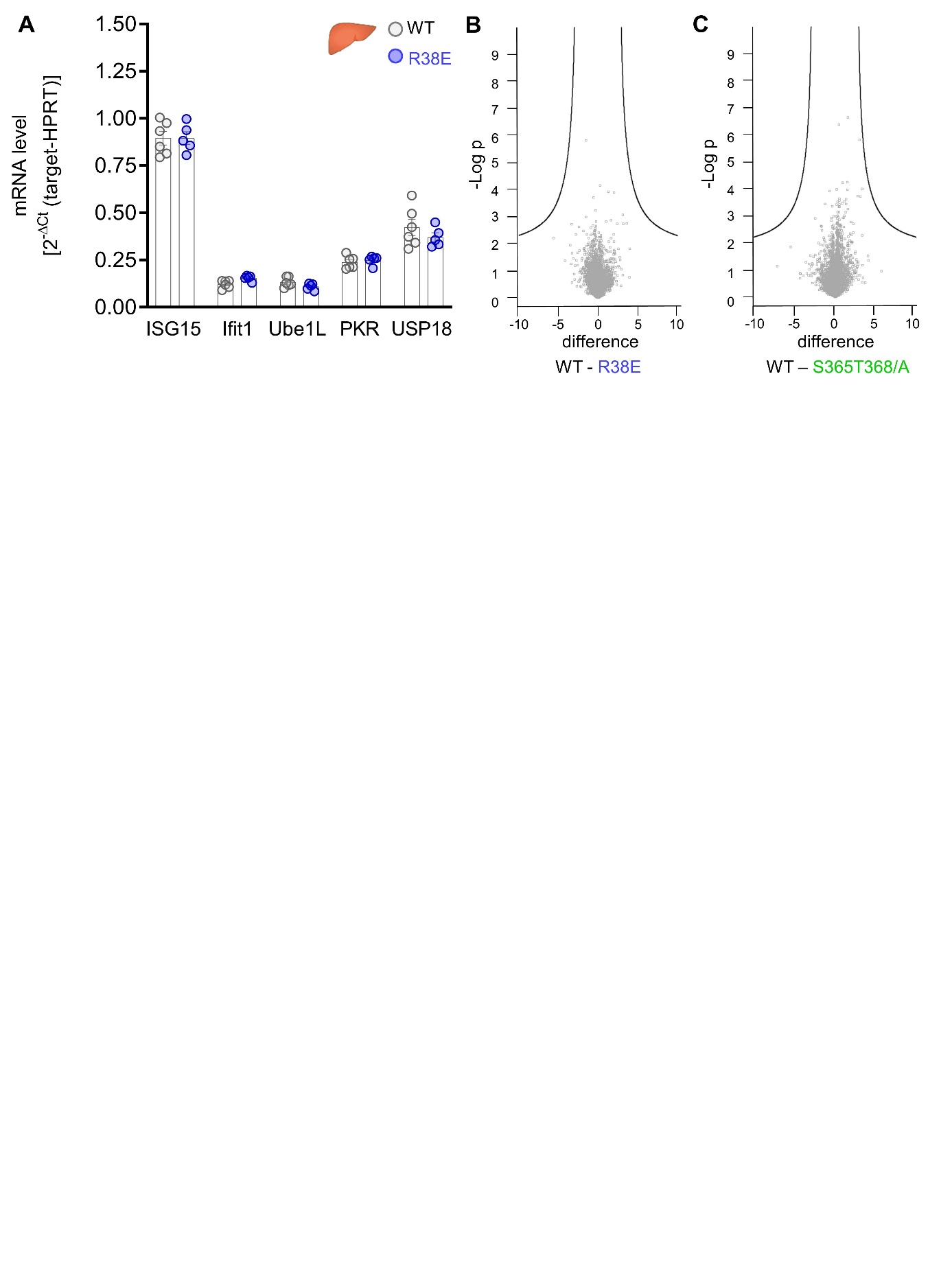
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**Figure S5: Gating strategy for infiltrating cells in heart tissue and for lymphoid cells in spleen.** For gating of infiltrating cells in infected heart tissue, please refer to Figure Supplemental S2 from Pinkert *et al.* [40](#_ENREF_40). For spleen, single cell suspensions of splenocytes were analyzed by flow cytometry. Doublets were discriminated by FSC and SSC and dead cells were excluded using a fixable cell viability dye. The following immune cell subsets were identified by surface staining of the indicated markers: CD4+ T cells: CD45+ CD19- CD3+ CD4+ CD8-. CD8+ T cells: CD45+ CD19- CD3+ CD8+ CD4- . Within the CD4+ and CD8+ T cell population, the activation status was determined by cell surface expression of CD44 and CD69.

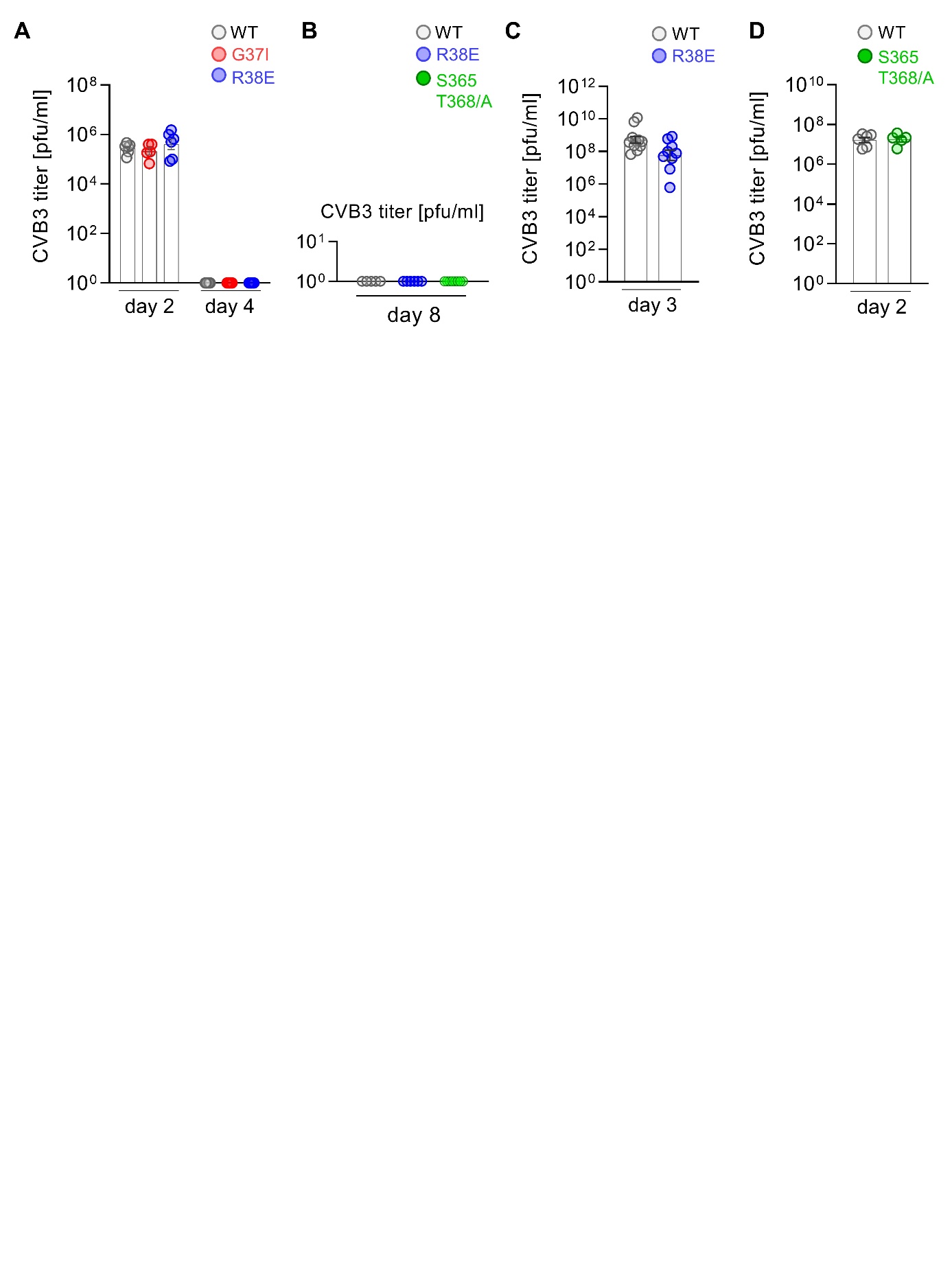
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**Figure S6:** **Liver proteome in PAR2-β-arrestin coupling incompetent mice.** WT and PAR2-β-arrestin-coupling incompetent PAR2-S365T368/A mice were infected with CVB3(H) and their liver proteomes were analyzed by shotgun liquid chromatography-tandem MS (LC-MS/MS) at the indicated time points (control: WT n=6, PAR2-S365T368/A n=3; day 8: WT n=14, PAR2-S365T368/A n=8). A heatmap displaying 2892 differentially regulated liver proteins is shown. Relative abundance is based on z-scored log2-transformed LFQ intensities. Red indicates proteins with a positive z-score, blue indicates proteins with a negative z-score, clearly clustering into up- and downregulated proteins eight days after infection.

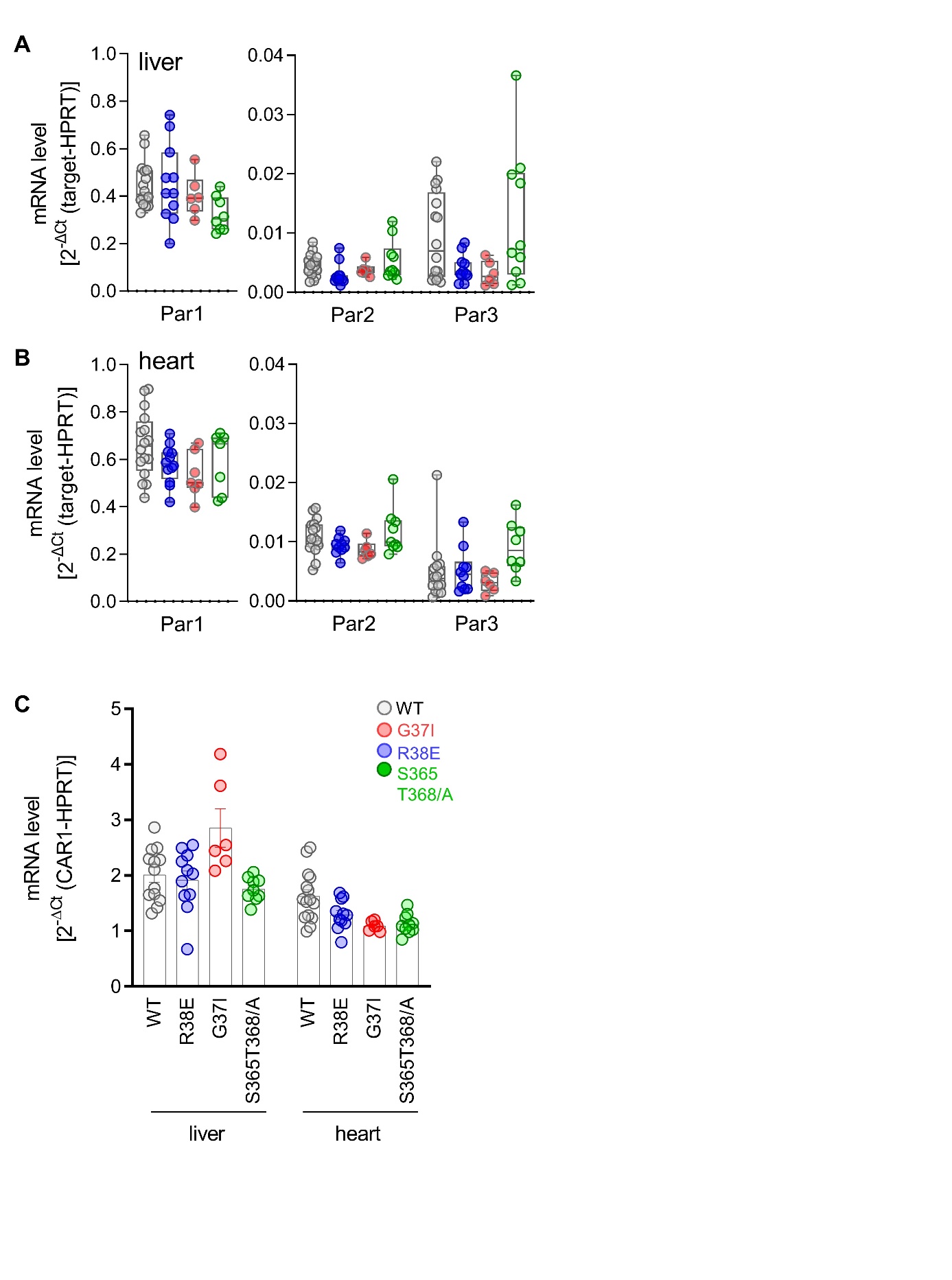
**Figure S7:** **Effect of NAPc2 on CVB3 infection. (A)** A/J mice were infected with 1x104 pfu of CVB3(N) and injected daily with NAPc2 (1 mg/kg body weight) or an equal volume of PBS (n=10 per group). **(B)** Infiltrating myeloid and lymphoid immune cells in heart tissue were quantified by flow cytometry after 8 days. **(C)** Viral genomes were quantified by TaqMan PCR analysis. **(D)** WT mice (Par2fl/fl mice on C57BL/6N background) infected with CVB3(H) and subjected to daily injections with NAPc2 (1 mg/kg body weight) initiated at the day of infection (n=9 PBS, n=8 NAPc2). Infiltrating myeloid and lymphoid immune cells in heart tissue were quantified by flow cytometry after 8 days. **(E)** Virus titers for liver, pancreas and heart were determined using plaque assays. PBS and NAPc2-treated WT mice did not statistically differ in terms of their CVB3 titers.

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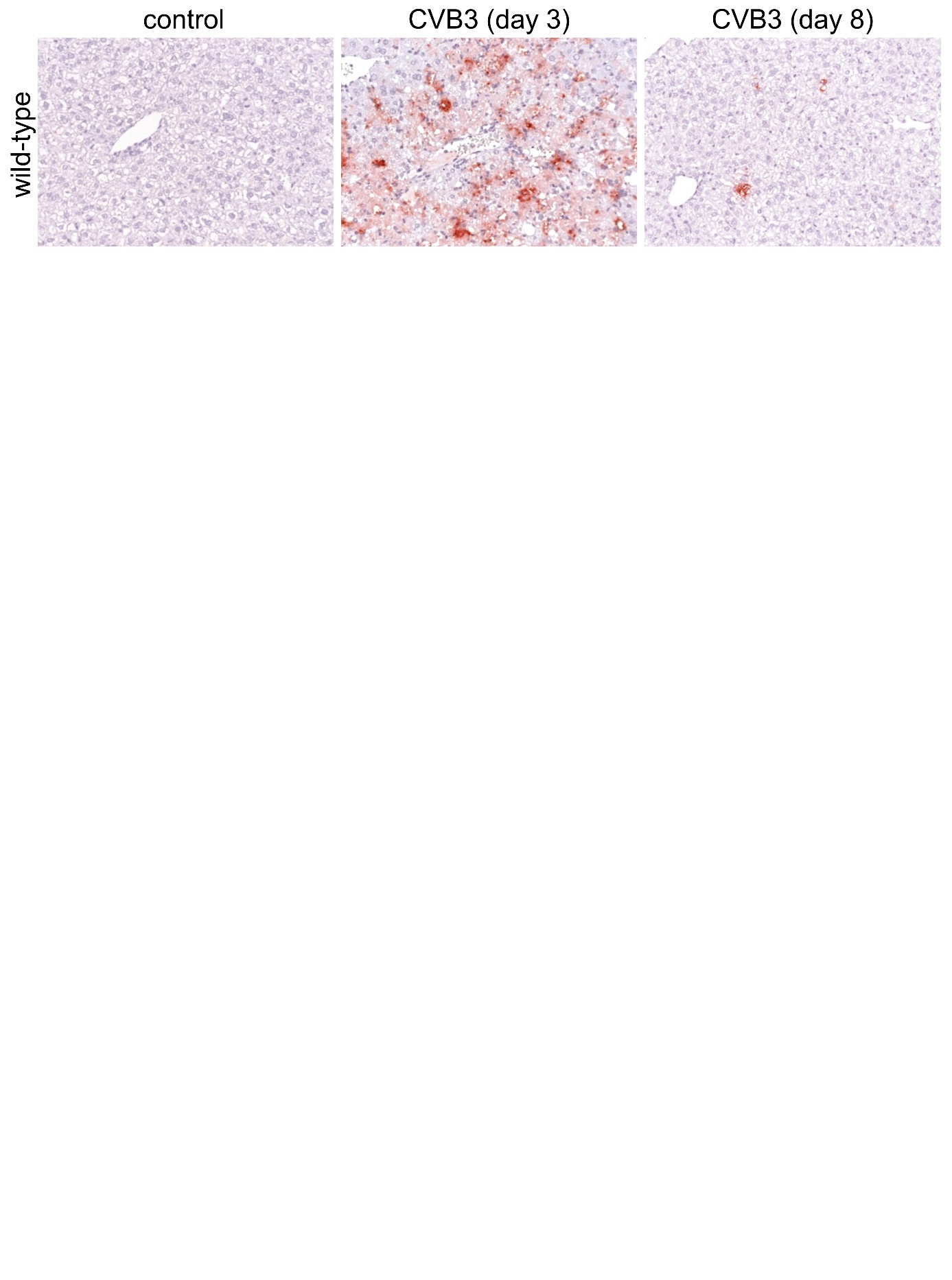
**Figure S8:** **IFN signatures in naive PAR2-R38E mice.** **(A)** RNA was extracted from liver tissue obtained from naive WT (n=6) and PAR2-R38E (n=5) and profiled for expression of IFN-related genes (ISG15: IFN induced gene of 15kDa; Ifit1: IFN induced protein with tetratricopeptide repeats 1; Ube1L: Ubiquitin-like modifier-activating enzyme 7; PKR: Protein kinase R; USP18: Ubiquitin specific peptidase 18) by quantitative PCR. **(B)((C)** Liver samples obtained from naive WT (n=6), PAR2-R38E mice (n=5) and PAR2-S365T368/A n=4 were subjected to LC-MS/MS analysis. In the depicted volcano plots, each protein identified in the proteome screen of liver tissue is represented by a dot. The x-axis specifies log2 fold changes and the y-axis specifies −log10 of P values obtained from t-test.

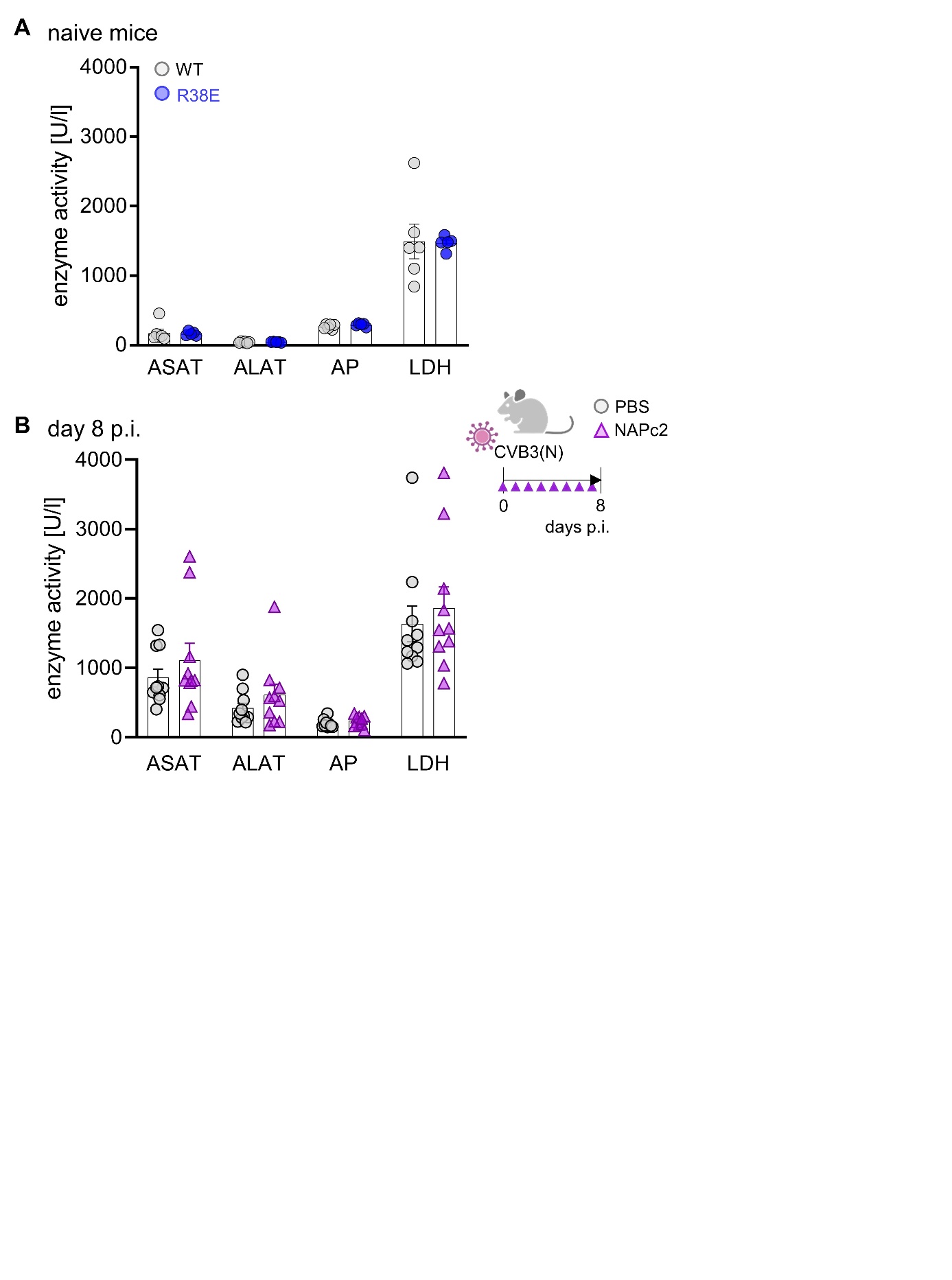
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**Figure S9: Virus concentrations in serum are not affected by canonical PAR2 cleavage. (A)** Wild-type (WT), PAR2-G37I (G37I) and PAR2-R38E (R38E) mice were infected with CVB3(N) and sacrificed after 2 and 4 days (n=6 per group). **(B)** Wild-type (WT, n=5), PAR2-R38E (R38E, n=6) and PAR2-S365T368/A (S365T368/A, n=8) mice were infected with CVB3(H) and sacrificed after 8 days. **(C)** Wild-type (WT, n=11) and PAR2-R38E mice (R38E, n=10) were infected with CVB3(H) and sacrificed after 3 days. **(D)** Wild-type (WT, n=6) and PAR2-S365T368/A (S365T368/A, n=5) mice were infected with CVB3(N) and sacrificed after 2 days. Virus titers in serum were determined using plaque assays.

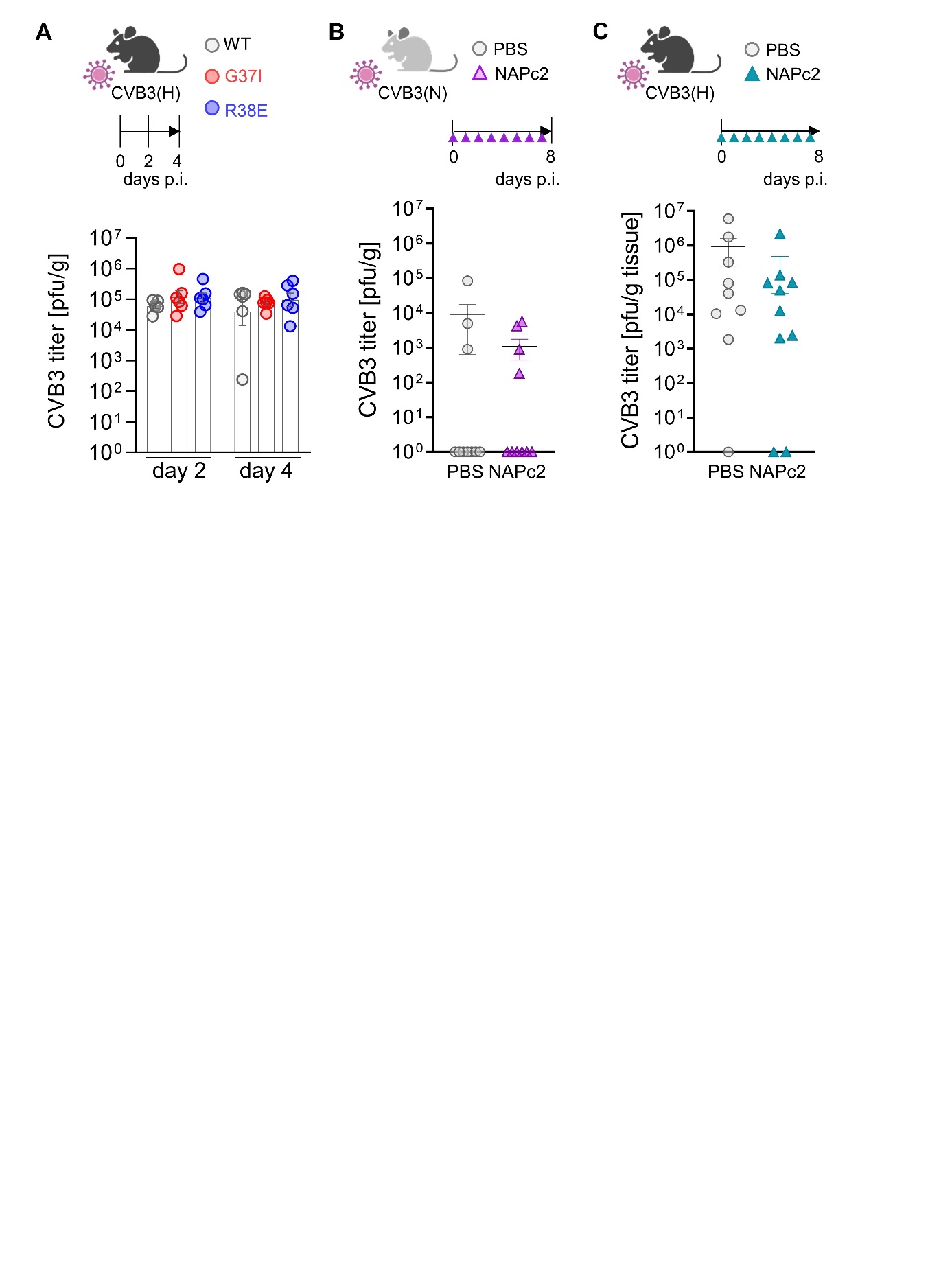
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**Figure S10: Quantification of PAR and CAR mRNA expression levels.** RNA was extracted from liver and heart tissue obtained from naive WT (n=15-16), PAR2-R38E (R38E, n=10-11), PAR2-G37I (G37I, n=6) and PAR2-S365T368/A (S365T368/A, n=8-10) mice profiled for expression of Par1, Par2 and Par3 **(A/B)** and Coxsackievirus-Adenovirus-Receptor 1 (CAR1) **(C)** by quantitative PCR.

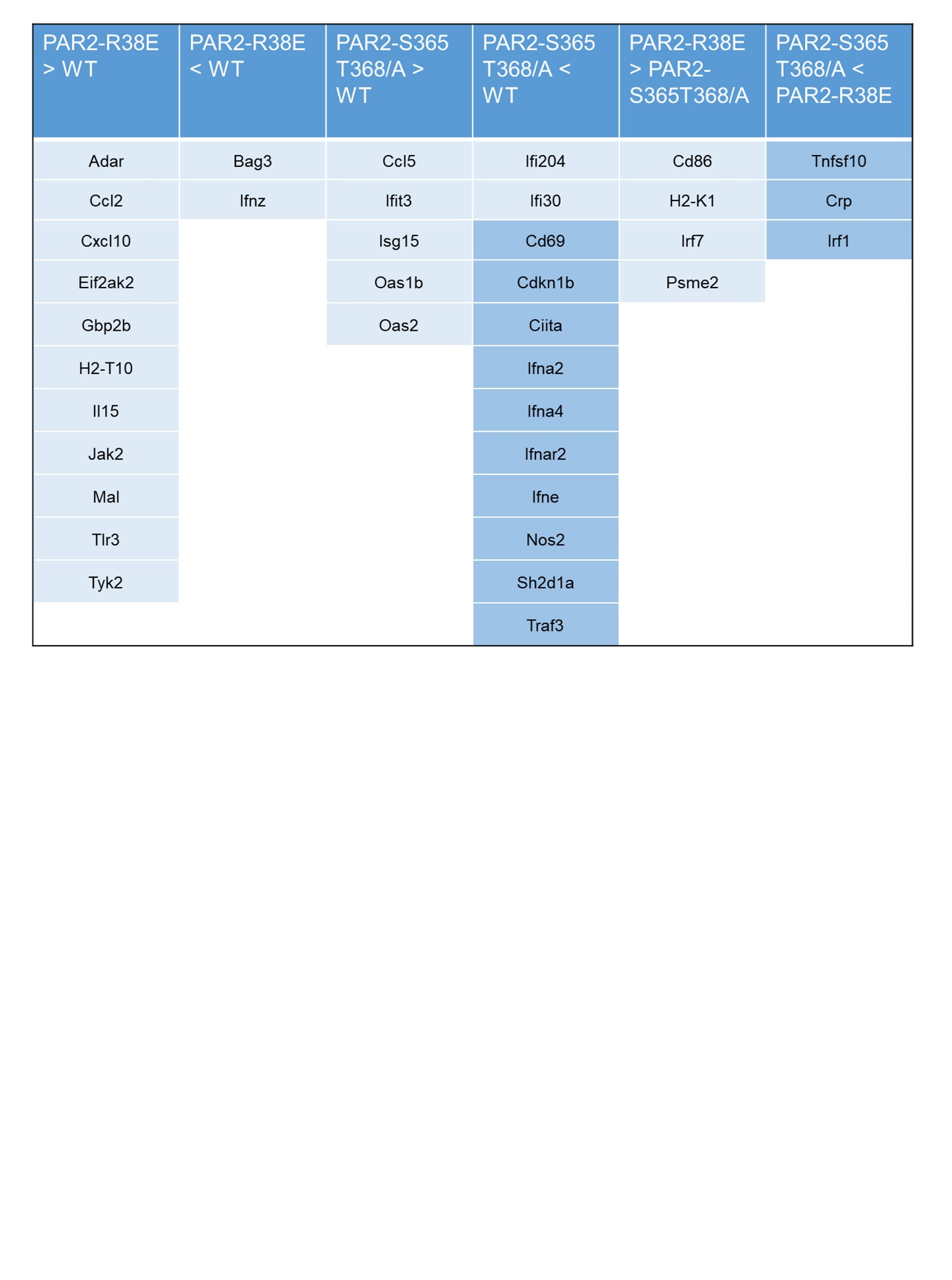
**Figure S11: Detection of CVB3 genome on liver tissue sections.** CVB3 genome visualized by RNA *in situ* hybridization on liver tissue sections. Representative micrographs for uninfected mice (control) and mice infected with CVB3(H), sacrificed after 3 and 8 days. To detect CVB3 RNA, liver tissue sections were hybridized using specific probes for CVB3 (ACD, Newark, CA, USA) followed by the RNAscope 2.5 HD Detection Kit Red from ACD (Newark, CA, USA) according to the manufacturer’s protocol.

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**Figure S12: Quantification of ASAT, ALAT, AP and LDH activities.** (A) Serum activity for ASAT, ALAT, AP and LDH (control: WT n=6, PAR2-R38E n=5). (B) A/J mice infected with 1x104 pfu of CVB3 (N), daily injections with NAPc2 (1 mg/kg body weight) starting at the day of infection. Serum activity for ASAT, ALAT, AP and LDH at day 3 (vehicle: n=10; Napc2: n=10).

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**Figure S13: Quantification of infectious viral particles in pancreas. (A)** Wild-type (WT), PAR2-G37I (G37I) and PAR2-R38E (R38E) mice were infected with CVB3(N). Mice were sacrificed after 2 and 4 days (WT n=6, PAR2-R38E: n=6). **(B)** A/J mice infected with 1x104 pfu of CVB3 (N), daily injections with NAPc2 (1 mg/kg body weight) starting at the day of infection. Mice were sacrificed after 8 days (n=10 per group). **(C)** WT (Par2fl/fl on C57BL/6N background) mice infected with CVB3(H), daily injections with NAPc2 (1 mg/kg body weight) starting at the day of infection. Mice were sacrificed after 8 days (PBS n=9; NAPc2: n=10). **(A-C)** The number of infectious viral particles determined by plaque assay for pancreatic tissue at the indicated time points.

**Figure S14: Comparison of IFN signatures in liver tissue from PAR2-R38E and PAR2-S365T368/A mice during CVB3 (N) infection.** RNA was extracted from liver tissue obtained from infected WT and PAR2-R38E mice (CV(N), day 2, n=6 per group) as well as from a separate cohort of infected WT and PAR2-S365T368/A mice (CV(N), day 2, WT: n=6, PAR2-S365T368/A: n=5). Liver RNA from uninfected mice was used as controls. The expression of IFN-related genes was profiled using a mouse T1IFN response profiler PCR array. Differential expression analysis revealed relative alterations of mRNA expression levels according to the 2-ΔΔCt method. Unpaired *t*-tests were used for two group comparisons of normally distributed data. Otherwise, Mann-Whitney-U tests for applied. The first two columns depict differentially regulated genes between PAR2-R38E mice and WT mice, with upregulated genes being shaded in light blue. 1st column: higher expression level in PAR2-R38E mice; 2nd column: lower expression level in PAR2-R38E mice. The third and fourth columns list genes with differential regulation between PAR2-S365T368/A mice and WT mice, with upregulated genes shaded in light blue or downregulated genes in PAR2-S365T368/A mice shaded in dark blue. 3rd column: higher expression level in PAR2-S365T368/A mice. 4th column: lower expression level in PAR2-S365T368/A mice. Genes with differential mRNA expression between PAR2-R38E and PAR2-S365T368/A liver tissue are shown in the fifth and sixth column. Upregulated genes (both mutant strains) shaded in light blue and downregulated genes (PAR2-S365T368/A mice) shaded in dark blue. 5th column: higher expression level in PAR2-R38E in comparison to PAR2-S365T368/A mice. 6th column: lower expression level in PAR2-S365T368/A mice in comparison to PAR2-R38E mice.

**Supplemental table legends**

**Table S1:**

Results of shotgun proteome analysis by LC-MS/MS of CVB3(H)-infected liver tissue. LC-MS/MS based proteome analysis of CVB3-infected liver tissue hearts of wild-type (WT) and PAR2-R38E mice. Control: WT n=6, R38E n=5; day 3: WT n=11, R38E n=9; day 8: WT n=14, R38E n=8). List of significantly regulated proteins as depicted in the heatmap shown in Figure 4B.

**Table S2:**

Gene Ontology enrichment analysis of shotgun proteome heatmap clusters in WT and R38E mice at day 3 and 8. Proteins of heatmap-based cluster “downregulated in CVB3 infection at day 3”, “downregulated in CVB3 infection at day 8”, “upregulated in CVB3 infection at day 3”, “upregulated in CVB3 infection at day 8” and cluster “upregulated in CVB3 infection at day 3 and 8” of shotgun proteome analysis were analyzed by Gene Ontology enrichment analysis with Fisher’s exact test and FDR correction.

**Table S3:**

Results of shotgun proteome analysis by LC-MS/MS of CVB3(H)-infected liver tissue. LC-MS/MS based proteome analysis of CVB3-infected liver tissue hearts of wild-type (WT) and PAR2 S365T368/A mice. List of significantly regulated proteins as depicted in the heatmap shown in Supplemental Figure 5.

**Table S4:**

Gene Ontology enrichment analysis of shotgun proteome heatmap clusters in WT and PAR2 S365T368/A mice at day 8. Proteins of heatmap-based cluster “downregulated in CVB3 infection at day 8” and cluster “upregulated in CVB3 infection at day 8” of shotgun proteome analysis were analyzed by Gene Ontology enrichment analysis with Fisher’s exact test and FDR correction.

**Major Resources Tables**

**Animals (in vivo studies)**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Species** | **Vendor or Source** | **Background Strain** | **Sex** | **Persistent ID / URL** |
| Mouse | Envigo | A/J | Male | [A inbred mice: A/JOlaHsd (inotivco.com)](https://www.inotivco.com/model/a-jolahsd) |

**Genetically Modified Animals**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **Species** | **Vendor or Source** | **Background Strain** | **Other Information** | **Persistent ID / URL** |
| **Parent – Male & Female** | Mouse | Johannes Gutenberg University Medical Center Mainz | PAR2fl/fl C57BL/6N |  | PMID: 31541031 |
| **Parent – Male & Female** | Mouse | Johannes Gutenberg University Medical Center Mainz | PAR2R38E  C57BL/6N |  | PMID: 31541031 |
| **Parent – Male & Female** | Mouse | Johannes Gutenberg University Medical Center Mainz | PAR2G37I  C57BL/6N |  | PMID: 31541031 |
| **Parent – Male &Female** | Mouse | Johannes Gutenberg University Medical Center Mainz | PAR2S365T368/A  C57BL/6N |  | PMID: 38096370 |

**Antibodies**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Target antigen** | **Vendor or Source** | **Catalog #** | **Working concentration** | **Lot # (preferred but not required)** | **Persistent ID / URL** |
| ADFP | Thermo Fisher Scientific, Waltham, MA, USA | PA5-79830 | 1:500 | --- | n.a. |
| CD8-PB (clone 53-6.7) | BD Biosciences | 558106 | 1:100 | --- | n.a. |
| MHCII I-A[b]-FITC (clone AF6-120.1) | BD Biosciences | 553551 | 1:150 | --- | n.a. |
| CD3-BUV737 (clone 145-2C11) | BD Biosciences | 612771 | 1:200 | --- | n.a. |
| B220-BUV395 (clone RA3-6B2) | BD Biosciences | 563793 | 1:200 | ---- | n.a. |
| NK1.1.-PE (clone Pk136) | Biolegend | 108707 | 1:300 | --- | n.a. |
| F4/80-APC (clone BM8) | Biolegend | 123115 | 1:100 | --- | n.a. |
| CD11b-BV510 (clone M1/70) | Biolegend | 101245 | 1:300 | --- | n.a. |
| Ly6G-BV605 (clone 1A8) | Biolegend | 127639 | 1:400 | --- | n.a. |
| CD11c-PE/Dazzle (clone N418) | Biolegend | 117347 | 1:200 | --- | n.a. |
| Ly6C-PE-Cy7 (clone HK1.4) | Biolegend | 128018 | 1:200 | --- | n.a. |
| CD4-PerCP-Cy5.5 (clone RM4-5) | Biolegend | 100539 | 1:300 | --- | n.a. |
| CD45.2-BV711 (clone 104) | Biolegend | 563685 | 1:200 | --- | n.a. |
| CD49b-PE (clone DX5) | Biolegend | 108907 | 1:300 | --- | n.a. |
| MHCII I-A[k] (clone 10-3.6) | Biolegend | 109905 | 1:150 | --- | n.a. |
| CD4-V500 (clone RM4-5) | BD Biosciences | 560783 | 1:100 | --- | n.a. |
| CD69-APC (clone H1.2F3) | Biolegend | 104513 | 1:300 | --- | n.a. |
| CD44-BV650 (clone IM7) | Biolegend | 103049 | 1:350 | --- | n.a. |

**DNA/cDNA Clones**

|  |  |  |  |
| --- | --- | --- | --- |
| **Clone Name** | **Sequence** | **Source / Repository** | **Persistent ID / URL** |
| HPRT fw primer | 5´-ATC ATT ATG CCG AGG ATT TGG AA-3´ | Eurofins NSC Finance Germany GmbH, Hamburg, Germany | n.a. |
| HPRT rev primer | 5’-TTG AGC ACA CAG AGG GCC A-3’ | Eurofins NSC Finance Germany GmbH, Hamburg, Germany | n.a. |
| HPRT probe | 5’FAM- TGG ACA GGA CTG AAA GAC TTG CTC GAG ATG -3’TAMRA | Eurofins NSC Finance Germany GmbH, Hamburg, Germany | n.a. |
| CVB3 fw primer | 5’-CCC TGA ATG CGG CTA ATC C-3’ | Eurofins NSC Finance Germany GmbH, Hamburg, Germany | n.a. |
| CVB3 rev primer | 5’-ATT GTC ACC ATA AGC AGC CA-3’ | Eurofins NSC Finance Germany GmbH, Hamburg, Germany | n.a. |
| CVB3 probe | 5’-FAM-TGC AGC GGA ACC G -MGB3’ | Eurofins NSC Finance Germany GmbH, Hamburg, Germany | n.a. |
| pBKCMV-H3 |  | Andreas Henke, Institute of Virology and Antiviral Therapy, Jena University Hospital, Friedrich Schiller University, Jena, Germany | n.a. |

**Cultured Cells**

|  |  |  |  |
| --- | --- | --- | --- |
| **Name** | **Vendor or Source** | **Sex (F, M, or unknown)** | **Persistent ID / URL** |
| Green monkey kidney cells | Andreas Henke, Institute of Virology and Antiviral Therapy, Jena University Hospital, Friedrich Schiller University, Jena, Germany |  |  |
| HeLa cells | ATCC |  |  |

**Data & Code Availability**

|  |  |  |
| --- | --- | --- |
| **Description** | **Source / Repository** | **Persistent ID / URL** |
| Mass spectrometry proteomics data | ProteomeXchange Consortium / PRIDEpartner repository | PXD042272 |
| Limma (used for QuantiNova LNA PCR Focus Panel analysis) | PMID: 25605792 |  |
| Hepatokin1 (model of central liver metabolism) | PMID: 29921957 |  |

**Other**

|  |  |  |
| --- | --- | --- |
| **Description** | **Source / Repository** | **Persistent ID / URL** |
| Fixable viability dye eFluor 780 | Invitrogen, Thermo Fisher Scientific | Product number:65-0865-14 |
| 123count eBeads | Invitrogen | Product number: 01-1234-42 |
| QuantiNova LNA PCR Focus Panel | Qiagen, Venlo, NL | Product number: SBMM-016Z |
| Bead‐based Bio‐Plex Pro Mouse Cytokine Assay and Chemokine Assay | Bio‐Rad, Hercules, CA, USA | --- |

**ARRIVE GUIDELINES**

The ARRIVE guidelines (<https://arriveguidelines.org/>) are a checklist of recommendations to improve the reporting of research involving animals. Key elements of the study design should be included below to better enable readers to scrutinize the research adequately, evaluate its methodological rigor, and reproduce the methods or findings.

**Study Design**

We used male mice for all experiments. Male mice aged 4-6 weeks (C57BL/6 background) or 7-8 weeks (A/J background). In each experiment, the sample size is provided in the figure legend. The breeding scheme is described below:

In C57BL/6(N) mice, PAR2 was rendered cleavage-insensitive to all proteases (R38E) or FXa selectively (G37I) by knock-in mouse technology that introduced site-specific mutations[36](#_ENREF_36),[37](#_ENREF_37). PAR2-β-arrestin coupling-deficient mice are based on findings shown by DeFea *et al.*[38](#_ENREF_38) and the generation is shown here [39](#_ENREF_39). Protein kinase C (PKC) phosphorylation sites S365 and T368 in the PAR2 carboxyl-terminal intracellular domain were mutated to A (PAR2 S365T368/A). All mouse strains were generated with ES cells or oocytes from C57BL/6N mice and experiments were performed on this background. PAR2 R38E and PAR2 G37I mice were generated with the same targeting strategy as PAR2fl/fl mice, which were used as WT controls. All mutant strains were transferred from Johannes Gutenberg University Medical Center Mainz to the animal facilities of the Charité-Universitätsmedizin Berlin for further breeding. For NAPc2 studies, in addition to WT controls (PAR2fl/fl on C57BL/6(N)), male A/J mice (7-8 weeks) were purchased from Envigo and infected with 1x104 pfu of CVB3(N).

Clinical inspection was performed at least daily and mice were scored for these parameters: body weight, activity, behavioral changes, movement. A drop of the body weight >25% resulted in sacrifice of mouse. On the final day of each infection study, after echocardiography, organs were collected for further analysis and immediately frozen in liquid nitrogen. Sample collection took place between 8am-11am.

**Sample Size:** Explorative data analysis, a *prior* sample size calculation was not performed.

**Inclusion Criteria**

Male mice aged 4-6 weeks (C57BL/6 background) or 7-8 weeks (A/J background).

**Exclusion Criteria**

We visualized cellular injury and inflammation on cross sections of the pancreas that were stained with hematoxylin and eosin. CVB3 infection results in acute virus- and inflammatory tissue destruction of the pancreas. Animals of a lack of this respective injury were classified as non-responder to CVB3 injection and considered as a dropout without further analysis in this study (<10 %).

**Randomization**

A/J mice were distributed randomly for PBS and Napc2 treatment.

**Blinding**

For histology and echocardiography analysis, specimen were blinded for the evaluator.