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Supplementary Materials for

A hexavalent Coxsackievirus B vaccine is highly immunogenic and has a strong protective capacity in mice and nonhuman primates

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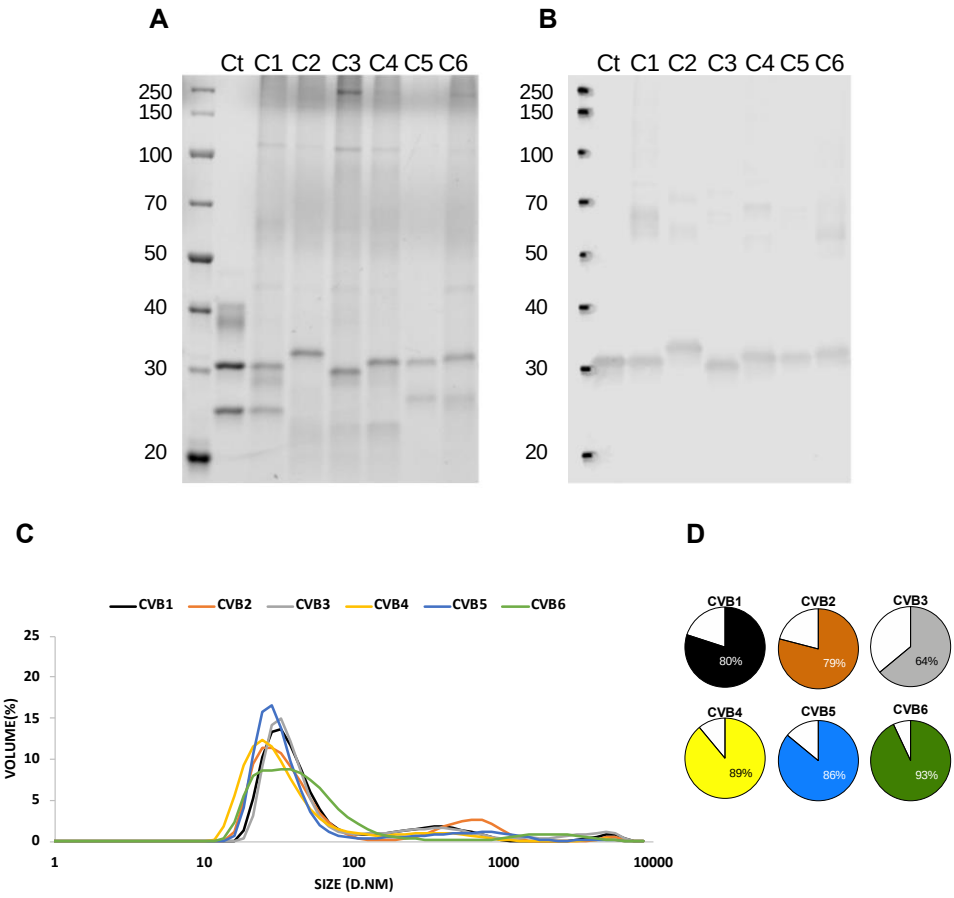
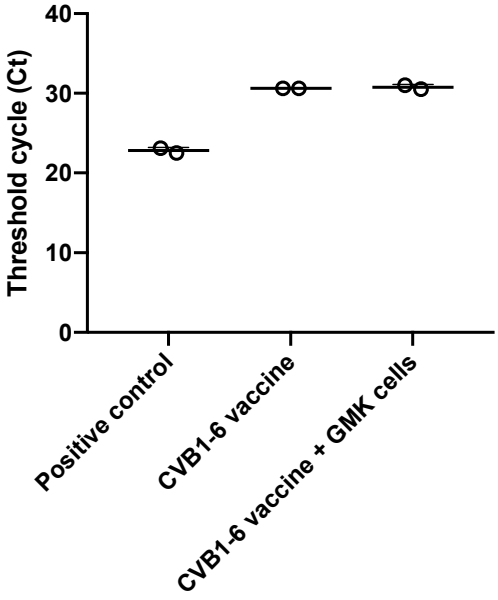


Supplementary Fig. 1. Characterisation of inactivated CVB1-6 viruses. CVB1-6 viruses were propagated in Vero cells, purified, inactivated with formalin, stored at -80°C for 27 months and then characterised as described in Materials and Methods. (**A**) Analysis of CVB1-6 total protein and virus protein content (2 µg virus/well) by SDS-PAGE followed by (**B**) Western blot analysis using an in-house produced rat monoclonal antibody 3A6, that binds to the CVB1-6 viral capsid protein VP1. Ct represents a control sample of 2 µg purified CVB1-VLP (virus like particle). (**C**) Dynamic Light Scattering (DLS) analysis of the inactivated CVB1-6 serotypes. (**D**) The volume distribution (percentages) of the most prominent particle populations in the vaccine preparations as measured by DLS analysis. C1, CVB1; C2, CVB2; C3, CVB3; C4, CVB4; C5, CVB5; C6, CVB6.

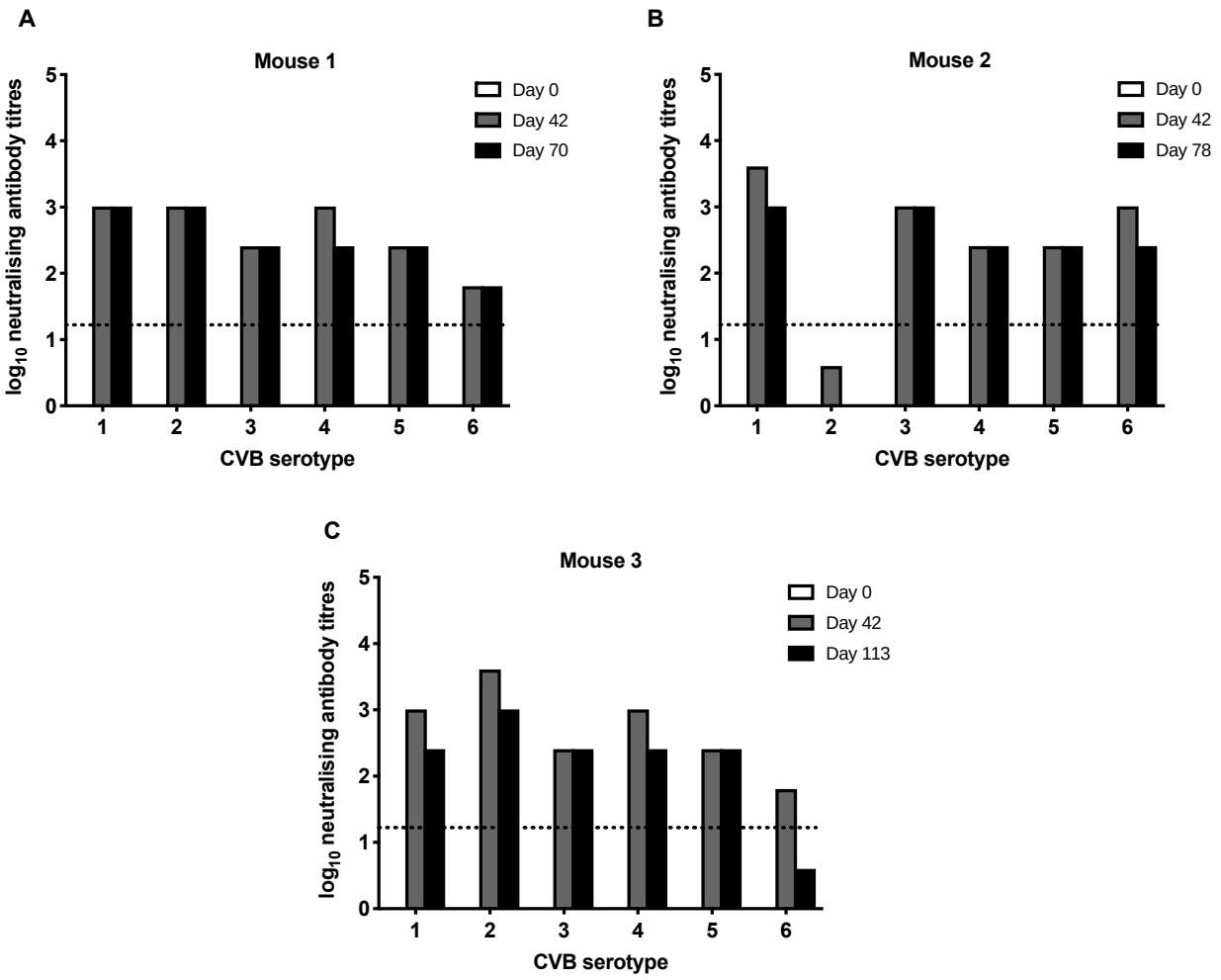
A



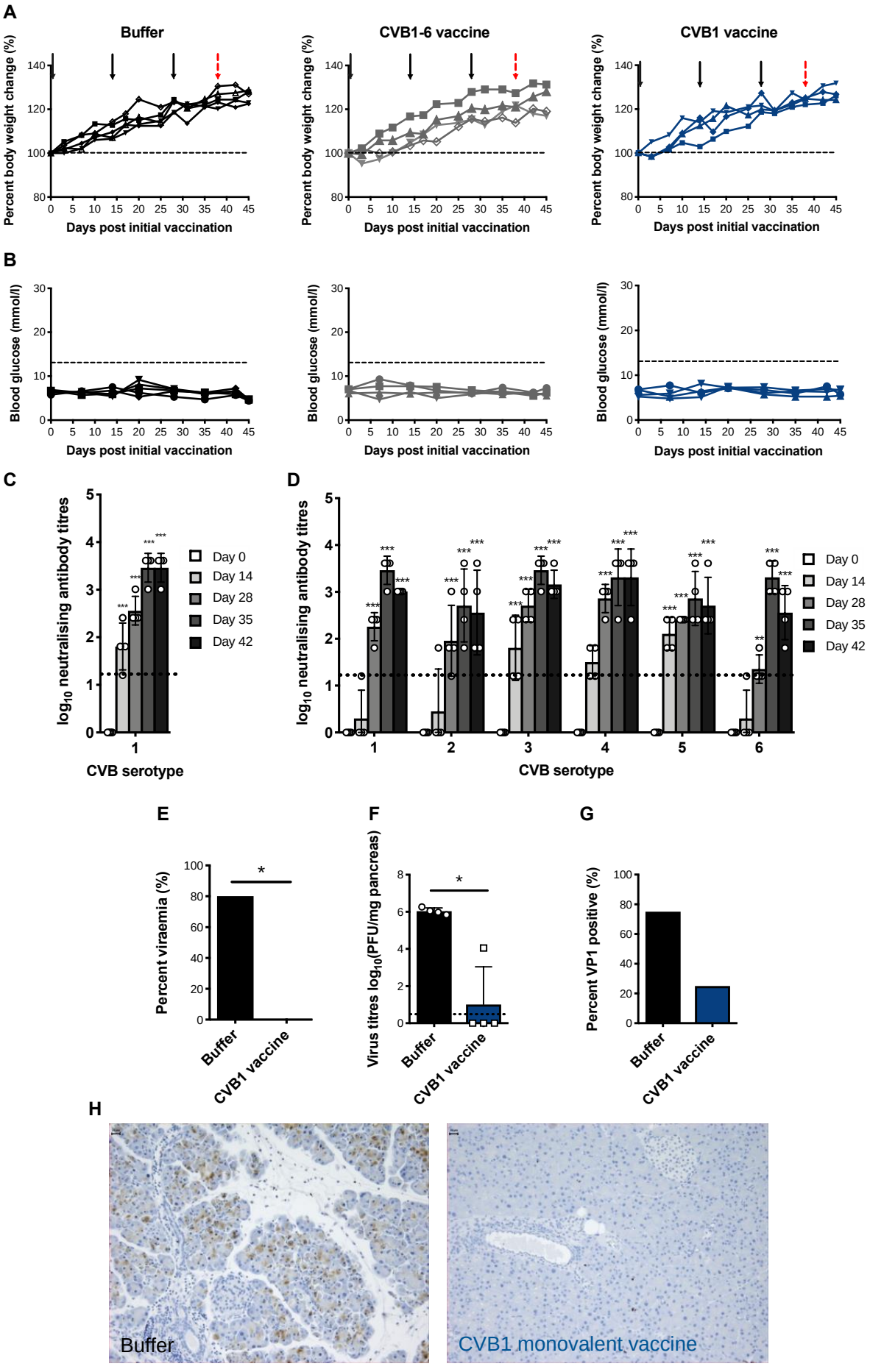
B

Group	Ct value		
	Replicate 1	Replicate 2	Average
Positive (CVB1 virus + GMK cells)	23.1	22.5	22.8
CVB1-6 vaccine	30.6	30.6	30.6
CVB1-6 vaccine + GMK cells	31	30.5	30.8

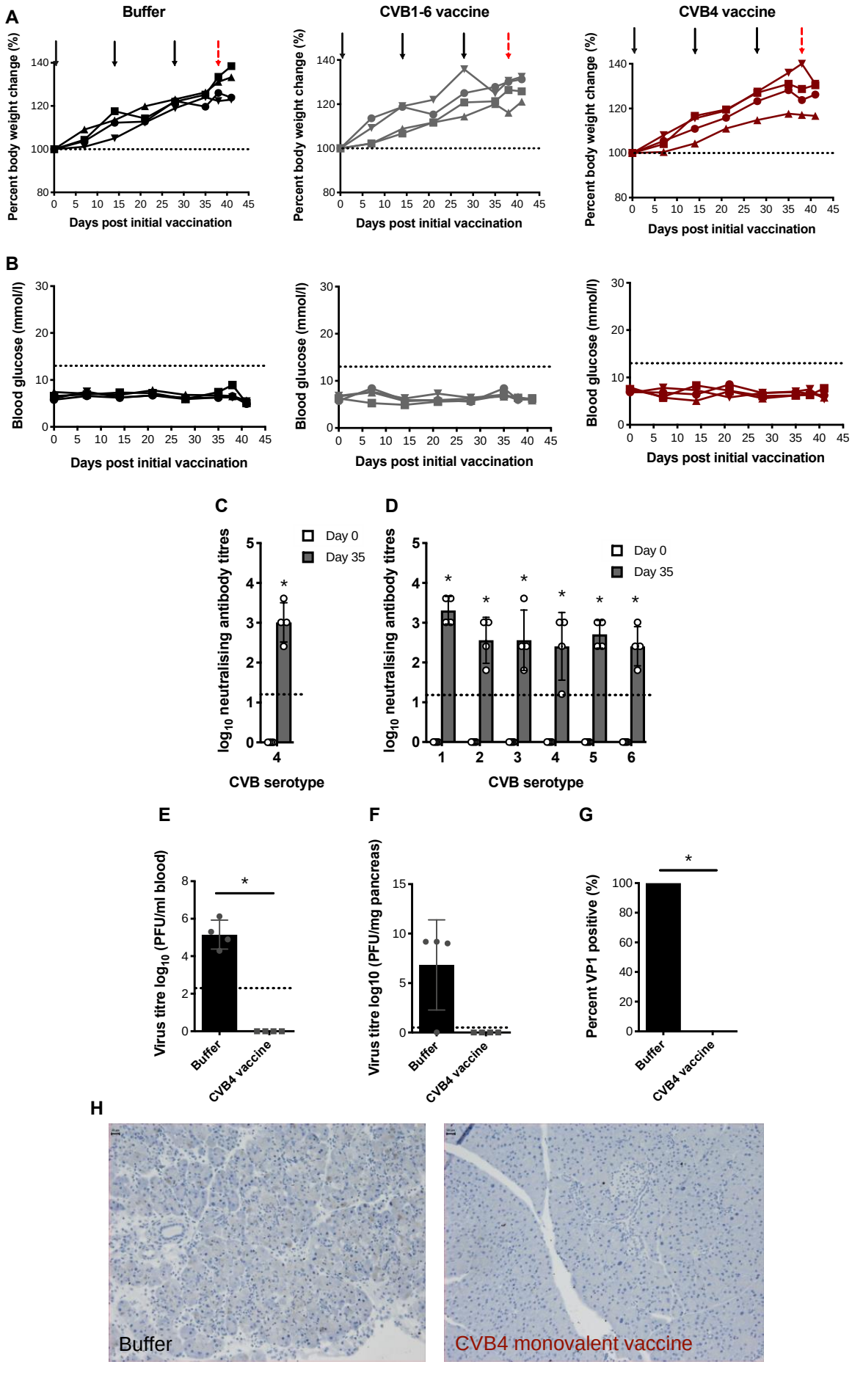
Supplementary Fig. 2. Real-time qPCR analysis of CVB1-6 vaccine inactivity in GMK cells treated with vaccine. (**A, B**) Viral RNA was assessed by enterovirus-specific RT-PCR in CVB1-6 vaccine, or in RNA extracted from GMK infected with CVB1 (MOI 0.001; positive control) or cultured with CVB1-6 vaccine (200 µl / well) for 5 days. (**A**) Ct threshold values for the respective groups and (**B**) table depicting the absolute Ct values (two RT-PCR replicates were run).



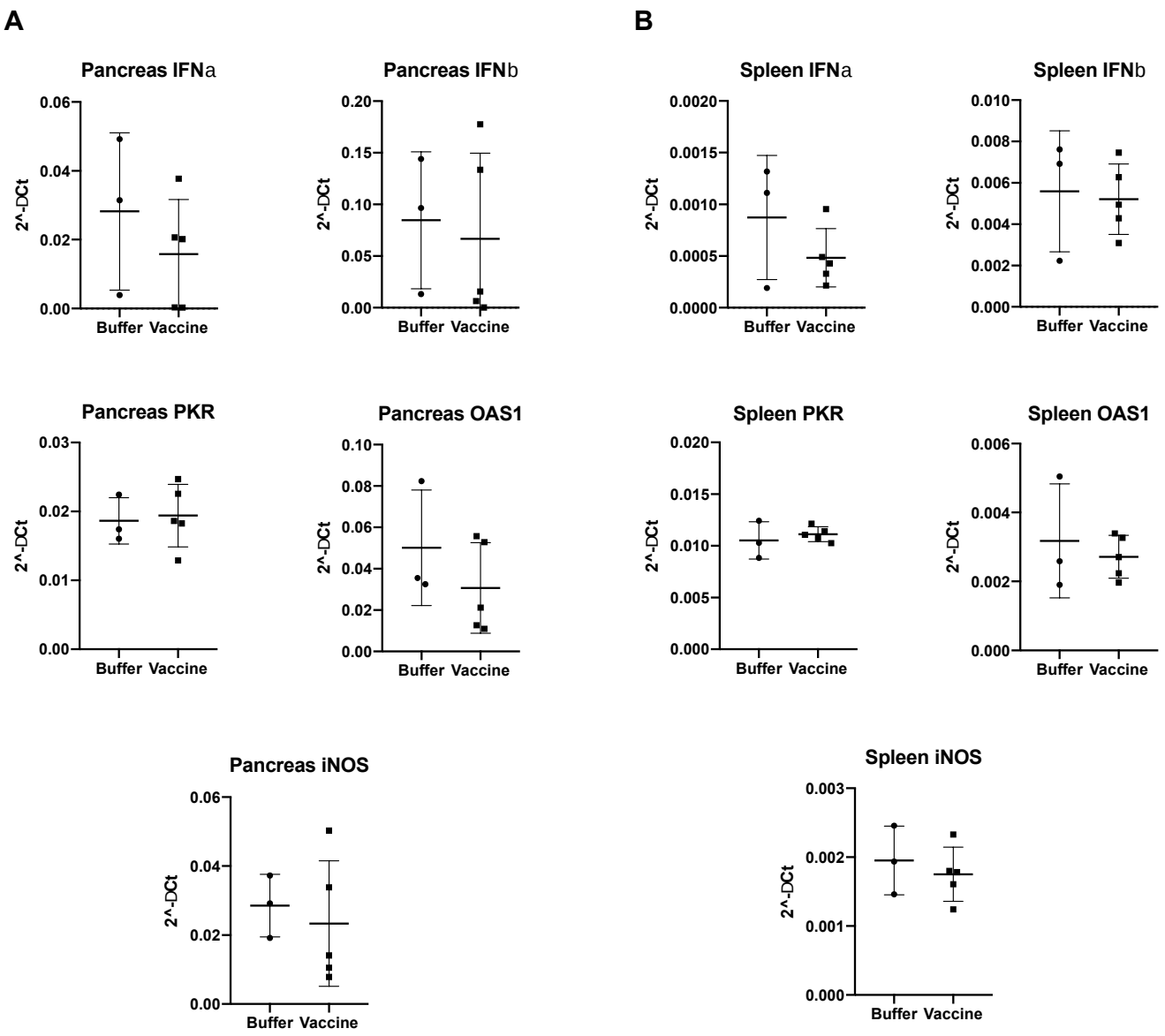
Supplementary Fig. 3 CVB1-6 vaccine induced neutralising antibody titres in NOD mice over time. Female NOD mice (4-6 weeks old) were immunised with CVB1-6 vaccine (1 μg, 150 μl, i.s.; n=3) on days 0, 21 and 35 and monitored until diabetes onset or 30 weeks of age at which point mice were sacrificed. Neutralising antibody titres were measured by standard neutralisation assay in serum collected on days 0, 42 and at the terminal time point. The results from individual mice are shown in **A-C** and the terminal serum samples were collected on days 70, 78 and 113 respectively. The dotted line shows the limit of detection.


A
Buffer
CVB1-6 vaccine
CVB1 vaccine
Percent body weight change (%)
Days post initial vaccination
B
Blood glucose (mmol/l)
Days post initial vaccination
C
log10 neutralising antibody titres
Day 0
Day 14
Day 28
Day 35
Day 42
CVB serotype
D
log10 neutralising antibody titres
CVB serotype
E
Percent viraemia (%)
Buffer
CVB1 vaccine
F
Virus titres log10(PFU/mg pancreas)
G
Percent VP1 positive (%)
H
Buffer
CVB1 monovalent vaccine

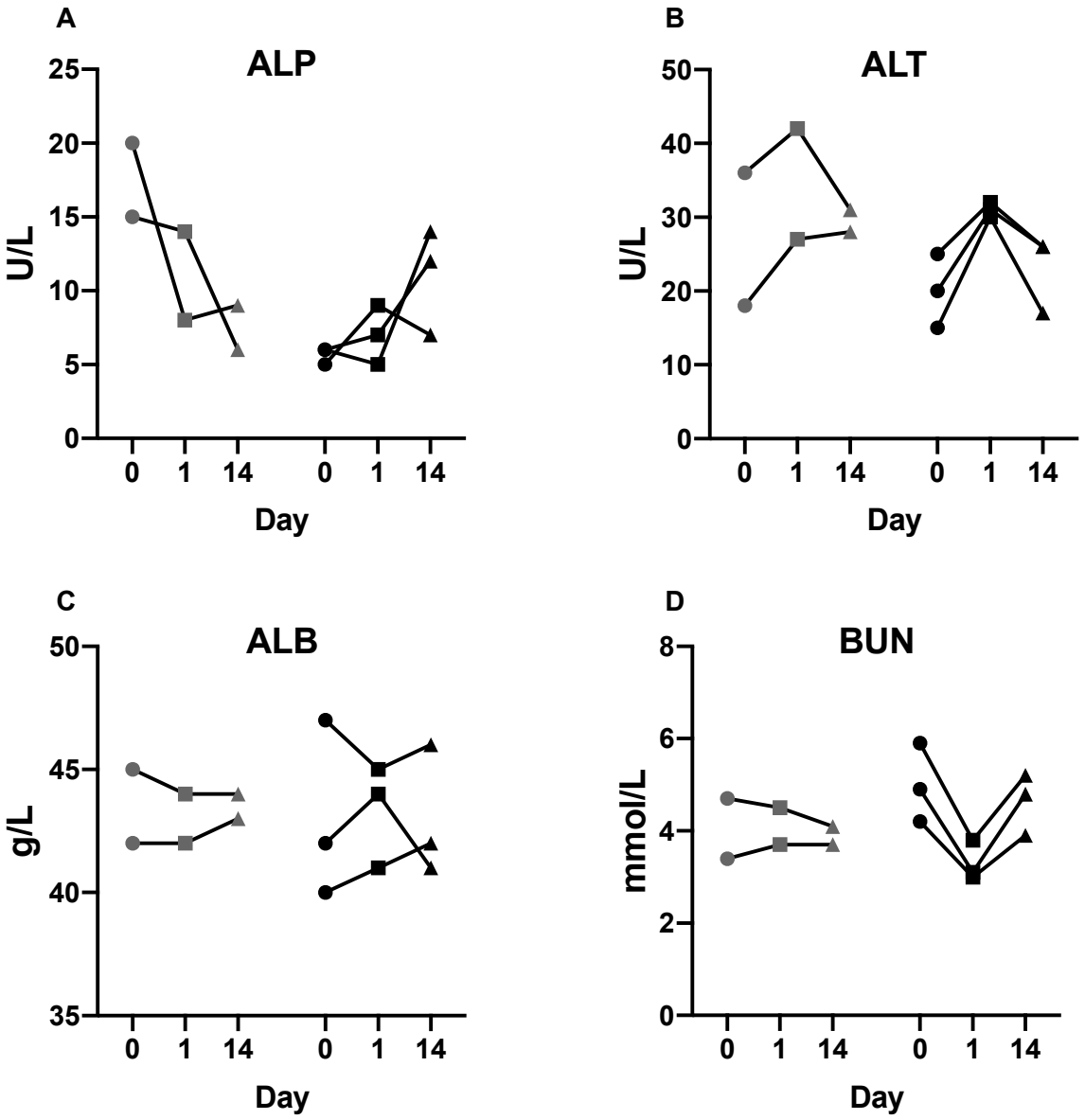
Supplementary Fig. 4. Monovalent CVB1 and hexavalent CVB1-6 vaccines are immunogenic in NOD mice and CVB1 monovalent vaccine prevents CVB1 induced viraemia. Female NOD mice (4 to 6 weeks old) were buffer-treated (150 µl, i.s; n=5; black), immunised with CVB1-6 vaccine (150 µl, i.s.; n=4; grey) or immunised with monovalent CVB1 vaccine (1 µg, 150 µl, i.s.; n=4; blue) on days 0, 14 and 28 and challenged with CVB1 (10^6 PFU / mouse, i.p.) on day 42. (**A**) Percentage weight change of buffer-treated (left, black), CVB1-6 vaccinated (middle, grey) or CVB1 vaccinated (blue, right) mice (100% equals the weight on day 0). The dotted line indicates the original weight, the black arrows show the vaccination time points and the hatched red arrow indicates CVB1 infection. (**B**) Blood glucose measurements of buffer-treated (left, black), CVB1-6 vaccinated (middle, grey) or CVB1 vaccinated (blue, right) mice. The dotted line shows cut off for diabetes. (**C**) Neutralising antibody titres against CVB1 in the sera of CVB1 vaccinated mice as determined by standard neutralisation assay. The dotted line shows the limit of detection. Mean values ± S.D. *** $p < 0.001$ compared to day 0 by one-way ANOVA with Bonferroni correction. (**D**) Neutralising antibody titres against the CVB1-6 serotypes in the sera of CVB1-6 vaccinated mice as determined by standard neutralisation assay. The dotted line shows the limit of detection. Mean values ± S.D. *** $p < 0.001$ compared to day 0 for the respective serotype by one-way ANOVA with Bonferroni correction. (**E**) Percentage of buffer treated or CVB1 vaccinated mice with viraemia on day 3 post CVB1 challenge as assessed by standard plaque assay. * $p < 0.05$ comparing the two groups as determined by Fisher's Exact Test. (**F**) Replicating virus in the pancreas on day 3 post CVB1 infection as determined by standard plaque assay in buffer treated mice (n=4) and CVB1 vaccinated mice (n=4). The virus titres for individual animals are shown with single symbols and the bars show the mean values ± S.D. The dotted line indicates the limit of detection for this assay. * $p < 0.05$ comparing the two groups by Mann Whitney U test. (**G**) Summary of VP1 positivity in the pancreas as determined by immunohistochemical staining in buffer treated (n=4) and CVB1 vaccinated (n=4) mice. (**H**) Representative images of VP1 staining in buffer treated and CVB1 vaccinated mice after CVB1 infection on day 3 post infection. VP1 positivity is indicated by the brown staining. 16X magnification. Scale bar equals 20 µm.


A
Buffer
CVB1-6 vaccine
CVB4 vaccine
Percent body weight change (%)
Days post initial vaccination
B
Blood glucose (mmol/l)
Days post initial vaccination
C
D
Day 0
Day 35
log10 neutralising antibody titres
CVB serotype
E
Virus titre log10 (PFU/ml blood)
F
Virus titre log10 (PFU/mg pancreas)
G
Percent VP1 positive (%)
Buffer
CVB4 vaccine
H
Buffer
CVB4 monovalent vaccine

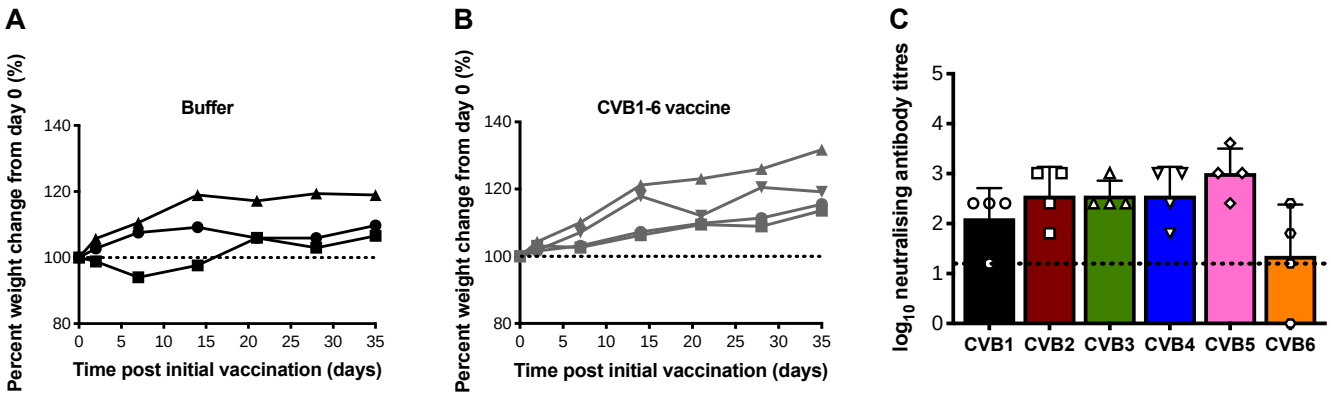
Supplementary Fig. 5. Monovalent CVB4 and hexavalent CVB1-6 vaccines are immunogenic in NOD mice and CVB4 monovalent vaccine prevents CVB4 induced viraemia. Female NOD mice (4-6 weeks old) were buffer-treated (150 µl, i.s; n=4; black), immunised with CVB1-6 vaccine (150 µl, i.s.; n=4; grey) or vaccinated with monovalent CVB4 vaccine (1 µg, 150 µl, i.s.; n=4; dark red) on days 0, 14 and 28 and challenged with CVB4 (10^5 PFU / mouse, i.p.) on day 38. (**A**) Percentage weight change of buffer-treated (left, black), CVB1-6 vaccinated (middle, grey) or CVB4 vaccinated (red, right) mice (100% equals the weight on day 0). The dotted line indicates the original weight, the black arrows show the vaccination time points and the hatched red arrow indicates CVB4 infection. (**B**) Blood glucose measurements of buffer-treated (left, black), CVB1-6 vaccinated (middle, grey) or CVB4 vaccinated (red, right) mice. The dotted line shows the cut off for diabetes. (**C**) Neutralising antibody titres against CVB4 in the sera of CVB4 vaccinated mice on day 0 and day 35 post initial vaccination as determined by standard neutralisation assay. The dotted line shows the limit of detection. The nAB titres for individual animals are shown with single symbols and the bars show the mean values ± S.D.. * $p < 0.05$ comparing the two groups with Mann Whitney U test. (**D**) Neutralising antibody titres against CVB1-6 serotypes in the sera of CVB1-6 vaccinated mice on day 0 and day 35 post initial vaccination as determined by standard neutralisation assay. The dotted line shows the limit of detection. Mean values ± S.D., * $p < 0.05$ comparing day 0 and day 35 by Mann Whitney U test. (**E**) Replicating virus in the blood of buffer treated or CVB4 vaccinated mice on day 3 post CVB4 infection, as measured by standard plaque assay. The virus titres for individual animals are shown with single symbols and the bars show the mean values ± S.D. The dotted line indicates the limit of detection for this assay. * $p < 0.05$ comparing the two groups with Mann Whitney U test. (**F**) Replicating virus in the pancreas on day 3 post infection as determined by standard plaque assay in buffer treated mice (n=4) and CVB4 vaccinated mice (n=4). The virus titres for individual animals are shown with single symbols and the bars show the mean values ± S.D. The dotted line indicates the limit of detection for this assay. (**G**) Summary of VP1 positivity in the pancreas as determined by immunohistochemical staining in buffer treated (n=4) and CVB4 vaccinated (n=4) mice. * $p < 0.05$ comparing the two groups as determined by Fisher's Exact Test. (**H**) Representative images of VP1 staining in buffer treated and CVB4 vaccinated mice after CVB4 infection on day 3 post infection. VP1 positivity is indicated by the brown staining. 16X magnification. Scale bar equals 20 µm.



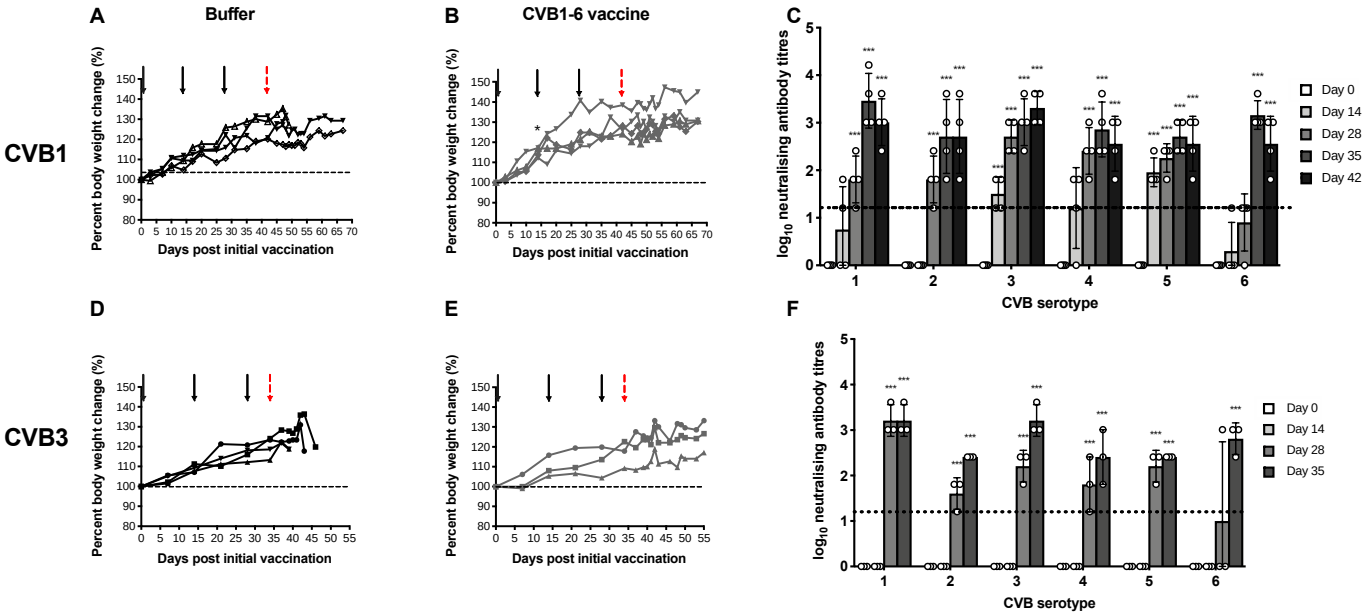
Supplementary Fig. 6. CVB1-6 vaccination does not alter the expression of genes involved in innate antiviral immunity. Male NOD mice (7 – 9.5 weeks old) were mock vaccinated with vaccine buffer (150 µl i.s.; n=3) or vaccinated with CVB1-6 vaccine (1 µg of each serotype, 150 µl i.s.; n=5) on days 0, 14 and 28 as demonstrated in the schematic shown in (Fig. 2A). Pancreas (**A**) and spleen (**B**) were collected two weeks after the last immunisation, RNA was extracted and the expression levels of interferon (IFN) -α, IFN-β, protein kinase R (PKR), 2'-5' oligoadenylate synthetase 1 (OAS1) and nitric oxide synthase (iNOS) were determined using RT-qPCR. Gene expression levels were normalised to GAPDH and are plotted as $2^{-(\Delta Ct)}$. Shown are the values from individual mice with the mean ± S.D.



Supplementary Fig. 7. CVB1-6 hexavalent vaccine is well tolerated in rhesus macaques. Rhesus macaques were immunised with CVB1-6 vaccine (5 μg of each serotype, i.m.) in the absence (grey symbols, n=2) or presence (black symbols, n=3) of Alum adjuvant (0.2% final concentration). Blood samples were collected on days 0, 1 and 14 after the prime vaccination and liver function tests were performed examining (**A**) alkaline phosphatase (ALP), (**B**) alanine amino transferase (ALT), (**C**) albumin (ALB) and (**D**) blood urea nitrogen (BUN). The values were all within the normal range for rhesus macaques [34]. Each line represents an individual animal.



Supplementary Fig. 8. Safety and immunogenicity of the CVB1-6 hexavalent vaccine in Balb/c mice. Male and female Balb/c mice (5.71 weeks old) were buffer treated (150 µl, i.s.; n=3) or immunised with CVB1-6 vaccine (1 µg of each serotype, 150 µl i.s.; n=4) on days 0 and 21. See Fig. 5A for the experimental set-up. (**A**, **B**) Percentage weight change of (**A**) buffer-treated or (**B**) CVB1-6 vaccinated mice compared to weight at day 0. The dotted line indicates the weight at the start of the study. No significant differences were found between the buffer treated and vaccinated group at each time point as determined by Mann Whitney U test. (**C**) Neutralising antibody titres against the six CVB serotypes in the serum of CVB1-6 immunised mice (n=4) on day 35 after the prime vaccination. Neutralising antibodies were not detected in the serum of mock-vaccinated mice. The dotted line in (**C**) shows the limit of detection. The neutralising antibody titres for individual animals are shown with single symbols and the bars show the mean values ± S.D.



Supplementary Fig. 9. Weights and neutralising antibody titres in *SOCS-1-tg* mice buffer treated or vaccinated with CVB1-6 vaccine and challenged with CVB1 or CVB3. Female *SOCS-1-tg* mice were buffer-treated (150 µl, i.s.) or vaccinated with CVB1-6 vaccine (1 µg of each serotype, 150 µl i.s.) and then challenged with CVB1 (10^6 PFU / mouse, i.p.; top panel; **A**, **B**, **C**) or CVB3 (10^6 PFU / mouse, i.p.; bottom panel; **D**, **E**, **F**). **(A, B**, **D**, **E**) Percentage weight change in buffer treated (**A**, **D**; black lines; n=4 for both groups) and CVB1-6 immunised (**B**, **E**; grey lines; n=4 and n=3 respectively) mice. The black arrows indicate the immunisation days and the hatched red arrows show the day of virus challenge. The dotted line shows the weight at day 0. No significant differences in weight were detected between the vaccinated groups and buffer treated groups except for the day 14 weights in the CVB1 group of mice (**A**, **B**; * $p < 0.05$) where the vaccinated mice had a greater body weight increase as determined by Mann Whitney U test. (**C**, **F**) Average neutralising antibody titres in the serum at the indicated time points against the six CVB serotypes as measured by standard neutralisation assay. Neutralising antibodies were not detected in the serum of buffer treated mice. Individual animals are shown by the single symbols. The dotted lines show the limit of detection. * $p < 0.05$ and *** $p < 0.001$ compared to day 0 by two-way ANOVA with Bonferroni correction.

Supplementary Table 1: Summary of mouse experiments

Experiment	Strain	# buffer treated mice	# CVB1-6 immunised mice	Age at start (weeks)	Immunisation days	Challenge CVB serotype	Day of CVB challenge	Endpoint	Figure(s)
Safety 1	C57Bl/6J	0	2	8.14	0, 14, 28	n/a	n/a	Day 84 (from prime immunisation)	Fig.1
Safety 2	NOD	3	5	7.14, 8.57, 9.14	0, 14, 28	n/a	n/a	Day 42 (from prime immunisation)	Fig.2 + Fig. S6
Safety 2	NOD	3	3	5.14, 5.57	0, 14, 28	n/a	n/a	Day 41 (from prime immunisation)	Fig.2
Safety 3	NOD	0	3	4.86, 5.43	0, 21, 35	n/a	n/a	Diabetes onset, days 70, 78 and 113	Fig. S3
CVB1 Acute	NOD	5	4 x CVB1, 4 x CVB1-6	5, 5.14, 5.29, 5.43	0, 14, 28	CVB1CDC7 (10*6 PFU/mouse)	Day 42	Day 3 p.i	Fig.3 + Fig. S4
CVB1 Diabetes	*SOCS-1-tg*	4	4	5, 5.14, 5.29, 5.43	0, 14, 28	CVB1CDC7 (10*6 PFU/mouse)	Day 42	Day 21 p.i / diabetes onset	Fig.6 + Fig. S9
CVB4 Acute	NOD	4	4 x CVB4, 4 x CVB1-6	4.43, 5,14, 5,29	0, 14, 28	CVB4-E2 (10*5 PFU/mouse)	Day 38	Day 3 p.i	Fig.3 + Fig. S5
CVB3 Diabetes	*SOCS-1-tg*	4	4	4.86, 5, 5.29, 5.43	0, 14, 28	CVB3-Nancy (10*6 PFU/mouse)	Day 34	Day 21 p.i / diabetes onset	Fig.6 + Fig. S9
CVB3 Myocarditis	Balb/c	3	4	5.71	0, 21	CVB3-Nancy (5x10*4 PFU/mouse)	Day 35	Day 5 p.i	Fig.5 + Fig. S8