Coxsackievirus B vaccines prevent infection-accelerated diabetes in NOD mice and have no disease-inducing effect

Journal:	*Diabetes*
Manuscript ID	DB21-0193.R2
Manuscript Type:	Original Article: Immunology and Transplantation
Date Submitted by the Author:	n/a
Complete List of Authors:	Stone, Virginia; Karolinska Institutet, Center for Infectious Medicine, Department of Medicine Huddinge, Karolinksa University Hospital Butrym, Marta; Karolinska Institutet, Center for Infectious Medicine, Department of Medicine Huddinge, Karolinska University Hospital Hankaniemi, Minna; Tampere University, Faculty of Medicine and Health Technology Sioofy-Khojine, Amir-Babak; Tampere University, Virology Hytönen, Vesa ; Tampere University, Faculty of Medicine and Health Technology ; Fimlab Laboratories Ltd Hyoty, Heikki; Tampere University Faculty of Medicine Flodström Tullberg, Malin; Karolinska Institutet, Center for Infectious Medicine, Department of Medicine Huddinge, Karolinska University Hospital

SCHOLARONE™
Manuscripts

Title: Coxsackievirus B vaccines prevent infection-accelerated diabetes in NOD mice and have no disease-inducing effect

Short running title: CVB vaccines do not alter diabetes onset but prevent virus-accelerated diabetes in NOD mice.

Authors names: Virginia M Stone[1], Marta Butrym[1], Minna M Hankaniemi[2], Amir-Babak Sioofy-Khojine[2], Vesa P Hytönen[2,3], Heikki Hyöty[2,3], Malin Flodström-Tullberg[1]

Institutions of origin:
[1] Center for Infectious Medicine, Department of Medicine Huddinge, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden.
[2] Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland.
[3] Fimlab Laboratories, Tampere, Finland.

Corresponding author:
Malin Flodström-Tullberg, Karolinska Institutet, +46 8 524 87625,
malin.flodstrom-tullberg@ki.se

Word count: 3624

Number of tables and figures: 3

Tweet: A Coxsackievirus B vaccine designed to address the role of viruses in human type 1 diabetes prevents virus-accelerated disease onset and does not alter diabetes onset in an autoimmune diabetes prone host @MFT_Diabetes @CIM_Sweden @karolinskainst @HytonenL @HHyoty Lab @TampereUni

Abstract:

Enteroviruses, including the Coxsackievirus Bs (CVB), have been implicated as causal agents in human type 1 diabetes. Immunization of at-risk individuals with a CVB vaccine provides an attractive strategy for elucidating the role of CVBs in the disease etiology. Previously we have shown that an inactivated whole-virus vaccine covering all CVB serotypes (CVB1-6) is safe to administer and highly immunogenic in preclinical models, including non-human primates. Before initiating clinical trials with this type of vaccine it was also important to address whether a) the vaccine itself induces adverse immune reactions including accelerating diabetes onset in a diabetes prone host and b) the vaccine can prevent CVB induced diabetes in a well-established disease model. Here we present results from studies in which female NOD mice were left untreated, mock-vaccinated or vaccinated with CVB1-6 vaccine and monitored for insulitis occurrence or diabetes development. We demonstrate that vaccination induces virus neutralizing antibodies without altering insulitis scores or the onset of diabetes. We also show that NOD mice vaccinated with a CVB1 vaccine are protected from CVB-induced accelerated disease onset. Taken together, these studies show that CVB vaccines do not alter islet inflammation or accelerate disease progression in an animal model that spontaneously develops autoimmune type 1 diabetes. However, they can prevent CVB-mediated disease progression in the same model.

Type 1 diabetes is a common autoimmune disease caused by the destruction of the insulin producing pancreatic beta cells. Genetic and environmental factors are contributory, but their precise roles remain unclarified (1). Amongst the possible environmental triggers, viral infections have been widely studied and mounting evidence suggests that enteroviruses, especially the Coxsackievirus B (CVB) serotypes, may contribute to the development of type 1 diabetes (1-3).

A few schools of thought exist regarding the mechanisms through which CVBs may cause type 1 diabetes. Results from some studies support the notion that CVBs could be involved in initiating the disease process. For instance, it was found in the TEDDY (The Environmental Determinants of Diabetes in the Young) study that prolonged enterovirus B infections were associated with the development of islet autoimmunity but not type 1 diabetes (3). Similar results were seen in the DIPP (Diabetes Prediction and Prevention) study where associations were also documented between enterovirus infections and islet autoimmunity (4-6). An alternative hypothesis is that CVBs accelerate an on-going autoimmune process. Data from the DAISY (Diabetes Auto Immunity Study in the Young) study implies that enterovirus infections in autoantibody positive individuals increase the speed of progression to diabetes (7). This observation has been supported by animal models in which CVB infection accelerates the onset of diabetes in pre-diabetic animals (8-10). It is of course feasible that both hypotheses hold true and enteroviruses may contribute to the development of type 1 diabetes in both manners.

To determine the causal role of CVBs in human type 1 diabetes, vaccine development initiatives have been undertaken (9; 11-14). A non-adjuvanted inactivated vaccine comprising of the six CVB1-6 serotypes was recently shown to be highly immunogenic in mice and non-human primates in preclinical studies (13). Furthermore, this vaccine did not alter weight gain and

blood glucose levels in both models and had no effect on temperature and hematological readouts in rhesus macaques, demonstrating an excellent safety profile (13).

The recent introduction of new vaccines in the human population has shown that adverse events may occur. These include associations between vaccination and the occurrence of autoimmune diseases (15; 16). As the current CVB vaccine is based on inactivated whole virus particles and CVB virus infections have been associated with both the initiation and progression of the processes that lead to type 1 diabetes (3; 5-7; 17), it is also paramount to ensure that vaccination itself doesn't affect the onset of autoimmune diabetes in a similar manner to infectious virus.

Here, we present the results from pre-clinical studies testing whether vaccination of young, CVB-naïve female NOD mice (a model prone to develop autoimmune diabetes (18)) with a multivalent CVB vaccine accelerates disease onset or increases diabetes incidence. Further to this, we also examined whether this type of vaccine can provide protection against the acceleration in diabetes onset seen after CVB infection of NOD mice that are in the pre-diabetic phase.

Research Design and Methods:

Animal husbandry and monitoring of animal health

NOD mice were bred in-house and housed in specific pathogen-free conditions at Karolinska Institutet, Stockholm, Sweden. A local ethics committee granted approval for all experiments which were performed in accordance with the NIH principles of Laboratory Animal Care and national laws in Sweden. Animals were housed in ventilated cages and provided with water and

food *ad libitum*. A maximum number of 5 mice were housed per cage and no mice were single housed. Extended health monitoring of mice was performed including examining changes in health status (weight changes, alterations in natural behaviour, porphyria, movement and posture, piloerection, respiration and skin). Animals were randomly assigned to treatment groups. Weight and blood glucose measurements were monitored weekly until the experimental endpoint (diabetes onset, a health score of 0.4 or higher, or when the animals had reached the defined end point of the experiment). The researchers were not blinded to the experimental groups during the experiments. At the experimental endpoint, mice were anaesthetised with isoflurane, a terminal heart puncture was performed for blood drawing and the animals were then euthanized by cervical dislocation.

Vaccine production

CVB1-6 and CVB1 vaccines were produced by formalin inactivation of the CVB1-6 or CVB1 serotypes (13). The vaccine was then formulated in Medium M199 (Gibco, Thermofisher Scientific, Vanda, Finland) containing 0.1% Tween 80 by mixing 1μg of each inactivated virus serotype per dose for the CVB1-6 vaccine or 1.8μg for the CVB1 vaccine.

Vaccination strategies

Female age-matched NOD mice (4.9 – 7.1 weeks old) were randomly assigned to treatment groups (untreated, mock-vaccinated or vaccinated). Animals were either left untreated, vaccinated with non-adjuvanted CVB1-6 vaccine on two or three occasions, 2-3 weeks apart, vaccinated with CVB1 vaccine on 3 occasions, 2-3 weeks apart, or mock-vaccinated with vaccine buffer alone (M199 Medium + 0.1% Tween 80 + 0.001% formalin, v/v). Each

vaccination was performed by subcutaneous (interscapular) injection (150µl). Serum samples were collected from the tail vein when indicated in the text. Animals were either euthanized 6 weeks later (Fig. 1), monitored for diabetes incidence up until the age of 30 weeks (Fig. 2), or infected with virus (Fig. 3) as described under CVB1 infection.

CVB1 infection

Female NOD mice (10.5 - 13.5 weeks old) were randomly assigned to either control (n=31) or CVB1 infection (n=14; 10^7 plaque forming units (PFU) CVB1 by intraperitoneal (i.p.) injection, total volume 200µl; Fig. 3a, b) groups. In other experiments (Fig. 3c-e), female NOD mice (6.3 – 6.9 weeks old) were assigned to untreated (n=16), mock-vaccination (n=16) or CVB1 vaccine (n=12) groups and vaccinated as described in Vaccination strategies above. Mice in the mock- and CVB1-vaccine groups were infected with CVB1 (10^7 PFU by i.p. injection, total volume 200µl) one week after the final vaccination (approximately 12-13 weeks old). In both experimental set ups diabetes incidence was followed up until 30 weeks of age/diabetes onset.

Blood glucose measuring and monitoring of diabetes incidence

Blood glucose concentrations were measured in blood drawn from the tail vein using a Bayer Contour XT blood glucose meter (Bayer, Basel, Switzerland). Diabetes was defined as a blood glucose value ≥ 18 mmol/l. If the blood glucose value was between 13 and 18 mmol/l the mouse was checked the next day and if it remained > 13mmol/l the mouse was deemed diabetic.

Neutralizing antibody measurements

CVB1-6 neutralizing antibody titers were measured by a standard virus plaque reduction assay using GMK cells (National Institute for Health and Welfare, Finland; mycoplasma negative; (4; 17; 19)). In short, serum was serially diluted starting with a 1:4 dilution and was mixed with 100 PFU of the respective CVB serotypes used to produce the vaccine (for details regarding the viruses, see (13; 20)). The serum-virus suspensions were incubated for 1 hour at 37°C and then overnight at room temperature. GMK-cells were grown to 95% confluency in 12-well plates and the virus-serum mixture was added to these cells and incubated at 37°C for 1h, then replaced with a semisolid medium (minimum essential medium supplemented with 0.67% carboxymethylcellulose – Merck, Sigma-Aldrich, Finland). Plates were incubated for 2 days at 37°C, then the cells were fixed and stained with formaldehyde-crystal violet solution. Plaque numbers were counted with the researchers blinded to the treatment groups, and serum samples which had a reduction in plaque numbers of 80% or more compared to an untreated virus control were deemed to be positive for neutralizing antibodies. This assay has a technical detection limit of 1:4 and serum sample positivity for neutralizing antibodies was set to a dilution ≥ 1:16.

Histology and immunohistochemistry

Mouse pancreases were collected, formalin-fixed in 4% paraformaldehyde overnight and embedded in paraffin. Organs were cut into 5-µm thick sections. For the insulitis scoring (Fig. 1), each pancreas was sectioned in two-three levels with >20 sections difference between each level (100-µm) and for the histological assessment in Fig. 3 and Supplementary Fig. 4, sections from one level of the pancreas were used. Sections were deparaffinized and stained with primary antibodies against insulin (1:20,000; A0564, Dako, Ely, UK) or glucagon (1:12,000; EP3070, Abcam, Cambridge UK; both validated in formalin-fixed paraffin-embedded murine

pancreas sections) and counter stained with hematoxylin using standard immunohistochemical techniques (as described in (9; 21)).

Insulitis scoring

Pancreas sections stained with insulin and glucagon were assessed (in a blinded manner) by light microscopy by two investigators and ranked for insulitis according to the following ranking method. 0- healthy islet with normal morphology with no mononuclear cells surrounding or infiltrating the islets; 1- peri-insulitis: mononuclear cells surrounding the islets on the periphery of the islets; 2- insulitis: infiltration of mononuclear cells into the islet; 3- infiltrated islet with no signs of insulin staining (denoted destroyed islet). See Fig. 1b for an example of islets with different scores. An insulitis score for each mouse was obtained by calculating the scores for each pancreas and dividing this total score by the number of islets examined. Data is presented as mean insulitis score ± SD for each treatment group.

Statistical analysis

Statistical analyses were performed using Prism 9 software (GraphPad, La Jolla, CA). Insulitis scores (index), CVB1 neutralizing antibody titers and age at diabetes onset (CVB1-infected mice) were analyzed by an unpaired t-test. Percentage of islets with differing insulitis scores was assessed by two-way ANOVA with Sidak's multiple comparison test. Age at diabetes onset (CVB1-6 vaccinated mice) was analyzed by one-way ANOVA with Tukey's multiple comparison test. Diabetes survival curves were assessed by Gehan-Breslow-Willcoxon test. In the studies examining virus-accelerated diabetes onset, the differences in the survival curves were assessed two weeks after infection when the acceleration in disease onset is expected to occur by Gehan-Breslow-Willcoxon test, as described in (22; 23). Age at diabetes onset in the

CVB1 vaccine studies was assessed by Kruskal Wallis test with Dunn's multiple comparisons. Data are expressed as mean ± SD. A p value ≤ 0.05 was considered statistically significant.

Data and Resource availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. No applicable resources were generated or analyzed during the current study.

Results:

A CVB1-6 vaccine does not aggravate insulitis in NOD mice

First, we studied whether the CVB1-6 vaccine alters pancreatic islet inflammation in age-matched female NOD mice. Young mice that had no previous exposure to CVBs were vaccinated three times (on days 0, 14 and 28, n=3 or on days 0, 21 and 35, n=5) with the CVB1-6 vaccine or with vaccine buffer (n=13) and their pancreases were assessed at around 12 weeks of age. As seen before (13), vaccinated mice had CVB1-6 neutralizing antibodies by day 41/42 after the initial vaccination dose (Fig. 1a) which were absent on day 0 (data not shown). Neutralizing antibody data shown in blue was previously presented in (13) but the pancreas had not been assessed for insulitis. Moreover, the CVB1-6 vaccine had no negative effects on animal weight and blood glucose levels up to 6 weeks post-initial vaccination (the endpoint of the study; Supplementary Fig. 1a-f). Pancreatic islet inflammation was assessed and the average number of islets scored per animal was 30 ± 14 (range: 9 – 64). All animals showed signs of pancreatic islet inflammation but no significant differences in pancreatic insulitis scores

between mock- and CVB1-6 vaccinated mice were observed (Fig. 1b-d). These results imply that the CVB1-6 vaccine does not alter immune cell infiltration in the pancreatic islets of Langerhans.

Diabetes onset is not affected in NOD mice vaccinated with a CVB1-6 vaccine

Next, we examined the safety of the CVB1-6 vaccine with regards to diabetes development in NOD mice. To address whether the vaccine changed the onset of diabetes in NOD mice, young animals were left untreated (n=10), mock-vaccinated (n=15) or vaccinated with CVB1-6 vaccine (n=14) two-three times on days 0, 21 and 35. Blood glucose levels were monitored until 30 weeks of age or until diabetes onset when the mice were removed. Vaccine immunogenicity was confirmed by CVB1-6 neutralizing antibody responses (Fig. 2a) which were absent at day 0 in all mice and at day 42 in untreated and mock-vaccinated mice (data not shown). No detrimental outcomes on weight (Supplementary Fig. 2) or general health status were seen. CVB1-6 vaccination did not alter the incidence of diabetes compared to the mock-vaccinated and untreated groups and the kinetics of diabetes onset did not differ between the groups (Fig. 2b). Likewise, no differences were seen in the mean age at diabetes onset when comparing animals from the three groups (Fig. 2c). Taken together, this data indicates that the CVB1-6 vaccine does not alter the development of autoimmune diabetes in NOD mice.

A CVB vaccine protects against CVB1-accelerated diabetes onset in NOD mice

CVB infections have been implicated in type 1 diabetes in humans and have also been shown to accelerate the onset of diabetes in pre-diabetic mice (7-10). As such, we next decided to examine whether vaccination can prevent the accelerating effect that CVB infection has on the development of diabetes in NOD mice. First, we confirmed that CVB infection accelerates the

onset of diabetes in pre-diabetic female NOD mice in our colony. Pre-diabetic animals were left untreated or infected with CVB1 and the incidence of diabetes was monitored up to 30 weeks of age. CVB1 infected mice developed diabetes faster than the control group (Fig. 3a) and the mean age at diabetes onset was significantly lower in infected animals (13.1 weeks old) compared to the controls (19.9 weeks old; Fig. 3b).

We subsequently wanted to see if a CVB vaccine could protect against this virus-mediated acceleration in diabetes onset. Female NOD mice were either left untreated, mock-vaccinated and then infected with CVB1 (mock + CVB1) or vaccinated and then infected with CVB1 (vaccine + CVB1). Diabetes incidence was monitored until the mice were 30 weeks old. To ensure the vaccine was immunogenic, virus neutralizing antibodies were measured in serum collected prior to infection (day 42). Mice vaccinated with the CVB1 vaccine induced a good neutralizing antibody response (Fig. 3c) which was absent in mock-vaccinated animals (data not shown). As expected, an acceleration in diabetes onset was seen in the mock-vaccinated (buffer) + CVB1 group compared to untreated mice (Fig. 3d). In comparison, the CVB1 vaccine protected against CVB1-mediated acceleration in diabetes onset and the survival curve in the vaccine + CVB1 group mirrored that of the untreated animals (Fig. 3d). Significant differences between the curves were detected in the 2 weeks after infection when the majority of acceleration occurs. Moreover, the mean age at diabetes onset was lower in the mock-vaccinated (buffer) + CVB1 group (16.3 weeks old; Fig. 3e) than in the vaccine + CVB1 groups (21.9 weeks old; Fig. 3e) and the untreated group (19.6 weeks old; Fig. 3e).

The protective capacity of the vaccine was further illustrated when pancreas integrity was compared between the untreated, mock-vaccinated (buffer) + CVB1 and vaccine + CVB1 groups at the onset of diabetes (Fig. 3f). Vaccinated animals had healthy exocrine tissue

morphology at the time of diabetes onset in a similar manner to untreated animals, whereas there was significant exocrine tissue destruction in the mock-vaccinated (buffer) group as shown by the representative images in Fig. 3f. Differences were also seen between these groups in the animals that did not develop diabetes by 30 weeks of age. There was evidence of exocrine tissue loss in the pancreas of mock-vaccinated animals as illustrated by the presence of islets in fat tissue (Supplementary Fig. 4 e,f), although some exocrine tissue had either remained healthy or regenerated in these animals (Supplementary Fig. 4 c,d). In contrast, normal pancreas histology was seen in the untreated and vaccinated groups (Supplementary Fig. 4 a,b,g,h). Collectively, these studies show that a CVB vaccine protects against CVB1-accelerated diabetes in NOD mice.

Discussion:

Pre-clinical studies are an important part of initial vaccine efficacy and safety assessments. These studies serve to identify elements that require further assessment and can also help to design vaccination schedules. Additionally, they may uncover adverse events including undesired immune reactions that can, for example, lead to, autoimmune diseases. Such diseases have occurred, albeit rarely, after immunization with other vaccines (15; 16). Our studies demonstrate that a multivalent CVB vaccine does not accelerate the onset of diabetes in NOD mice, a commonly used animal model for type 1 diabetes. We confirmed that early vaccination with this vaccine induces virus neutralizing antibodies and showed that immunity to CVBs is achieved without altering islet inflammation or changing the average time to diabetes onset. These results are in line with our previous observation that vaccination of pre-diabetic NOD mice with a monovalent CVB1 vaccine did not increase the production of insulin autoantibodies (9). This also suggests that inactivation of the viruses abolishes the diabetogenic properties of

the CVBs, which have previously been observed in the NOD mouse (8-10) and which are suspected in humans (4; 7).

Human cohort studies focused on understanding the triggers of type 1 diabetes have produced results suggesting that CVBs could be critically involved at different stages of the disease. In the TEDDY and DIPP studies, enterovirus infections were associated with the development of islet-specific autoantibodies (3-6). In contrast, the DAISY study reported that enterovirus infections accelerated the speed of progression to overt diabetes in autoantibody positive individuals (7). Different animal models exist that may replicate how CVBs could contribute to type 1 diabetes development in humans, as alluded to in the cohort studies. Direct infection of the beta-cell by CVBs is a possible mechanism through which beta-cell autoimmunity could be induced. In our previous studies using the SOCS-1-tg mouse model, where the beta-cells are susceptible to CVB infection leading to diabetes (21; 24), we have shown that CVB vaccines can prevent virus-induced diabetes (13; 14). It is also possible to mimic virus-acceleration of an on-going autoimmune process by infecting pre-diabetic NOD mice with CVBs (8-10). In this study we report for the first time that a CVB vaccine is also capable of preventing virus-mediated acceleration in diabetes onset. Type 1 diabetes appears to be a highly heterogenous disease and it is feasible that both virus-induced autoimmunity and acceleration in the rate of diabetes onset in autoantibody-positive individuals after virus infection could occur in different groups. The ability of CVB vaccines to prevent both forms of virus-mediated diabetes in relevant pre-clinical models provides excellent proof-of-concept evidence for the use of such a vaccine to elucidate the multiple potential roles of CVBs in human type 1 diabetes.

Based on the aforementioned studies (amongst others) that suggest enteroviruses may have an important role in type 1 diabetes and from promising results using the mono- and current

multivalent CVB vaccine in pre-clinical studies (9; 13; 14; 25), the production and clinical testing of a similar multivalent CVB vaccine was recently initiated (11; 12; 26). Our previous work with experimental CVB vaccines demonstrates that such vaccines show strong potential for use in the prevention of CVB infections and diseases associated with these infections in humans (13; 14). We also found that there were no adverse effects on glucose regulation (13) and no conspicuous infiltration of immune cells in the pancreas (Stone et al. unpublished observation) in rhesus macaques immunized with the multivalent CVB vaccine. The present study builds on these foundations by suggesting that this type of vaccine does not alter islet inflammation or diabetes onset in a preclinical mouse model for autoimmune type 1 diabetes. In summary, this study provides data that supports the use of an equivalent vaccine in human clinical trials to establish whether CVBs are involved in type 1 diabetes. Such trials will involve the immunization of young children with a genetic predisposition for the disease who are yet to experience a CVB infection. If the involvement of CVBs in type 1 diabetes is confirmed, the vaccine could provide a viable preventative measure for this disease.

Article information

Acknowledgements. We would like to acknowledge S. Parvin, Karolinska Institutet, Stockholm, Sweden, for her help with processing and staining histological samples, the M.F-T. group for scientific discussions and laboratory assistance, and the animal staff at the PKL facility at Karolinska Institutet, Stockholm, Sweden, for their assistance with the animal studies. M. Jokela from the Faculty of Medicine and Health Technology (Tampere University, Finland) is acknowledged for their assistance with the vaccine production and M. Kekäläinen and M. Ovaskainen from the Faculty of Medicine and Health Technology (Tampere University, Finland) for the analysis of virus antibodies. We also would like to acknowledge Biocenter Finland for their infrastructure support.

Funding. We would like to acknowledge financial support from the Swedish Child Diabetes Foundation, the Swedish Diabetes Foundation, Karolinska Institutet including the Strategic Research Program in Diabetes, the Business Finland (formerly TEKES; THERDIAB project, diary no. 1843/31/2014), the Academy of Finland (grant 309455 awarded to MMH and grant 288671 awarded to HH), the Sigrid Jusélius Foundation, the Reino Lahtikari Foundation and the JDRF (2-SRA-2017-A-N).

Conflict of Interest Statement. HH owns stocks and is the chairman of the board of Vactech Ltd, which develops vaccines against picornaviruses. HH and MFT serve on the scientific advisory board of Provention Bio Inc., which is developing a clinical CVB vaccine in collaboration with Vactech Ltd. The other authors have no conflict of interest to declare.

Author Contributions. VMS, MMH, VPH, ABSK, HH and MFT designed the study, MMH produced and performed quality control analyses of the vaccine, VMS and MB performed experiments, VMS, MB, ABSK and MFT analyzed results, VMS, MB and MFT wrote and edited the manuscript. All authors read, edited and approved the final manuscript. MFT and HH are the guarantors of this work, and as such had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. This study was presented, in part, in abstract form at the 12th Annual and 13th Annual nPOD Meetings (23rd-26th February 2020 and 22nd-24th February 2021).

References

1. DiMeglio LA, Evans-Molina C, Oram RA: Type 1 diabetes. Lancet 2018;391:2449-2462
2. Richardson SJ, Morgan NG: Enteroviral infections in the pathogenesis of type 1 diabetes: new insights for therapeutic intervention. Curr Opin Pharmacol 2018;43:11-19
3. Vehik K, Lynch KF, Wong MC, Tian X, Ross MC, Gibbs RA, Ajami NJ, Petrosino JF, Rewers M, Toppari J, Ziegler AG, She JX, Lernmark A, Akolkar B, Hagopian WA, Schatz DA, Krischer JP, Hyoty H, Lloyd RE, Group TS: Prospective virome analyses in young children at increased genetic risk for type 1 diabetes. Nat Med 2019;25:1865-1872
4. Laitinen OH, Honkanen H, Pakkanen O, Oikarinen S, Hankaniemi MM, Huhtala H, Ruokoranta T, Lecouturier V, Andre P, Harju R, Virtanen SM, Lehtonen J, Almond JW, Simell T, Simell O, Ilonen J, Veijola R, Knip M, Hyoty H: Coxsackievirus B1 is associated with induction of beta-cell autoimmunity that portends type 1 diabetes. Diabetes 2014;63:446-455
5. Oikarinen S, Martiskainen M, Tauriainen S, Huhtala H, Ilonen J, Veijola R, Simell O, Knip M, Hyoty H: Enterovirus RNA in blood is linked to the development of type 1 diabetes. Diabetes 2011;60:276-279
6. Salminen K, Sadeharju K, Lonnrot M, Vahasalo P, Kupila A, Korhonen S, Ilonen J, Simell O, Knip M, Hyoty H: Enterovirus infections are associated with the induction of beta-cell autoimmunity in a prospective birth cohort study. J Med Virol 2003;69:91-98
7. Stene LC, Oikarinen S, Hyoty H, Barriga KJ, Norris JM, Klingensmith G, Hutton JC, Erlich HA, Eisenbarth GS, Rewers M: Enterovirus infection and progression from islet autoimmunity to type 1 diabetes: the Diabetes and Autoimmunity Study in the Young (DAISY). Diabetes 2010;59:3174-3180
8. Horwitz MS, Bradley LM, Harbertson J, Krahl T, Lee J, Sarvetnick N: Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. Nat Med 1998;4:781-785
9. Larsson PG, Lakshmikanth T, Laitinen OH, Utorova R, Jacobson S, Oikarinen M, Domsgen E, Koivunen MR, Chaux P, Devard N, Lecouturier V, Almond J, Knip M, Hyoty H, Flodstrom-Tullberg M: A preclinical study on the efficacy and safety of a new vaccine against Coxsackievirus B1 reveals no risk for accelerated diabetes development in mouse models. Diabetologia 2015;58:346-354
10. Serreze DV, Ottendorfer EW, Ellis TM, Gauntt CJ, Atkinson MA: Acceleration of type 1 diabetes by a coxsackievirus infection requires a preexisting critical mass of autoreactive T-cells in pancreatic islets. Diabetes 2000;49:708-711
11. Dunne JL, Richardson SJ, Atkinson MA, Craig ME, Dahl-Jorgensen K, Flodstrom-Tullberg M, Hyoty H, Insel RA, Lernmark A, Lloyd RE, Morgan NG, Pugliese A: Rationale for enteroviral vaccination and antiviral therapies in human type 1 diabetes. Diabetologia 2019;
12. Hyoty H, Leon F, Knip M: Developing a vaccine for Type 1 diabetes by targeting coxsackievirus B. Expert Rev Vaccines 2018;
13. Stone VM, Hankaniemi MM, Laitinen OH, Sioofy-Khojine AB, Lin A, Diaz Lozano IM, Mazur MA, Marjomaki V, Lore K, Hyoty H, Hytonen VP, Flodstrom-Tullberg M: A hexavalent Coxsackievirus B vaccine is highly immunogenic and has a strong protective capacity in mice and nonhuman primates. Sci Adv 2020;6:eaaz2433
14. Stone VM, Hankaniemi MM, Svedin E, Sioofy-Khojine A, Oikarinen S, Hyoty H, Laitinen OH, Hytonen VP, Flodstrom-Tullberg M: A Coxsackievirus B vaccine protects against virus-induced diabetes in an experimental mouse model of type 1 diabetes. Diabetologia 2018;61:476-481

15. Schultz NH, Sorvoll IH, Michelsen AE, Munthe LA, Lund-Johansen F, Ahlen MT, Wiedmann M, Aamodt AH, Skattor TH, Tjonnfjord GE, Holme PA: Thrombosis and Thrombocytopenia after ChAdOx1 nCoV-19 Vaccination. N Engl J Med 2021;
16. Segal Y, Shoenfeld Y: Vaccine-induced autoimmunity: the role of molecular mimicry and immune crossreaction. Cell Mol Immunol 2018;15:586-594
17. Roivainen M, Knip M, Hyoty H, Kulmala P, Hiltunen M, Vahasalo P, Hovi T, Akerblom HK: Several different enterovirus serotypes can be associated with prediabetic autoimmune episodes and onset of overt IDDM. Childhood Diabetes in Finland (DiMe) Study Group. J Med Virol 1998;56:74-78
18. Mullen Y: Development of the Nonobese Diabetic Mouse and Contribution of Animal Models for Understanding Type 1 Diabetes. Pancreas 2017;46:455-466
19. Sioofy-Khojine AB, Lehtonen J, Nurminen N, Laitinen OH, Oikarinen S, Huhtala H, Pakkanen O, Ruokoranta T, Hankaniemi MM, Toppari J, Vaha-Makila M, Ilonen J, Veijola R, Knip M, Hyoty H: Coxsackievirus B1 infections are associated with the initiation of insulin-driven autoimmunity that progresses to type 1 diabetes. Diabetologia 2018;61:1193-1202
20. Hamalainen S, Nurminen N, Ahlfors H, Oikarinen S, Sioofy-Khojine AB, Frisk G, Oberste MS, Lahesmaa R, Pesu M, Hyoty H: Coxsackievirus B1 reveals strain specific differences in plasmacytoid dendritic cell mediated immunogenicity. J Med Virol 2014;86:1412-1420
21. Flodstrom M, Maday A, Balakrishna D, Cleary MM, Yoshimura A, Sarvetnick N: Target cell defense prevents the development of diabetes after viral infection. Nat Immunol 2002;3:373-382
22. McCall KD, Thuma JR, Courreges MC, Benencia F, James CB, Malgor R, Kantake N, Mudd W, Denlinger N, Nolan B, Wen L, Schwartz FL: Toll-like receptor 3 is critical for coxsackievirus B4-induced type 1 diabetes in female NOD mice. Endocrinology 2015;156:453-461
23. Serreze DV, Wasserfall C, Ottendorfer EW, Stalvey M, Pierce MA, Gauntt C, O'Donnell B, Flanagan JB, Campbell-Thompson M, Ellis TM, Atkinson MA: Diabetes acceleration or prevention by a coxsackievirus B4 infection: critical requirements for both interleukin-4 and gamma interferon. J Virol 2005;79:1045-1052
24. Flodstrom M, Tsai D, Fine C, Maday A, Sarvetnick N: Diabetogenic potential of human pathogens uncovered in experimentally permissive beta-cells. Diabetes 2003;52:2025-2034
25. Hankaniemi MM, Laitinen OH, Stone VM, Sioofy-Khojine A, Maatta JAE, Larsson PG, Marjomaki V, Hyoty H, Flodstrom-Tullberg M, Hytonen VP: Optimized production and purification of Coxsackievirus B1 vaccine and its preclinical evaluation in a mouse model. Vaccine 2017;35:3718-3725
26. ProventionBio: Provention Bio Initiates First-in-Human Study of Coxsackievirus B Vaccine Candidate PRV-101 [Press Release]. 2020;

Figure legends:

Figure 1: CVB1-6 vaccine does not increase pancreatic islet inflammation (insulitis) in pre-diabetic NOD mice. Female NOD mice (mean age 5.5 weeks, range: 5.1 - 6.3 weeks) were

mock-vaccinated (buffer, n=13) or vaccinated with CVB1-6 vaccine (n=8) by i.s. injection on three occasions (on days 0, 14 and 28, n=3 or on days 0, 21 and 35, n=5). Mice were followed until 12 weeks of age (6-8 weeks after the first vaccination). (a) Average virus neutralizing antibody titers in the serum of CVB1-6 vaccinated mice against the six CVB serotypes on day 41/42 post the first vaccination dose. Sera from mock-vaccinated mice had no virus neutralizing capacity (data not shown). Shown are the mean neutralizing antibody titers ± SD with individual mice represented by a single symbol. Blue symbols represent neutralizing antibody titer data that was also published in (13) (b-d) Sections of formalin fixed paraffin embedded pancreas were scored in a blinded manner for islet immune infiltration as described in the Research Design and Methods section. (b) Representative images of islets with different scores as described in the Research Design and Methods and ESM Methods. (c) The total score per pancreas was divided by the total number of islets scored. Shown are the mean scores ± SD with. Each score from an individual animal is represented by a single symbol; buffer (black squares; n=13) or CVB1-6 vaccine (black circles; n=8). No statistically significant difference was found between the groups using an unpaired t test. (d) Data show the percentage of islets from each mouse that fall into each insulitis category assessed as illustrated in (b). Islets were scored as intact (0; black circles), peri-insulitis (1; black squares), insulitis (2; black triangles) or destroyed (3; black diamonds). No statistically significant differences were found between the groups using two-way ANOVA with Sidak's multiple comparison test.

Figure 2: Diabetes onset is not altered in NOD mice immunized with a CVB1-6 vaccine. (a-c) Female NOD mice (mean age 5.7 weeks, range 4.9 - 7.1 weeks) were left untreated (n=10), mock-vaccinated (n=15) or vaccinated (n=14) with CVB1-6 vaccine by i.s. injection on either two (days 0 and 21; n=6 for buffer, n=10 for CVB1-6 vaccine) or three (days 0, 21 and 35; n=9 for buffer, n=4 for CVB1-6 vaccine) occasions. (a) Average neutralizing antibody titers in the

serum of CVB1-6 vaccinated mice against the six CVB serotypes on day 42 post the first vaccination dose. Sera from untreated and mock-vaccinated mice had no neutralizing capacity. Shown are the mean virus neutralizing antibody titers ± SD with individual mice represented by a single symbol. Blue symbols represent virus neutralizing antibody titer data that was also published in (13). (b) Cumulative diabetes incidence and (c) average age at diabetes onset in the three groups. The dotted lines in (b) and (c) show the average age at vaccination. The mean age at diabetes onset ± SD is shown in (c) and the ages at which individual animals developed diabetes are displayed as single symbols. No statistically significant differences were found between the groups using Gehan-Breslow-Willcoxon test (b) one-way ANOVA with Tukey's multiple comparison test (c).

Figure 3: CVB1 vaccine protects against CVB1 accelerated disease in NOD mice. (a,b) Female NOD mice were left untreated (control; dotted line; n=31) or infected with CVB1 (10^7 PFU by i.p. injection, total volume 200µl) between 10.5 - 13.5 weeks of age (solid line; n=14) and diabetes incidence was followed up to 30 weeks of age. (a) Diabetes incidence curves of the two groups. The red arrow indicates the mean age at infection. The grey box shows the two-week period after virus infection, $p<0.001$ when comparing the diabetes incidence curves during this period by Gehan-Breslow-Wilcoxon test. The p value, $p=0.0103$, comes from the comparison of the two curves up to 30 weeks of age by Gehan-Breslow-Wilcoxon test. (b) Age at diabetes onset. Individual mice are represented by a single symbol and the horizontal line shows the mean age at diabetes onset ± SD. $p<0.0001$, unpaired t-test. (c-e) Female mice (6.3 – 6.9 weeks old) were left untreated (n=16), mock-vaccinated with vaccine buffer and infected with CVB1 virus (buffer + CVB1; n=16) or vaccinated with CVB1 vaccine and infected with CVB1 virus (vaccine + CVB1; n=12). Vaccinations were performed on days 0, 21 and 35 and the mice were infected with virus (10^7 PFU by i.p. injection, total volume 200µl) on day 42 (12.3 -12.9 weeks of age). Diabetes incidence was followed up to 30 weeks of age. (c) Neutralizing antibody titers on days 0 and 42 in mice vaccinated with the CVB1 vaccine as measured by standard plaque reduction assay. Neutralizing antibodies were not detected in the mock-vaccinated and untreated groups (data not shown). Individual mice are represented by a single symbol and the horizontal line shows the mean neutralizing antibody titer ± SD. $p<0.005$, unpaired t-test. (d) Diabetes incidence curves in the untreated (dotted line), buffer + CVB1 (dashed line) and vaccine + CVB1 (solid line) groups. The black arrows indicate the approximate vaccination ages and the red arrow indicates the approximate age when the mice were infected. The grey box shows the two-week period after virus infection, $p=0.008$ when comparing the diabetes incidence curves by Gehan-Breslow-Wilcoxon test. (e) Age at diabetes onset. Individual mice are represented by a single symbol and the horizontal line shows the mean age at diabetes onset ± SD. Groups compared by Kruskal-Wallis test with Dunn's multiple

comparison. In brackets are the p values generated when one mouse which was borderline diabetic from 15 weeks of age but didn't develop overt diabetes until 25 weeks of age was excluded (open square; buffer + CVB1), see Supplementary Fig. 3b for the blood glucose values. (f) Representative images of sequential pancreas sections stained with insulin (top row) and glucagon (bottom row) from mice that developed diabetes in the untreated (left hand column), mock vaccinated (buffer) + CVB1 (middle column) and vaccine + CVB1 (right hand column) groups. Positive areas are stained brown. Scale bars are present in the bottom left-hand corner of each image.

Title: Coxsackievirus B vaccines prevent infection-accelerated diabetes in NOD mice and have no disease-inducing effect~~have no disease accelerating effect but prevent infection-accelerated diabetes onset in NOD mice~~

Short running title: CVB vaccines do not alter diabetes onset but prevent virus-accelerated diabetes in NOD mice.

Authors names: Virginia M Stone[1], Marta Butrym[1], Minna M Hankaniemi[2], Amir-Babak Sioofy-Khojine[2], Vesa P Hytönen[2,3], Heikki Hyöty[2,3], Malin Flodström-Tullberg[1]

Institutions of origin:

[1] Center for Infectious Medicine, Department of Medicine Huddinge, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden.

[2] Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland.

[3] Fimlab Laboratories, Tampere, Finland.

Corresponding author:

Malin Flodström-Tullberg, Karolinska Institutet, +46 8 524 87625, malin.flodstrom-tullberg@ki.se

Word count: 3624~~058~~

Number of tables and figures: 3

Tweet: ~~Study exploring the efficacy and safety of a~~A Coxsackievirus B vaccine ~~made~~ designed to address the role of viruses in human type 1 diabetes prevents virus-accelerated disease onset and ~~shows that it~~ does not~~n't~~ alter diabetes onset in an autoimmune diabetes prone host ~~and prevents virus-accelerated disease onset~~ @MFT_Diabetes @CIM_Sweden @karolinskainst @HytonenL @HHyoty Lab @TampereUni

Abstract:

Enteroviruses, including the Coxsackievirus Bs (CVB), have been implicated as causal agents in human type 1 diabetes. Immunization of at-risk individuals with a CVB vaccine provides an attractive strategy for elucidating the role of CVBs in the disease etiology. Previously we have shown that an inactivated whole-virus vaccine covering all CVB serotypes (CVB1-6) is safe to administer and highly immunogenic in preclinical models, including non-human primates. Before initiating clinical trials with this type of vaccine it was also important to address whether a) the vaccine itself induces adverse immune reactions including accelerating diabetes onset in a diabetes prone host and b) the vaccine can prevent CVB induced diabetes in a well-established disease model. Here we present results from studies in which female NOD mice were left untreated, mock-vaccinated~~treated~~ or vaccinated with CVB1-6 vaccine and monitored for insulitis occurrence or diabetes development. We demonstrate that vaccination induces virus neutralizing antibodies without altering insulitis scores or the onset of diabetes. We also show that NOD mice vaccinated with a CVB1 vaccine are protected from CVB-induced accelerated disease onset. Taken together, these studies show that CVB vaccines do not alter islet inflammation or accelerate disease progression in an animal model that spontaneously develops autoimmune type 1 diabetes. However, they can ~~are capable of~~ prevent~~ing~~ CVB-mediated disease progression in the same model.

Type 1 diabetes is a common autoimmune disease caused by the destruction of the insulin producing pancreatic beta cells. Genetic and environmental factors are contributory, but their precise roles remain unclarified (1). Amongst the possible environmental triggers, viral infections have been widely studied and mounting evidence suggests that enteroviruses, especially the Coxsackievirus B (CVB) serotypes, may contribute to the development of type 1 diabetes (1-3).

A few schools of thought exist regarding the mechanisms through which CVBs may cause type 1 diabetes. Results from some studies support the notion that CVBs could be involved in initiating the disease process. For instance, it was found in the TEDDY (The Environmental Determinants of Diabetes in the Young) study that prolonged enterovirus B infections were associated with the development of islet autoimmunity but not type 1 diabetes (3). Similar results were seen in the DIPP (Diabetes Prediction and Prevention) study where associations were also documented between enterovirus infections and islet autoimmunity (4-6). An alternative hypothesis is that CVBs accelerate an on-going autoimmune process. Data from the DAISY (Diabetes Auto Immunity Study in the Young) study implies that enterovirus infections in autoantibody positive individuals increase the speed of progression to diabetes (7). This observation has been supported by animal models in which CVB infections accelerates the onset of diabetes in pre-diabetic animals (8-10). It is of course feasible that both ~~of these~~ hypotheses ~~could~~ hold true and enteroviruses may contribute to the development of type 1 diabetes in both manners.

To determine the causal role of CVBs in human type 1 diabetes, vaccine development initiatives have been undertaken (9; 11-14). A non-adjuvanted inactivated vaccine comprising of the six CVB1-6 serotypes was recently shown to be highly immunogenic in mice and non-human

primates in preclinical studies (13). Furthermore, this vaccine did not alter weight gain and blood glucose levels in both models and had no effect on temperature and hematological readouts in rhesus macaques, demonstrating an excellent safety profile (13).

The recent introduction of new vaccines in the human population has shown that adverse events may occur. These include associations between vaccination and the occurrence of autoimmune diseases (15; 16). As the current CVB vaccine is based on inactivated whole virus particles and CVB virus infections have been associated with both the initiation and progression of the processes that lead to type 1 diabetes (3; 5-7; 17), it is also paramount to ensure that vaccination itself doesn't affect the onset of autoimmune diabetes in a similar manner to infectious virus.

Here, we present the results from pre-clinical studies testing whether vaccination of young, CVB-naïve female NOD mice (a model prone to develop autoimmune diabetes (18)) with a multivalent CVB vaccine accelerates disease onset or increases diabetes incidence. Further to this, we also examined whether this type of vaccine ~~is capable of providing~~can provide protection against the acceleration in diabetes onset seen after CVB infection of NOD mice that are in the pre-diabetic phase.

Research Design and Methods:

Animal husbandry and monitoring of animal health

NOD mice were bred in-house and housed in specific pathogen-free conditions at Karolinska Institutet, Stockholm, Sweden. A local ethics committee granted approval for all experiments which were performed in accordance with the NIH principles of Laboratory Animal Care and

national laws in Sweden. Animals were housed in ventilated cages and provided with water and food *ad libitum*. A maximum number of 5 mice were housed per cage and no mice were single housed. Extended health monitoring of mice was performed including examining changes in health status (weight changes, alterations in natural behaviour, porphyria, movement and posture, piloerection, respiration and skin). Animals were randomly assigned to treatment groups. Weight and blood glucose measurements were monitored weekly until the experimental endpoint (diabetes onset, a health score of 0.4 or higher, or when the animals had reached the defined ~~end defined as the~~ end point of the experiment). The researchers were not blinded to the experimental groups during the experiments. At the experimental endpoint, mice were anaesthetised with isoflurane, a terminal heart puncture was performed for blood drawing and the animals were then euthanized by cervical dislocation.

Vaccine production

CVB1-6 and CVB1 vaccines were produced by formalin inactivation of the CVB1-6 or CVB1 ~~-6~~ serotypes (13). The vaccine was then formulated in Medium M199 (Gibco, Thermofisher Scientific, Vanda, Finland) ~~with~~ containing 0.1% Tween 80 by mixing 1µg of each inactivated virus serotype per dose for the CVB1-6 vaccine or 1.8µg for the CVB1 vaccine.

Vaccination strategies

Female age-matched NOD mice (4.9 – 7.1 weeks old) were randomly assigned to treatment groups (untreated, mock-~~–~~vaccinated or vaccinated). Animals were either left untreated, vaccinated with non-adjuvanted CVB1-6 vaccine on two or three occasions, 2-3 weeks apart, vaccinated with CVB1 vaccine on 3 occasions, 2-3 weeks apart, or mock-vaccinated with

For Peer Review Only

vaccine buffer alone (M199 Medium + 0.1% Tween 80 + 0.001% formalin, v/v). Each vaccination was performed by subcutaneous (interscapular) injection (150µl). Serum samples were collected from the tail vein when indicated in the text. Animals were either euthanized 6 weeks later (Fig. 1), monitored for diabetes incidence up until the age of 30 weeks (Fig. 2) or infected with virus (Fig. 3) as described under CVB1 infection.

CVB1 infection

Female NOD mice (10.5 - 13.5 weeks old) were randomly assigned to either control (n=31) or CVB1 infection (n=14; 10^7 plaque forming units (PFU) CVB1 by intraperitoneal (i.p.) injection, total volume 200µl; Fig. 3a, b) groups. In other experiments (Fig. 3c-e), female NOD mice (6.3 – 6.9 weeks old) were assigned to untreated (n=16), mock-vaccination (n=16) or CVB1 vaccine (n=12) groups and vaccinated as described in Vaccination strategies above. Mice in the mock- and CVB1-vaccine groups were infected with CVB1 (10^7 PFU by i.p. injection, total volume 200µl) one week after the final vaccination (approximately 12-13 weeks old). In both experimental set ups diabetes incidence was followed up until 30~~25~~ weeks of age/diabetes onset.

Blood glucose measuring and monitoring of diabetes incidence

Blood glucose concentrations were measured in blood drawn from the tail vein using a Bayer Contour XT blood glucose meter (Bayer, Basel, Switzerland). Diabetes was defined as a blood glucose value ≥ 18 mmol/l. If the blood glucose value was between 13 and 18 mmol/l the mouse was checked the next day and if it remained > 13mmol/l the mouse was deemed diabetic.

Neutralizing antibody measurements

CVB1-6 neutralizing antibody titers were measured by a standard virus plaque reduction assay using GMK cells (National Institute for Health and Welfare, Finland; mycoplasma negative; (4; 17; 19)). In short, serum was serially diluted starting with a 1:4 dilution and was mixed with 100 PFU of the respective CVB serotypes used to produce the vaccine (for details regarding the viruses, see (13; 20)). The serum-virus suspensions were incubated for 1 hour at 37°C and then overnight at room temperature. GMK-cells were grown to 95% confluency in 12-well plates and the virus-serum mixture was added to these cells and incubated at 37°C for 1h, then replaced with a semisolid medium (minimum essential medium supplemented with 0.67% carboxymethylcellulose – Merck, Sigma-Aldrich, Finland). Plates were incubated for 2 days at 37°C, then the cells were fixed and stained with formaldehyde-crystal violet solution. Plaque numbers were counted with the researchers blinded to the treatment groups, and serum samples which had a reduction in plaque numbers of 80% or more compared to an untreated virus control were deemed to be positive for neutralizing antibodies. This assay has a technical detection limit of 1:4 and serum sample positivity for neutralizing antibodies was set to a dilution ≥ 1:16.

Histology and immunohistochemistry

Mouse pancreases were collected, formalin-fixed in 4% paraformaldehyde overnight and embedded in paraffin. Organs were cut into 5-µm thick sections. For the insulitis scoring (Fig. 1), eEach pancreas was sectioned in two-three levels with >20 sections difference between each level (100-µm) and for the histological assessment in Fig. 3 and Supplementary Fig. 4, sections from one level of the pancreas were used. . Sections were deparaffinized and stained with primary antibodies against insulin (1:20,000; A0564, Dako, Ely, UK) or glucagon (1:12,000; EP3070, Abcam, Cambridge UK; both validated in formalin-fixed paraffin--embedded murine

pancreas sections) and counter stained with hematoxylin using standard immunohistochemical techniques (as described in (9; 21)).

Insulitis scoring

Pancreas sections stained with insulin and glucagon were assessed (in a blinded manner) by light microscopy by two investigators and ranked for insulitis according to the following ranking method. 0- healthy islet with normal morphology with no mononuclear cells surrounding or infiltrating the islets; 1- peri-insulitis: mononuclear cells surrounding the islets on the periphery of the islets; 2- insulitis: infiltration of mononuclear cells into the islet; 3- infiltrated islet with no signs of insulin staining (denoted destroyed islet). See Fig. 1b for an example of islets with different scores. An insulitis score for each mouse was obtained by calculating the scores for each pancreas and dividing this total score by the number of islets examined. Data is presented as mean insulitis score ± SD for each treatment group.

Statistical analysis

Statistical analyses were performed using Prism 9 software (GraphPad, La Jolla, CA). Insulitis scores (index), CVB1 neutralizing antibody titers and age at diabetes onset (CVB1-infected mice) were analyzed by an unpaired t-test. Percentage of islets with differing insulitis scores was assessed by two-way ANOVA with Sidak's multiple comparison test. Age at diabetes onset (CVB1-6 vaccinated mice) was analyzed by one-way ANOVA with Tukey's multiple comparison test. Diabetes survival curves were assessed by Gehan-Breslow-Willcoxon test. In the studies examining virus-accelerated diabetes onset, the differences in the survival curves were assessed two weeks after infection when the acceleration in disease onset is expected to occur by Gehan-Breslow-Willcoxon test, as described in (22; 23). Age at diabetes onset in the

CVB1 vaccine studies was assessed by Kruskal Wallis test with Dunn's multiple comparisons. Data are expressed as mean ± SD. A p value ≤ 0.05 was considered statistically significant.

Data and Resource availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. No applicable resources were generated or analyzed during the current study.

Results:

A CVB1-6 vaccine does not aggravate insulitis in NOD mice

First, we studied whether the CVB1-6 vaccine alters pancreatic islet inflammation in ~~pre-diabetic,~~ age-matched female NOD mice. Young mice that had no previous exposure to CVBs were vaccinated three times (on days 0, 14 and 28, n=3 or on days 0, 21 and 35, n=5) with the CVB1-6 vaccine or with vaccine buffer ~~alone~~ (n=13) and their pancreases were assessed at around 12 weeks of age. As seen before (13), ~~immunized~~ vaccinated mice had CVB1-6 neutralizing antibodies by day 41/42 after the initial vaccination dose (Fig. 1a) which were absent on day 0 (data not shown). Neutralizing antibody data shown in blue was previously presented in (13) but the pancreas ~~were~~ had not been assessed for insulitis. Moreover, the CVB1-6 vaccine had no negative effects on animal weight and blood glucose levels up to 6 weeks post-initial vaccination (the endpoint of the study; Supplementary Fig. 1a-f). Pancreatic islet inflammation was ~~assessed~~assessed and the average number of islets scored per animal was 30 ± 14 (range: 9 – 64). All animals showed signs of pancreatic islet inflammation but no

significant differences in pancreatic insulitis scores between mock- and CVB1-6 ~~immunized~~vaccinated mice were observed (Fig. 1b-d). These results imply that the CVB1-6 vaccine does not alter immune cell infiltration in the pancreatic islets of Langerhans.

Diabetes onset is not affected in NOD mice vaccinated with a CVB1-6 vaccine

Next, we examined the safety of the CVB1-6 vaccine with regards to diabetes development in NOD mice. To address whether the vaccine changed the onset of diabetes in NOD mice, young animals were left untreated (n=10), mock-vaccinated (n=15) or vaccinated with CVB1-6 vaccine (n=14) two-three times on days 0, 21 and 35. Blood glucose levels were monitored until 30 weeks of age or until diabetes onset when the mice were removed. Vaccine immunogenicity was confirmed by CVB1-6 neutralizing antibody responses (Fig. 2a) which were absent at day 0 in all mice and at day 42 in untreated and mock-vaccinated~~immunized~~ mice (data not shown). No detrimental outcomes on weight (Supplementary Fig. 2) or general health status were seen. CVB1-6 vaccination did not alter the incidence of diabetes compared to the mock-vaccinated and untreated groups and the kinetics of diabetes onset did not differ between the groups (Fig. 2b). Likewise, no differences were seen in the mean age at diabetes onset when comparing animals from the three groups (Fig. 2c). Taken together, this data indicates that the CVB1-6 vaccine does not alter the development of autoimmune diabetes in NOD mice.

A CVB vaccine protects against CVB1-accelerated diabetes onset in NOD mice

CVB infections have been implicated in type 1 diabetes in humans and have also been shown to accelerate the onset of diabetes in pre-diabetic mice (7-10). As such, we next decided to examine whether vaccination can prevent the accelerating effect that CVB infection has on the

development of diabetes in NOD mice. ~~We f~~First, we confirmed that CVB infection accelerates the onset of diabetes in pre-diabetic female NOD mice in our colony. Pre-diabetic animals were left untreated or infected with CVB1 and the incidence of diabetes was monitored up to 30~~25~~ weeks of age. CVB1 infected mice developed diabetes faster than the control group (Fig. 3a) and the mean age at diabetes onset was significantly lower in infected animals (13.1 weeks old) compared to the controls (19.9~~1~~ weeks old; Fig. 3b).

We subsequently ~~next~~ wanted to see if a CVB vaccine could protect against this virus-mediated acceleration in diabetes onset. Female NOD mice were either left untreated, mock-~~-~~vaccinated and then infected with CVB1 (mock + CVB1~~), or~~) or vaccinated and then infected with CVB1 (vaccine + CVB1). Diabetes incidence was monitored until the mice were 30~~25~~ weeks old. To ensure the vaccine was immunogenic, virus neutralizing antibodies were measured in serum collected prior to infection (day 42). Mice ~~immunized~~ vaccinated with the CVB1 vaccine induced a good neutralizing antibody response (Fig. 3c) which was absent in mock-vaccinated animals (data not shown). As expected, an acceleration in diabetes onset was seen in the ~~buffer~~ mock-vaccinated (buffer) + CVB1 group compared to untreated mice (Fig. 3d). In comparison, the CVB1 vaccine protected against CVB1-mediated acceleration in diabetes onset and the survival curve in the vaccine + CVB1 group mirrored that of the untreated animals (Fig. 3d). Significant differences between the curves were detected in the 2 weeks after infection when the majority of ~~the~~ acceleration occurs. Moreover, the mean age at diabetes onset was lower in the mock-vaccinated (buffer) + CVB1 group (16.3 weeks old; Fig. 3e) than in the vaccine + CVB1 group (21.9 weeks old; Fig. 3e) and the untreated group (19.6 weeks old; Fig. 3e). ~~similarthe same in the untreated and vaccine + CVB1 groups (both(19.620.1 weeks and 21.9 weeks old respectively) but was lower in the mock-vaccinated (buffer) + CVB1 group (16.35 weeks old; Fig. 3e).~~

The protective capacity of the vaccine was further illustrated when pancreas integrity was compared between the untreated, mock-vaccinated (buffer) + CVB1 and vaccine + CVB1 groups at the onset of diabetes (Fig. 3f). Vaccinated animals had healthy exocrine tissue morphology at the time of diabetes onset in a similar manner to untreated animals, whereas there was significant exocrine tissue destruction in the mock-vaccinated (buffer) group as shown by the representative images in Fig. 3f. Differences were also seen between these groups in the animals that did not develop diabetes by 30 weeks of age. There was evidence of exocrine tissue loss in the pancreas of mock-vaccinated animals as illustrated by the presence of islets in fat tissue (Supplementary Fig. 4 e,f), although some exocrine tissue ~~had~~had either remained healthy ~~or regenerated~~or regenerated in these animals (Supplementary Fig. 4 c,d). In contrast, normal pancreas histology was seen in the untreated and vaccinated groups (Supplementary Fig. 4 a,b,g,h). Collectively, t~~T~~hese studies show that a CVB vaccine protects against CVB1-accelerated diabetes in NOD mice.

Discussion:

Pre-clinical studies are an important part of initial vaccine efficacy and safety assessments. These studies serve to identify elements that require further assessment and can also help to design vaccination schedules. Additionally, ~~These studies may additionally~~they may uncover adverse events including undesired immune reactions that can, for example, lead to, autoimmune diseases. Such diseases have occurred, albeit rarely, after immunization with other vaccines (15; 16). Our studies demonstrate that a multivalent CVB vaccine does not accelerate the onset of diabetes in NOD mice, a commonly used animal model for type 1 diabetes. We confirmed that early vaccination with this vaccine induces virus neutralizing antibodies and

showed that immunity to CVBs is achieved without altering islet inflammation or changing the average time to diabetes onset. These results are in line with our previous observation that vaccination of pre-diabetic NOD mice with a monovalent CVB1 vaccine did not increase the production of insulin autoantibodies (9). This also suggests that inactivation of the viruses abolishes the diabetogenic properties of the CVBs, which have previously been observed in the NOD mouse (8-10) and which are suspected in humans (4; 7).

Human cohort studies focused on understanding the triggers of type 1 diabetes have produced results suggesting that CVBs could be critically involved at different stages of the disease. In the TEDDY and DIPP studies, enterovirus infections were associated with the development of islet-specific autoantibodies (3-6). In contrast, the DAISY study reported that enterovirus infections accelerated the speed of progression to overt diabetes in autoantibody positive individuals (7). Different animal models exist that may replicate how CVBs could contribute to type 1 diabetes development in humans, as alluded to in the cohort studies. Direct infection of the beta-cell by CVBs is a possible mechanism through which beta-cell autoimmunity could be induced. In our previous studies using the SOCS-1-tg mouse model, where the beta-cells are susceptible to CVB infection leading to diabetes (21; 24), we have shown that CVB vaccines can prevent virus-induced diabetes (13; 14). It is also possible to mimic virus-acceleration of an on-going autoimmune process by infecting pre-diabetic NOD mice with CVBs (8-10). In this study we report for the first time that a CVB vaccine is also capable of preventing virus-mediated acceleration in diabetes onset. Type 1 diabetes appears to be a highly heterogenous disease and it is feasible that both virus-induced autoimmunity and acceleration in the rate of diabetes onset in autoantibody-positive individuals after virus infection could occur in different groups. The ability of CVB vaccines to prevent both forms of virus-mediated diabetes in

For Peer Review Only

relevant pre-clinical models provides excellent proof-of-concept evidence for the use of such a vaccine to elucidate the multiple potential roles of CVBs in human type 1 diabetes.

Based on the aforementioned studies (amongst others) that suggest enteroviruses may have an important role in type 1 diabetes and from promising results using the mono- and current multivalent CVB vaccine in pre-clinical studies (9; 13; 14; 25), the production and clinical testing of a similar multivalent CVB vaccine was recently initiated (11; 12; 26). Our previous work with experimental CVB vaccines demonstrates that such vaccines show strong potential for use in the prevention of CVB infections and diseases associated with these infections in humans (13; 14). We also found that there were no adverse effects on glucose regulation (13) and no conspicuous infiltration of immune cells in the pancreas (Stone et al. unpublished observation) in rhesus macaques immunized with the multivalent CVB vaccine. The present study builds on these foundations by suggesting that this type of vaccine does not alter islet inflammation or diabetes onset in a preclinical mouse model for autoimmune type 1 diabetes. In summary, this study provides data that supports the use of an equivalent vaccine in human clinical trials to establish whether CVBs are involved in type 1 diabetes. Such trials will involve the immunization of young children with a genetic predisposition for the disease who are yet to experience a CVB infection. If the involvement of CVBs in type 1 diabetes is confirmed, the vaccine could provide a viable preventative measure~~treatment~~ for this disease.

Article information

Acknowledgements. We would like to acknowledge S. Parvin, Karolinska Institutet, Stockholm, Sweden, for her help with processing and staining histological samples, the M.F-T. group for scientific discussions and laboratory assistance, and the animal staff at the PKL facility at Karolinska Institutet, Stockholm, Sweden, for their assistance with the animal studies.

M. Jokela from the Faculty of Medicine and Health Technology (Tampere University, Finland) is acknowledged for their assistance with the vaccine production and M. Kekäläinen and M. Ovaskainen from the Faculty of Medicine and Health Technology (Tampere University, Finland) for the analysis of virus antibodies. We also would like to acknowledge Biocenter Finland for their infrastructure support.

Funding. We would like to acknowledge financial support from the Swedish Child Diabetes Foundation, the Swedish Diabetes Foundation, Karolinska Institutet including the Strategic Research Program in Diabetes, the Business Finland (formerly TEKES; THERDIAB project, diary no. 1843/31/2014), the Academy of Finland (grant 309455 awarded to MMH and grant 288671 awarded to HH), the Sigrid Jusélius Foundation, the Reino Lahtikari Foundation and the JDRF (2-SRA-2017-A-N).

~~**Duality of interest**~~**Conflict of Interest Statement.** HH owns stocks and is the chairman of the board of Vactech Ltd, which develops vaccines against picornaviruses. HH and MFT serve on the scientific advisory board of Provention Bio Inc., which is developing a clinical CVB vaccine in collaboration with Vactech Ltd. The other authors have no conflict of interest to declare.

Author Contributions. VMS, MMH, VPH, ABSK, HH and MFT designed the study, MMH produced and performed quality control analyses of the vaccine, VMS and MB performed experiments, VMS, MB, ABSK and MFT analyzed results, VMS, MB and MFT wrote and edited the manuscript. All authors read, edited and approved the final manuscript. MFT and HH are the guarantors of this work, and as such had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. This study was presented, in part, in abstract form at the 12th Annual and 13th Annual nPOD Meetings (23rd-26th February 2020 and 22nd-24th February 2021).

References

1. DiMeglio LA, Evans-Molina C, Oram RA: Type 1 diabetes. Lancet 2018;391:2449-2462
2. Richardson SJ, Morgan NG: Enteroviral infections in the pathogenesis of type 1 diabetes: new insights for therapeutic intervention. Curr Opin Pharmacol 2018;43:11-19
3. Vehik K, Lynch KF, Wong MC, Tian X, Ross MC, Gibbs RA, Ajami NJ, Petrosino JF, Rewers M, Toppari J, Ziegler AG, She JX, Lernmark A, Akolkar B, Hagopian WA, Schatz DA, Krischer JP, Hyoty H, Lloyd RE, Group TS: Prospective virome analyses in young children at increased genetic risk for type 1 diabetes. Nat Med 2019;25:1865-1872
4. Laitinen OH, Honkanen H, Pakkanen O, Oikarinen S, Hankaniemi MM, Huhtala H, Ruokoranta T, Lecouturier V, Andre P, Harju R, Virtanen SM, Lehtonen J, Almond JW, Simell T, Simell O, Ilonen J, Veijola R, Knip M, Hyoty H: Coxsackievirus B1 is associated with induction of beta-cell autoimmunity that portends type 1 diabetes. Diabetes 2014;63:446-455
5. Oikarinen S, Martiskainen M, Tauriainen S, Huhtala H, Ilonen J, Veijola R, Simell O, Knip M, Hyoty H: Enterovirus RNA in blood is linked to the development of type 1 diabetes. Diabetes 2011;60:276-279
6. Salminen K, Sadeharju K, Lonnrot M, Vahasalo P, Kupila A, Korhonen S, Ilonen J, Simell O, Knip M, Hyoty H: Enterovirus infections are associated with the induction of beta-cell autoimmunity in a prospective birth cohort study. J Med Virol 2003;69:91-98
7. Stene LC, Oikarinen S, Hyoty H, Barriga KJ, Norris JM, Klingensmith G, Hutton JC, Erlich HA, Eisenbarth GS, Rewers M: Enterovirus infection and progression from islet autoimmunity to type 1 diabetes: the Diabetes and Autoimmunity Study in the Young (DAISY). Diabetes 2010;59:3174-3180
8. Horwitz MS, Bradley LM, Harbertson J, Krahl T, Lee J, Sarvetnick N: Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. Nat Med 1998;4:781-785
9. Larsson PG, Lakshmikanth T, Laitinen OH, Utorova R, Jacobson S, Oikarinen M, Domsgen E, Koivunen MR, Chaux P, Devard N, Lecouturier V, Almond J, Knip M, Hyoty H, Flodstrom-Tullberg M: A preclinical study on the efficacy and safety of a new vaccine against Coxsackievirus B1 reveals no risk for accelerated diabetes development in mouse models. Diabetologia 2015;58:346-354
10. Serreze DV, Ottendorfer EW, Ellis TM, Gauntt CJ, Atkinson MA: Acceleration of type 1 diabetes by a coxsackievirus infection requires a preexisting critical mass of autoreactive T-cells in pancreatic islets. Diabetes 2000;49:708-711
11. Dunne JL, Richardson SJ, Atkinson MA, Craig ME, Dahl-Jorgensen K, Flodstrom-Tullberg M, Hyoty H, Insel RA, Lernmark A, Lloyd RE, Morgan NG, Pugliese A: Rationale for enteroviral vaccination and antiviral therapies in human type 1 diabetes. Diabetologia 2019;
12. Hyoty H, Leon F, Knip M: Developing a vaccine for Type 1 diabetes by targeting coxsackievirus B. Expert Rev Vaccines 2018;

13. Stone VM, Hankaniemi MM, Laitinen OH, Sioofy-Khojine AB, Lin A, Diaz Lozano IM, Mazur MA, Marjomaki V, Lore K, Hyoty H, Hytonen VP, Flodstrom-Tullberg M: A hexavalent Coxsackievirus B vaccine is highly immunogenic and has a strong protective capacity in mice and nonhuman primates. Sci Adv 2020;6:eaaz2433
14. Stone VM, Hankaniemi MM, Svedin E, Sioofy-Khojine A, Oikarinen S, Hyoty H, Laitinen OH, Hytonen VP, Flodstrom-Tullberg M: A Coxsackievirus B vaccine protects against virus-induced diabetes in an experimental mouse model of type 1 diabetes. Diabetologia 2018;61:476-481
15. Schultz NH, Sorvoll IH, Michelsen AE, Munthe LA, Lund-Johansen F, Ahlen MT, Wiedmann M, Aamodt AH, Skattor TH, Tjonnfjord GE, Holme PA: Thrombosis and Thrombocytopenia after ChAdOx1 nCoV-19 Vaccination. N Engl J Med 2021;
16. Segal Y, Shoenfeld Y: Vaccine-induced autoimmunity: the role of molecular mimicry and immune crossreaction. Cell Mol Immunol 2018;15:586-594
17. Roivainen M, Knip M, Hyoty H, Kulmala P, Hiltunen M, Vahasalo P, Hovi T, Akerblom HK: Several different enterovirus serotypes can be associated with prediabetic autoimmune episodes and onset of overt IDDM. Childhood Diabetes in Finland (DiMe) Study Group. J Med Virol 1998;56:74-78
18. Mullen Y: Development of the Nonobese Diabetic Mouse and Contribution of Animal Models for Understanding Type 1 Diabetes. Pancreas 2017;46:455-466
19. Sioofy-Khojine AB, Lehtonen J, Nurminen N, Laitinen OH, Oikarinen S, Huhtala H, Pakkanen O, Ruokoranta T, Hankaniemi MM, Toppari J, Vaha-Makila M, Ilonen J, Veijola R, Knip M, Hyoty H: Coxsackievirus B1 infections are associated with the initiation of insulin-driven autoimmunity that progresses to type 1 diabetes. Diabetologia 2018;61:1193-1202
20. Hamalainen S, Nurminen N, Ahlfors H, Oikarinen S, Sioofy-Khojine AB, Frisk G, Oberste MS, Lahesmaa R, Pesu M, Hyoty H: Coxsackievirus B1 reveals strain specific differences in plasmacytoid dendritic cell mediated immunogenicity. J Med Virol 2014;86:1412-1420
21. Flodstrom M, Maday A, Balakrishna D, Cleary MM, Yoshimura A, Sarvetnick N: Target cell defense prevents the development of diabetes after viral infection. Nat Immunol 2002;3:373-382
22. McCall KD, Thuma JR, Courreges MC, Benencia F, James CB, Malgor R, Kantake N, Mudd W, Denlinger N, Nolan B, Wen L, Schwartz FL: Toll-like receptor 3 is critical for coxsackievirus B4-induced type 1 diabetes in female NOD mice. Endocrinology 2015;156:453-461
23. Serreze DV, Wasserfall C, Ottendorfer EW, Stalvey M, Pierce MA, Gauntt C, O'Donnell B, Flanagan JB, Campbell-Thompson M, Ellis TM, Atkinson MA: Diabetes acceleration or prevention by a coxsackievirus B4 infection: critical requirements for both interleukin-4 and gamma interferon. J Virol 2005;79:1045-1052
24. Flodstrom M, Tsai D, Fine C, Maday A, Sarvetnick N: Diabetogenic potential of human pathogens uncovered in experimentally permissive beta-cells. Diabetes 2003;52:2025-2034
25. Hankaniemi MM, Laitinen OH, Stone VM, Sioofy-Khojine A, Maatta JAE, Larsson PG, Marjomaki V, Hyoty H, Flodstrom-Tullberg M, Hytonen VP: Optimized production and purification of Coxsackievirus B1 vaccine and its preclinical evaluation in a mouse model. Vaccine 2017;35:3718-3725
26. ProventionBio: Provention Bio Initiates First-in-Human Study of Coxsackievirus B Vaccine Candidate PRV-101 [Press Release]. 2020;

Figure legends:

Figure 1: CVB1-6 vaccine does not increase pancreatic islet inflammation (insulitis) in pre-diabetic NOD mice. Female NOD mice (mean age 5.5 weeks, range: 5.1 - 6.3 weeks) were mock--vaccinated (buffer, n=13) or vaccinated with CVB1-6 vaccine (n=8) by i.s. injection on three occasions (on days 0, 14 and 28, n=3 or on days 0, 21 and 35, n=5). Mice were followed until 12 weeks of age (6-8 weeks after the first vaccination). (a) Average virus neutralizing antibody titers in the serum of CVB1-6 vaccinated mice against the six CVB serotypes on day 41/42 post the first vaccination dose. Sera from mock-vaccinated mice had no virus neutralizing capacity (data not shown). Shown are the mean neutralizing antibody titers ± SD with individual mice represented by a single symbol. Blue symbols represent neutralizing antibody titer data that was also published in (13) (b-d) Sections of formalin fixed paraffin embedded pancreas were scored in a blinded manner for islet immune infiltration as described in the Research Design and Methods section. (b) Representative images of islets with different scores as described in the Research Design and Methods and ESM Methods. (c) The total score per pancreas was divided by the total number of islets scored. Shown are the mean scores ± SD with. Each score from an individual animal is represented by a single symbol; buffer (black squares; n=13) or CVB1-6 vaccine (black circles; n=8). No statistically significant difference was found between the groups using an unpaired t test. (d) Data show the percentage of islets from each mouse that fall into each insulitis category assessed as illustrated in (b). Islets were scored as intact (0; black circles), peri-insulitis (1; black squares), insulitis (2; black triangles) or destroyed (3; black diamonds). No statistically significant differences were found between the groups using two-way ANOVA with Sidak's multiple comparison test.

Figure 2: Diabetes onset is not altered in NOD mice immunized with a CVB1-6 vaccine. (a-c) Female NOD mice (mean age 5.7 weeks, range 4.9 - 7.1 weeks) were left untreated (n=10), mock--vaccinated (n=15) or vaccinated (n=14) with CVB1-6 vaccine by i.s. injection on either two (days 0 and 21; n=6 for buffer, n=10 for CVB1-6 vaccine) or three (days 0, 21 and 35; n=9 for buffer, n=4 for CVB1-6 vaccine) occasions. (a) Average neutralizing antibody titers in the serum of CVB1-6 vaccinated mice against the six CVB serotypes on day 42 post the first vaccination dose. Sera from untreated and mock-vaccinated mice had no neutralizing capacity. Shown are the mean virus neutralizing antibody titers ± SD with individual mice represented by a single symbol. Blue symbols represent virus neutralizing antibody titer data that was also published in (13). (b) Cumulative diabetes incidence and (c) average age at diabetes onset in the three groups. The dotted lines in (b) and (c) show the average age at vaccination. The mean age at diabetes onset ± SD is shown in (c) and the ages at which individual animals developed diabetes are displayed as single symbols. No statistically significant differences were found between the groups using Gehan-Breslow-Willcoxon test (b) one-way ANOVA with Tukey's multiple comparison test (c).

For Peer Review Only

Figure 3: CVB1 vaccine protects against CVB1 accelerated disease in NOD mice. (a,b) Female NOD mice were left untreated (control; dotted line; n=31) or infected with CVB1 (10^7 PFU by i.p. injection, total volume 200µl) between 10.5 - 13.5 weeks of age (solid line; n=14) and diabetes incidence was followed up to 30~~25~~ weeks of age. (a) Diabetes incidence curves of the two groups. The red arrow indicates the mean age at infection. The grey box shows the two-week period after virus infection, $p<0.001$ when comparing the diabetes incidence curves during this period by Gehan-Breslow-Wilcoxon test. The p value, p=0.0103~~05~~, comes from the comparison of the two curves up to 30~~25~~ weeks of age by Gehan-Breslow-Wilcoxon test. (b) Age at diabetes onset. Individual mice are represented by a single symbol and the horizontal line shows the mean age at diabetes onset ± SD. $p<0.0001$, unpaired t-test. (c-e) Female mice (6.3 – 6.9 weeks old) were left untreated (n=16~~5~~), mock-~~-~~vaccinated with vaccine buffer and infected with CVB1 virus (buffer + CVB1; n=16) or vaccinated with CVB1 vaccine and infected with CVB1 virus (vaccine + CVB1; n=12). Vaccinations were performed on days 0, 21 and 35 and the mice were infected with virus (10^7 PFU by i.p. injection, total volume 200µl) on day 42 (12.3 -12.9 weeks of age). Diabetes incidence was followed up to 30 weeks of age. (c) Neutralizing antibody titers on days 0 and 42 in mice vaccinated with the CVB1 vaccine as measured by standard plaque reduction assay. Neutralizing antibodies were not detected in the ~~buffer treated~~mock-vaccinated and untreated groups (data not shown). Individual mice are represented by a single symbol and the horizontal line shows the mean neutralizing antibody titer ± SD. $p<0.005$, unpaired t-test. (d) Diabetes incidence curves in the untreated (dotted line), buffer + CVB1 (dashed line) and vaccine + CVB1 (solid line) groups. The black arrows indicate the approximate vaccination ages and the red arrow indicates the approximate age when the mice were infected. The grey box shows the two-week period after virus infection, p=0.008 when comparing the diabetes incidence curves by Gehan-Breslow-Wilcoxon test. (e) Age at diabetes onset. Individual mice are represented by a single symbol and the horizontal line shows the mean age at diabetes onset ± SD. Groups compared by Kruskal-Wallis test with

Dunn's multiple comparison. In brackets are the p values generated when one mouse which was borderline diabetic from 15 weeks of age but didn't develop overt diabetes until 25 weeks of age was excluded (open square; buffer + CVB1), see Supplementary Fig. 3b for the blood glucose values. (f) Representative images of sequential pancreas sections stained with insulin (top row) and glucagon (bottom row) from mice that developed diabetes in the untreated (left hand column), mock vaccinated (buffer) + CVB1 (middle column) and vaccine + CVB1 (right hand column) groups. Positive areas are stained brown. Scale bars are present in the bottom left-hand corner of each image.

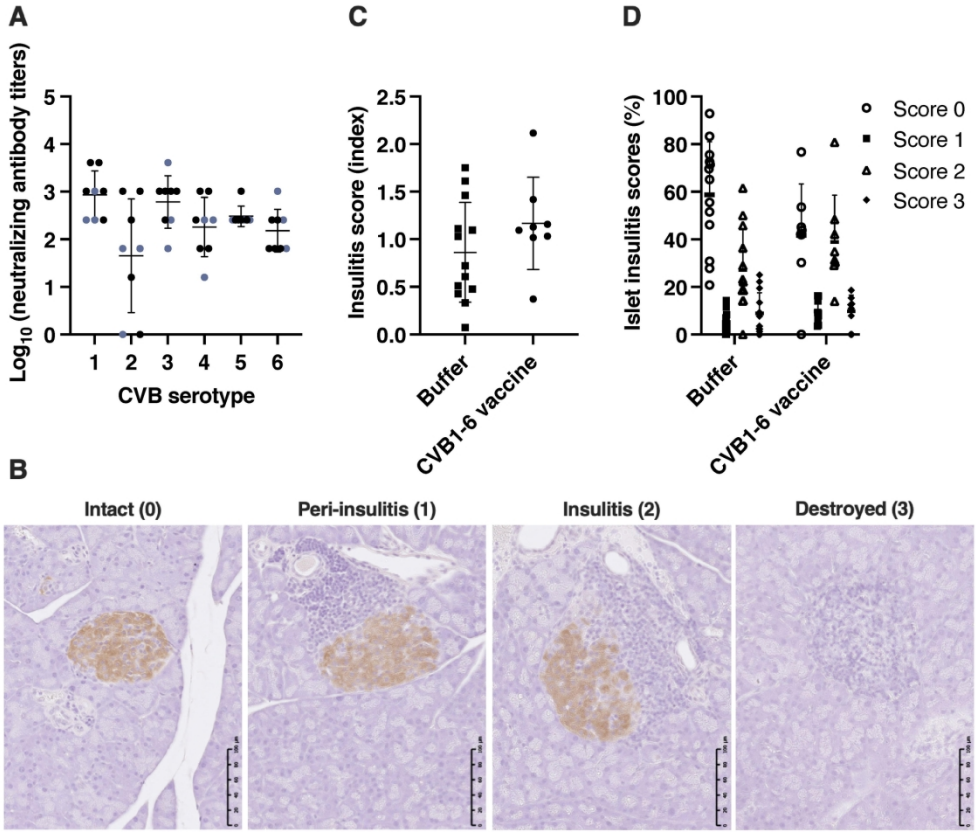


Figure 1: CVB1-6 vaccine does not increase pancreatic islet inflammation (insulitis) in pre-diabetic NOD mice. Female NOD mice (mean age 5.5 weeks, range: 5.1 - 6.3 weeks) were mock-vaccinated (buffer, n=13) or vaccinated with CVB1-6 vaccine (n=8) by i.s. injection on three occasions (on days 0, 14 and 28, n=3 or on days 0, 21 and 35, n=5). Mice were followed until 12 weeks of age (6-8 weeks after the first vaccination). (a) Average virus neutralizing antibody titers in the serum of CVB1-6 vaccinated mice against the six CVB serotypes on day 41/42 post the first vaccination dose. Sera from mock-vaccinated mice had no virus neutralizing capacity (data not shown). Shown are the mean neutralizing antibody titers ± SD with individual mice represented by a single symbol. Blue symbols represent neutralizing antibody titer data that was also published in (13) (b-d) Sections of formalin fixed paraffin embedded pancreas were scored in a blinded manner for islet immune infiltration as described in the Research Design and Methods section. (b) Representative images of islets with different scores as described in the Research Design and Methods and ESM Methods. (c) The total score per pancreas was divided by the total number of islets scored. Shown are the mean scores ± SD with. Each score from an individual animal is represented by a single symbol; buffer (black squares; n=13) or CVB1-6 vaccine (black circles; n=8). No statistically significant difference was found between the groups using an unpaired t test. (d) Data show the percentage of islets from each mouse that fall into each insulitis category assessed as illustrated in (b). Islets were scored as intact (0; black circles), peri-insulitis (1; black squares), insulitis (2; black triangles) or destroyed (3; black diamonds). No statistically significant differences were found between the groups using two-way ANOVA with Sidak's multiple comparison test.

181x153mm (300 x 300 DPI)

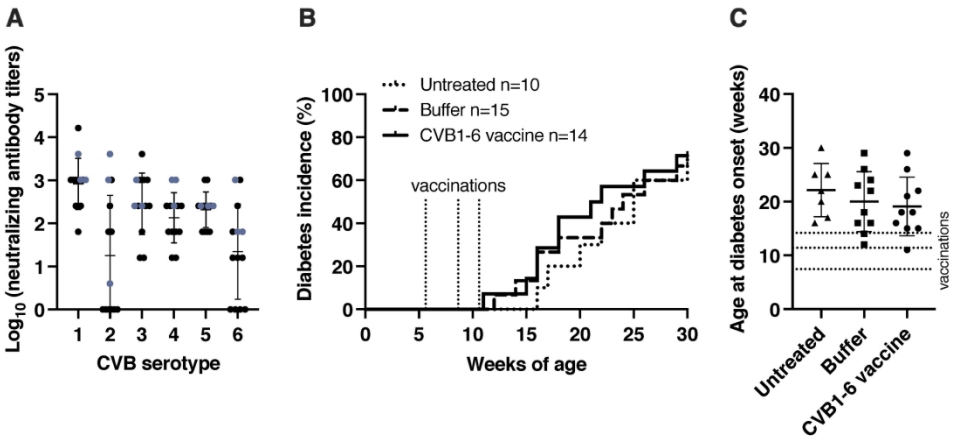


Figure 2: Diabetes onset is not altered in NOD mice immunized with a CVB1-6 vaccine. (a-c) Female NOD mice (mean age 5.7 weeks, range 4.9 - 7.1 weeks) were left untreated (n=10), mock-vaccinated (n=15) or vaccinated (n=14) with CVB1-6 vaccine by i.s. injection on either two (days 0 and 21; n=6 for buffer, n=10 for CVB1-6 vaccine) or three (days 0, 21 and 35; n=9 for buffer, n=4 for CVB1-6 vaccine) occasions. (a) Average neutralizing antibody titers in the serum of CVB1-6 vaccinated mice against the six CVB serotypes on day 42 post the first vaccination dose. Sera from untreated and mock-vaccinated mice had no neutralizing capacity. Shown are the mean virus neutralizing antibody titers ± SD with individual mice represented by a single symbol. Blue symbols represent virus neutralizing antibody titer data that was also published in (13). (b) Cumulative diabetes incidence and (c) average age at diabetes onset in the three groups. The dotted lines in (b) and (c) show the average age at vaccination. The mean age at diabetes onset ± SD is shown in (c) and the ages at which individual animals developed diabetes are displayed as single symbols. No statistically significant differences were found between the groups using Gehan-Breslow-Willcoxon test (b) one-way ANOVA with Tukey's multiple comparison test (c).

181x84mm (300 x 300 DPI)

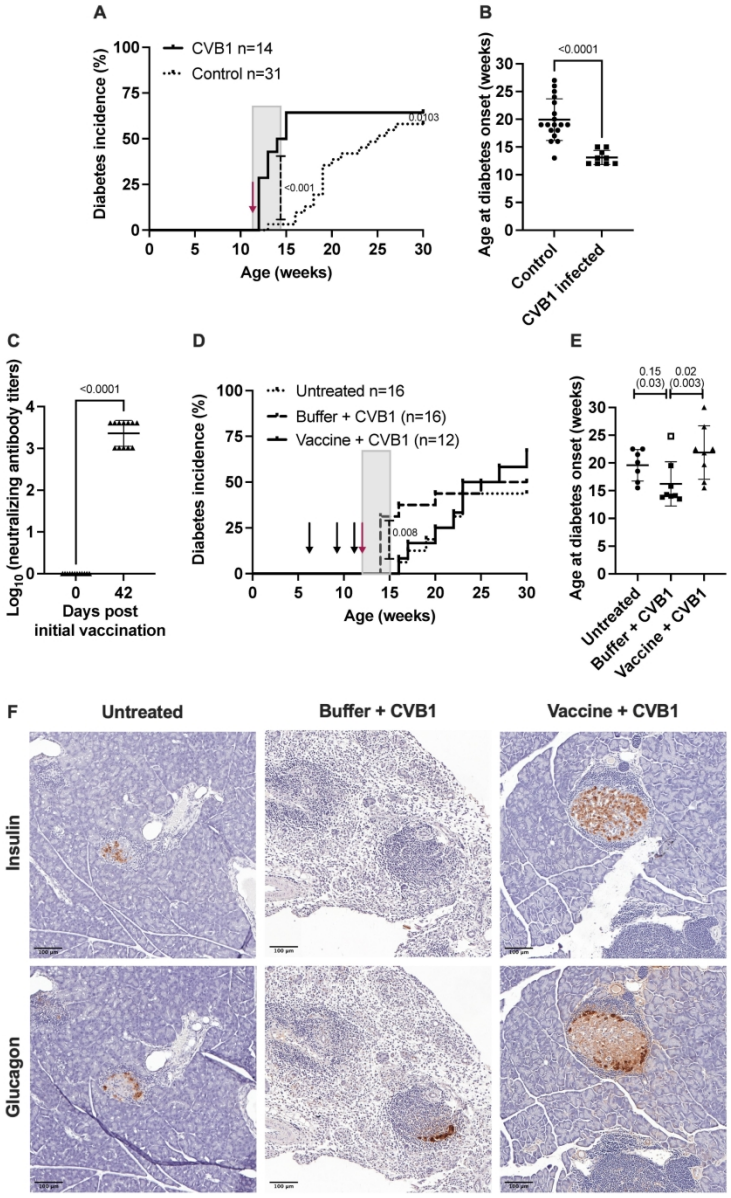


Figure 3: CVB1 vaccine protects against CVB1 accelerated disease in NOD mice. (a,b) Female NOD mice were left untreated (control; dotted line; n=31) or infected with CVB1 (10^7 PFU by i.p. injection, total volume 200µl) between 10.5 - 13.5 weeks of age (solid line; n=14) and diabetes incidence was followed up to 30 weeks of age. (a) Diabetes incidence curves of the two groups. The red arrow indicates the mean age at infection. The grey box shows the two-week period after virus infection, $p<0.001$ when comparing the diabetes incidence curves during this period by Gehan-Breslow-Wilcoxon test. The p value, $p=0.0103$, comes from the comparison of the two curves up to 30 weeks of age by Gehan-Breslow-Wilcoxon test. (b) Age at diabetes onset. Individual mice are represented by a single symbol and the horizontal line shows the mean age at diabetes onset ± SD. $p<0.0001$, unpaired t-test. (c-e) Female mice (6.3 – 6.9 weeks old) were left untreated (n=16), mock-vaccinated with vaccine buffer and infected with CVB1 virus (buffer + CVB1; n=16) or vaccinated with CVB1 vaccine and infected with CVB1 virus (vaccine + CVB1; n=12). Vaccinations were performed on days 0, 21 and 35 and the mice were infected with virus (10^7 PFU by i.p. injection, total volume 200µl) on day 42 (12.3 -12.9 weeks of age). Diabetes incidence was followed up to 30 weeks of age.

(c) Neutralizing antibody titers on days 0 and 42 in mice vaccinated with the CVB1 vaccine as measured by standard plaque reduction assay. Neutralizing antibodies were not detected in the mock-vaccinated and untreated groups (data not shown). Individual mice are represented by a single symbol and the horizontal line shows the mean neutralizing antibody titer ± SD. $p<0.005$, unpaired t-test. (d) Diabetes incidence curves in the untreated (dotted line), buffer + CVB1 (dashed line) and vaccine + CVB1 (solid line) groups. The black arrows indicate the approximate vaccination ages and the red arrow indicates the approximate age when the mice were infected. The grey box shows the two-week period after virus infection, $p=0.008$ when comparing the diabetes incidence curves by Gehan-Breslow-Wilcoxon test. (e) Age at diabetes onset. Individual mice are represented by a single symbol and the horizontal line shows the mean age at diabetes onset ± SD. Groups compared by Kruskal-Wallis test with Dunn's multiple comparison. In brackets are the p values generated when one mouse which was borderline diabetic from 15 weeks of age but didn't develop overt diabetes until 25 weeks of age was excluded (open square; buffer + CVB1), see Supplementary Fig. 3b for the blood glucose values. (f) Representative images of sequential pancreas sections stained with insulin (top row) and glucagon (bottom row) from mice that developed diabetes in the untreated (left hand column), mock vaccinated (buffer) + CVB1 (middle column) and vaccine + CVB1 (right hand column) groups. Positive areas are stained brown. Scale bars are present in the bottom left-hand corner of each image.

181x280mm (300 x 300 DPI)

Online Supplemental Materials for:

Coxsackievirus B vaccines prevent infection-accelerated diabetes in NOD mice and have no disease inducing effect

Authors: Virginia M Stone, Marta Butrym, Minna M Hankaniemi, Amir-Babak Sioofy-Khojine, Vesa P Hytönen, Heikki Hyöty, Malin Flodström-Tullberg

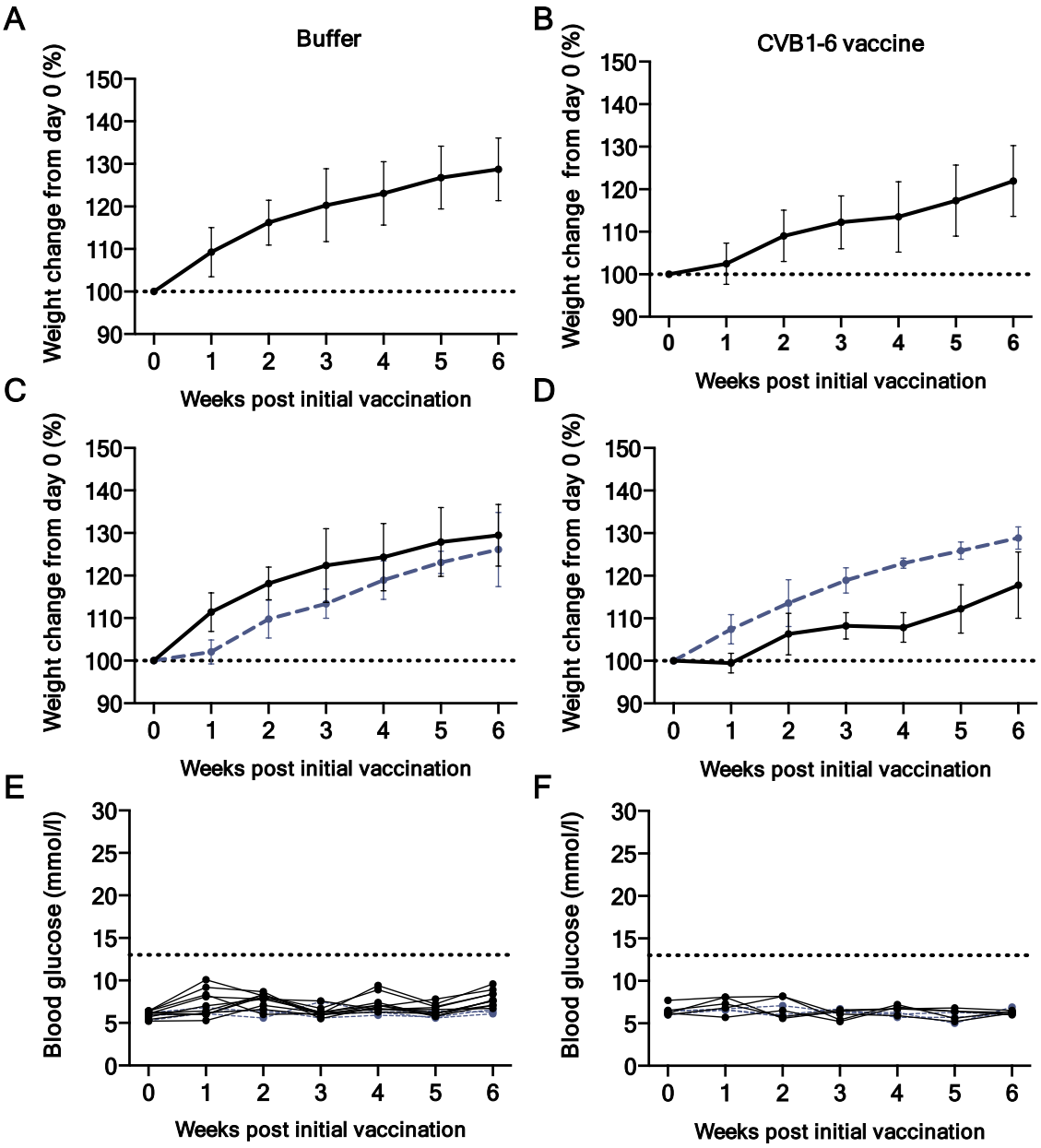
Contents:

Contents: 1
Supplementary Table 1: Reagents and suppliers.....2
Supplementary Figure 1: CVB1-6 vaccine has no adverse effects on weight or blood glucose......3
Supplementary Figure 2: CVB1-6 vaccine has no adverse effects on weight or blood glucose......4
Supplementary Figure 3: CVB1 vaccine protects against CVB1 accelerated diabetes......5
Supplementary Figure 4: CVB1 vaccine prevents CVB1-mediated exocrine tissue destruction......6
References.....6

Supplementary Table 1: Reagents and suppliers

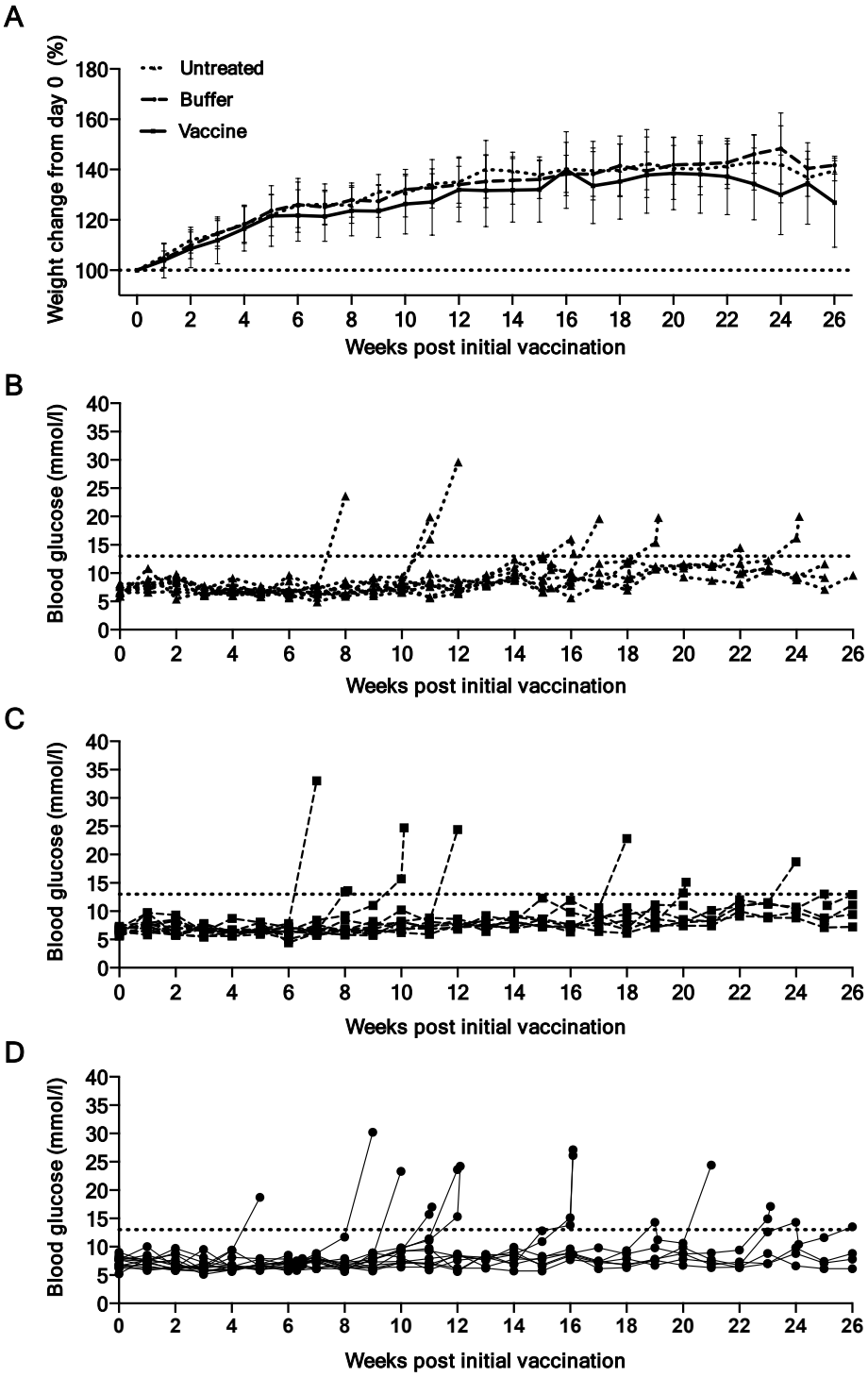
REAGENT or RESOURCE	SOURCE	REFERENCE
Antibodies		
Guinea pig anti-insulin 1:20,000	DakoCytomation	A0564, N1542
Rabbit anti-Glucagon 1:12000	Abcam	EP3070, Ab92517
Goat anti-guinea pig 1:200	Vector Laboratories	W0762, BA-7000
Goat anti-rabbit 1:200	Dako	E0432
Biological Samples		
Formalin fixed paraffin embedded mouse pancreas		
Chemicals, Peptides, and Recombinant Proteins		
M199 Medium	Gibco	11043-023
Carboxymethylcellulose	Sigma-Aldrich	C5013
Immunohistochemistry PAP pen	Dako	S2002
Normal Goat Serum (used concentrations 10% and 2%)	Dako	X0907
Elite ABC HRP Detection Kit	Vectastain	PK-6100
DAB Peroxidase Substrate Kit	Vector	SK-4100
Hematoxylin Mayer's	Sigma-Aldrich	MHS32

For Peer Review Only

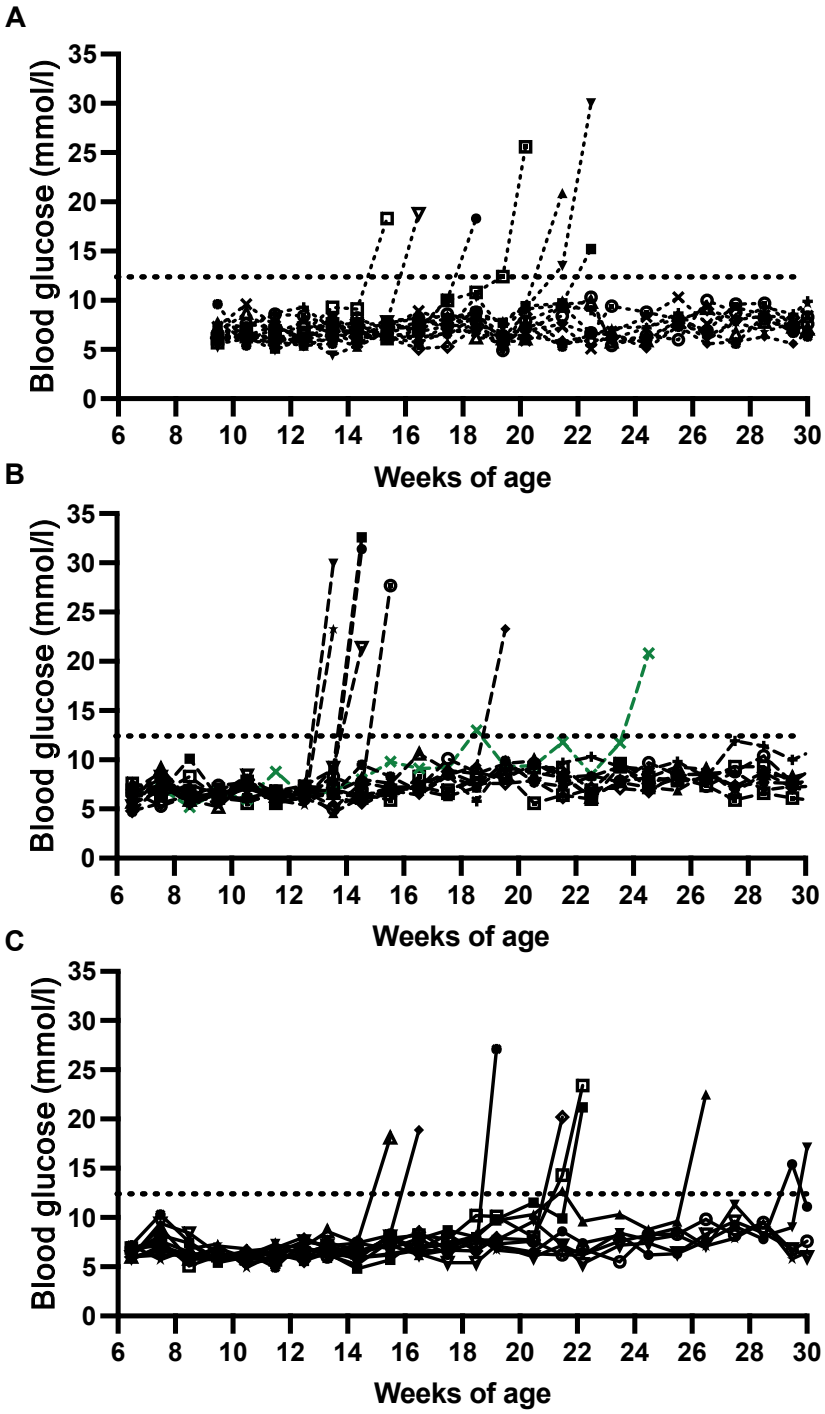


Supplementary Figure 1: CVB1-6 vaccine has no adverse effects on weight or blood glucose. Female NOD mice (5.1 - 6.3 weeks old) were mock vaccinated (buffer, n=13) or vaccinated with CVB1-6 vaccine (n=8) by interscapular (subcutaneous) injection on three occasions (on days 0, 14 and 28, n=3 or on days 0, 21 and 35, n=5). (a, b) Percentage weight change from day 0 in buffer treated (left) and CVB1-6 vaccinated mice (right). Shown are the mean values ± SD. The dotted line indicates the weight prior to the first vaccination on day 0. In (b) the weight data has been separated into new data (black lines) and data previously published in (1). (c) Blood glucose values for the buffer treated (left) and CVB1-6 vaccinated (right) mice from day 0. The dotted line indicates the diabetes threshold. The blue lines were previously published in (1).

For Peer Review Only

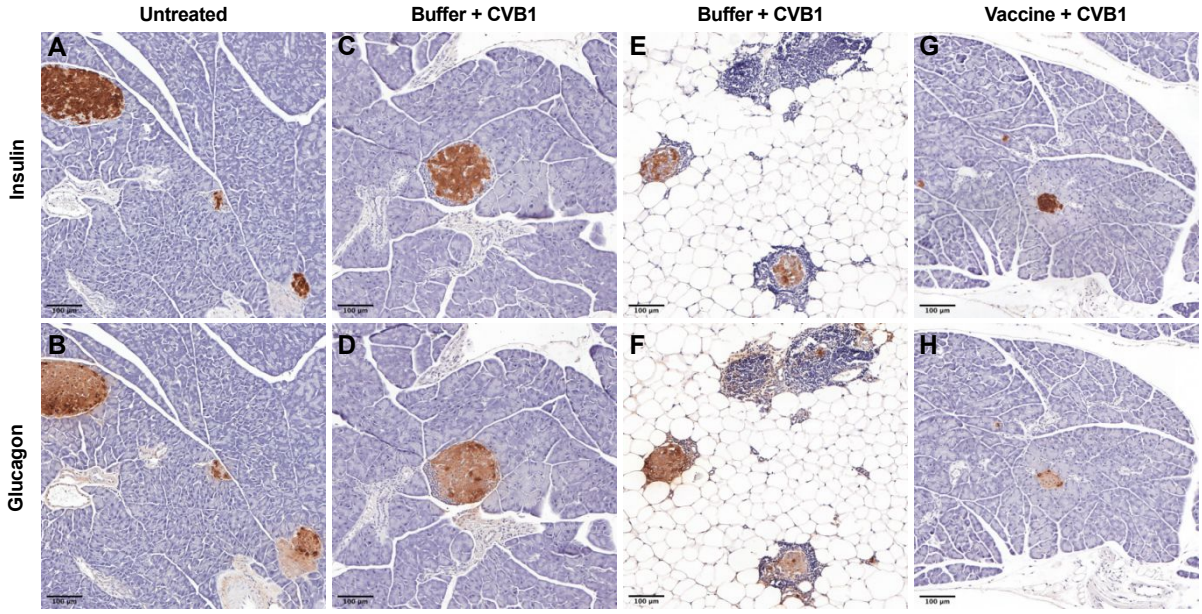


Supplementary Figure 2: CVB1-6 vaccine has no adverse effects on weight or blood glucose. Female NOD mice (4.9 - 7.1 weeks old) were left untreated (n=10), mock-vaccinated (n=15) or vaccinated (n=14) with CVB1-6 vaccine by interscapular (subcutaneous) injection on either two (days 0 and 21; n=6 for buffer, n=10 for CVB1-6 vaccine) or three (days 0, 21 and 35; n=9 for buffer, n=4 for CVB1-6 vaccine) occasions. (a) Percentage weight change from day 0 in untreated, mock-vaccinated (buffer) and CVB1-6 vaccinated mice. Shown are the mean values ± SD. The dotted line indicates the weight prior to the first vaccination on day 0. (b) Blood glucose values for the untreated (b), mock-vaccinated (buffer) (c) and CVB1-6 vaccinated (d) mice from day 0 post initial vaccination. The dotted line indicates the diabetes threshold.



Supplementary Figure 3: CVB1 vaccine protects against CVB1 accelerated diabetes. Female NOD mice were left (a) untreated (dotted lines; n=16; blood glucose levels monitored from 8 weeks of age), (b) mock-vaccinated with vaccine buffer and infected with CVB1 virus (dashed lines; buffer + CVB1; n=16; 6.3 – 6.9 weeks old) or (c) vaccinated with CVB1 vaccine and infected with CVB1 virus (solid lines; vaccine + CVB1; n=12; 6.3 – 6.9 weeks old). Vaccinations (buffer or vaccine injections) were performed on days 0, 21 and 35 and the mice were infected with virus (10^7 PFU by i.p. injection, total volume 200µl) on day 42 (12.3 -12.9 weeks of age). (a-c) Blood glucose levels were monitored up to 30 weeks of age. The dotted line indicates the diabetes threshold. In (b) the mouse in green was borderline diabetic until 25 weeks of age and excluded from some of the statistical analyses performed in Fig. 3e in the main article text.

For Peer Review Only



Supplementary Figure 4: CVB1 vaccine prevents CVB1-mediated exocrine tissue destruction. Female NOD mice were left untreated (a, b; n=15; blood glucose levels monitored from 8 weeks of age), mock-vaccinated with vaccine buffer and infected with CVB1 virus (c-f; buffer + CVB1; n=16; 6.3 – 6.9 weeks old) or vaccinated with CVB1 vaccine and infected with CVB1 virus (g, h; vaccine + CVB1; n=12; 6.3 – 6.9 weeks old). Vaccinations (buffer or vaccine injections) were performed on days 0, 21 and 35 and the mice were infected with virus (10^7 PFU by i.p. injection, total volume 200µl) on day 42 (12.3 -12.9 weeks of age). Mice were followed until diabetes onset or 25 weeks of age and at the terminal timepoints pancreas was collected for histological analysis. (a-h) Representative images of pancreas histology from mice that did not develop diabetes by the terminal endpoint. Sequential sections were stained with insulin (a, c, e, g) or glucagon (b, d, f, h) and assessed by light microscopy. The images in c-f come from the same mouse and show a part of the exocrine tissue with healthy appearance (c, d) and another part with extensive fatty replacement of acinar cells by fat (e, f). Scale bars are shown in the bottom left-hand corner of each image.

References

1. Stone VM, Hankaniemi MM, Laitinen OH, Sioofy-Khojine AB, Lin A, Diaz Lozano IM, Mazur MA, Marjomaki V, Lore K, Hyoty H, Hytonen VP, Flodstrom-Tullberg M: A hexavalent Coxsackievirus B vaccine is highly immunogenic and has a strong protective capacity in mice and nonhuman primates. Sci Adv 2020;6:eaaz2433

Online Supplemental~~ry~~ Materials~~Information~~ for:

Coxsackievirus B vaccines prevent infection-accelerated diabetes in NOD mice and have no disease inducing effect

~~Coxsackievirus B vaccines have no accelerating effect on disease progression but prevent infection-induced diabetes onset in NOD mice.~~

Authors: Virginia M Stone, Marta Butrym, Minna M Hankaniemi, Amir-Babak Sioofy-Khojine, Vesa P Hytönen, Heikki Hyöty, Malin Fl~~l~~odström-Tullberg

Contents:

Contents: 1
Supplementary Table 1: Reagents and suppliers 2
Supplementary Figure 1: CVB1-6 vaccine has no adverse effects on weight or blood glucose. 3
Supplementary Figure 2: CVB1-6 vaccine has no adverse effects on weight or blood glucose. 4
Supplementary Figure 3: CVB1 vaccine protects against CVB1 accelerated diabetes. 5
Supplementary Figure 4: CVB1 vaccine prevents CVB1-mediated exocrine tissue destruction. 6
References 6
Error! Hyperlink reference not valid.~~Contents: ....Error! Bookmark not defined.1~~
Error! Hyperlink reference not valid.~~Supplementary Table 1: Reagents and suppliers ....Error! Bookmark not defined.2~~
Error! Hyperlink reference not valid.~~Supplementary Figure 1: CVB1-6 vaccine has no adverse effects on weight or blood glucose. ....Error! Bookmark not defined.3~~
Error! Hyperlink reference not valid.~~Supplementary Figure 2: CVB1-6 vaccine has no adverse effects on weight or blood glucose. ....Error! Bookmark not defined.4~~
Error! Hyperlink reference not valid.~~Supplementary Figure 3: CVB1 vaccine protects against CVB1 accelerated diabetes. ....Error! Bookmark not defined.5~~
Error! Hyperlink reference not valid.~~Supplementary Figure 4: CVB1 vaccine prevents CVB1-mediated exocrine tissue destruction. ....Error! Bookmark not defined.6~~
Error! Hyperlink reference not valid.~~Contents: ....Error! Bookmark not defined.1~~

Error! Hyperlink reference not valid.Supplementary Table 1: Reagents and suppliers .....Error! Bookmark not defined.2

Error! Hyperlink reference not valid.Supplementary Figure 1: CVB1-6 vaccine has no adverse effects on weight or blood glucose. .....Error! Bookmark not defined.3

Error! Hyperlink reference not valid.Supplementary Figure 2: CVB1-6 vaccine has no adverse effects on weight or blood glucose. .....Error! Bookmark not defined.4

Error! Hyperlink reference not valid.Supplementary Figure 3: CVB1 vaccine protects against CVB1 accelerated diabetes. .....Error! Bookmark not defined.5

Error! Hyperlink reference not valid.Supplementary Figure 4: CVB1 vaccine prevents CVB1-mediated exocrine tissue destruction. .....Error! Bookmark not defined.6

Error! Hyperlink reference not valid.References.....Error! Bookmark not defined.7

Contents: 1

Supplementary Table 1 2

Supplementary Figure 1: CVB1-6 vaccine has no adverse effects on weight or blood glucose.....3

Supplementary Figure 2: CVB1-6 vaccine has no adverse effects on weight or blood glucose.....4

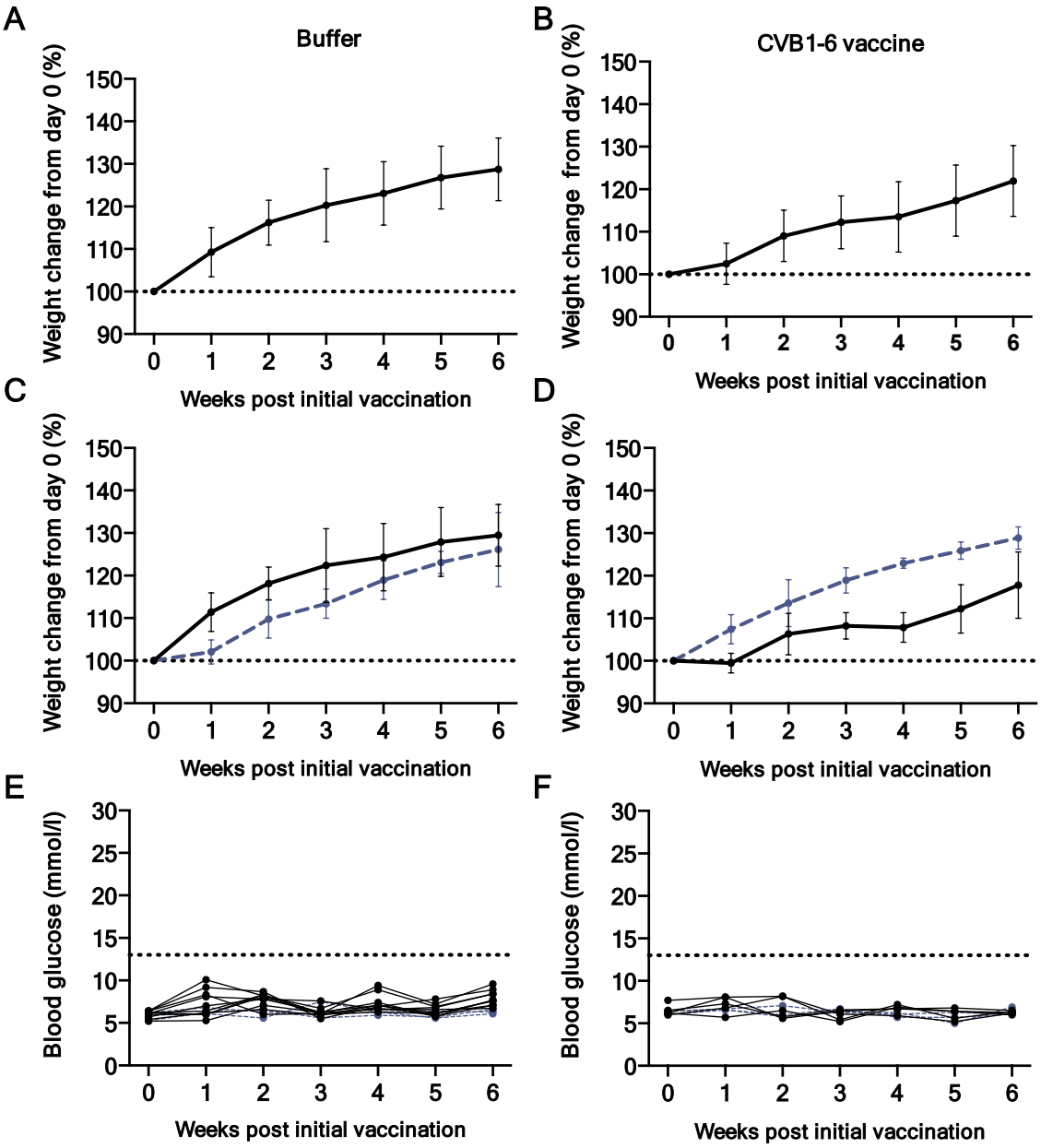
Supplementary Figure 3: CVB1 vaccine protects against CVB1 accelerated diabetes.....5

References.....6

Supplementary Table 1: Reagents and suppliers

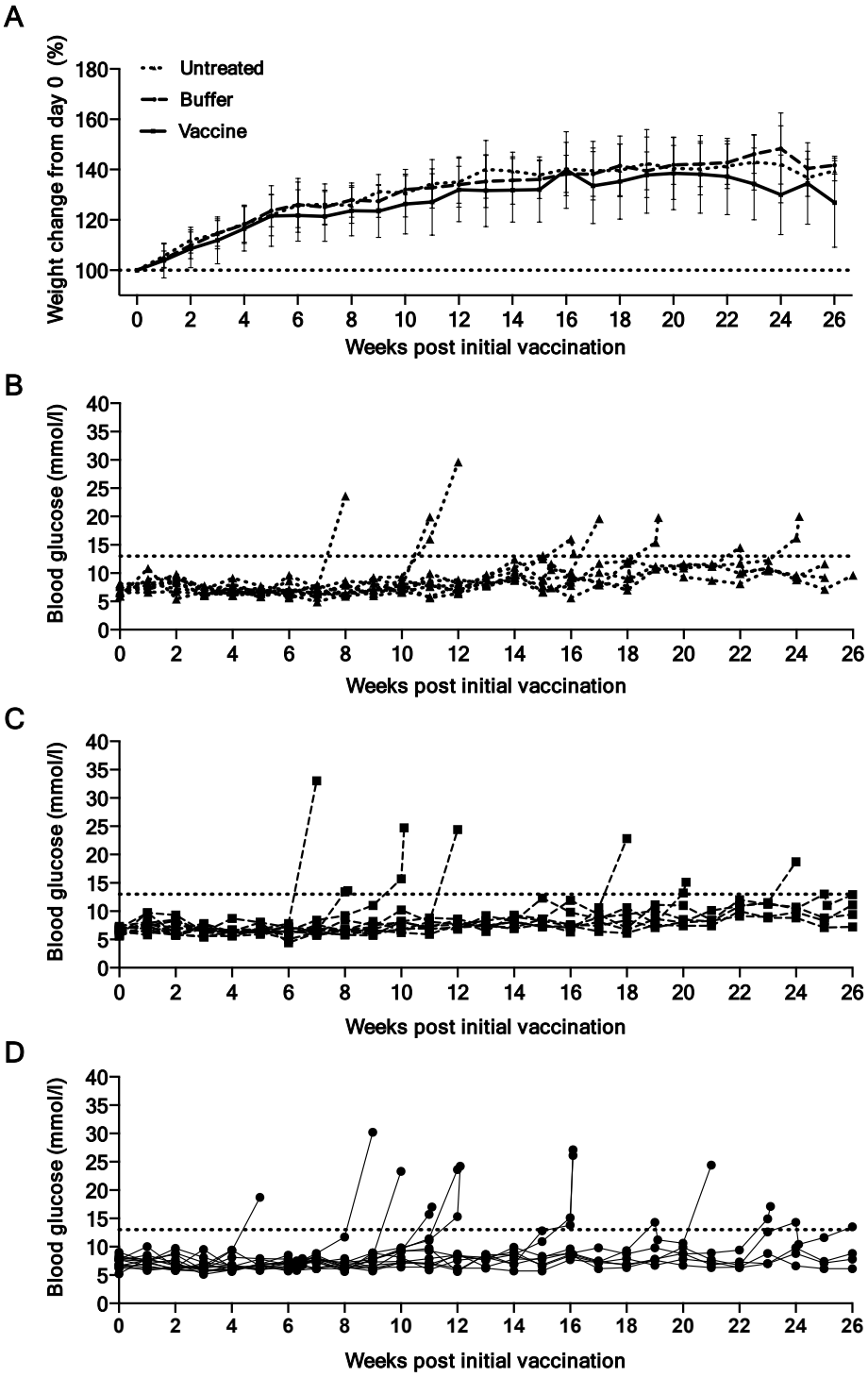
REAGENT or RESOURCE	SOURCE	REFERENCE
Antibodies		
Guinea pig anti-insulin 1:20,000	DakoCytomation	A0564, N1542
Rabbit anti-Glucagon 1:12000	Abcam	EP3070, Ab92517
Goat anti-guinea pig 1:200	Vector Laboratories	W0762, BA-7000
Goat anti-rabbit 1:200	Dako	E0432
Biological Samples		
Formalin fixed paraffin embedded mouse pancreas		
Chemicals, Peptides, and Recombinant Proteins		
M199 Medium	Gibco	11043-023
Carboxymethylcellulose	Sigma-Aldrich	C5013
Immunohistochemistry PAP pen	Dako	S2002
Normal Goat Serum (used concentrations 10% and 2%)	Dako	X0907
Elite ABC HRP Detection Kit	Vectastain	PK-6100
DAB Peroxidase Substrate Kit	Vector	SK-4100
Hematoxylin Mayer's	Sigma-Aldrich	MHS32

For Peer Review Only



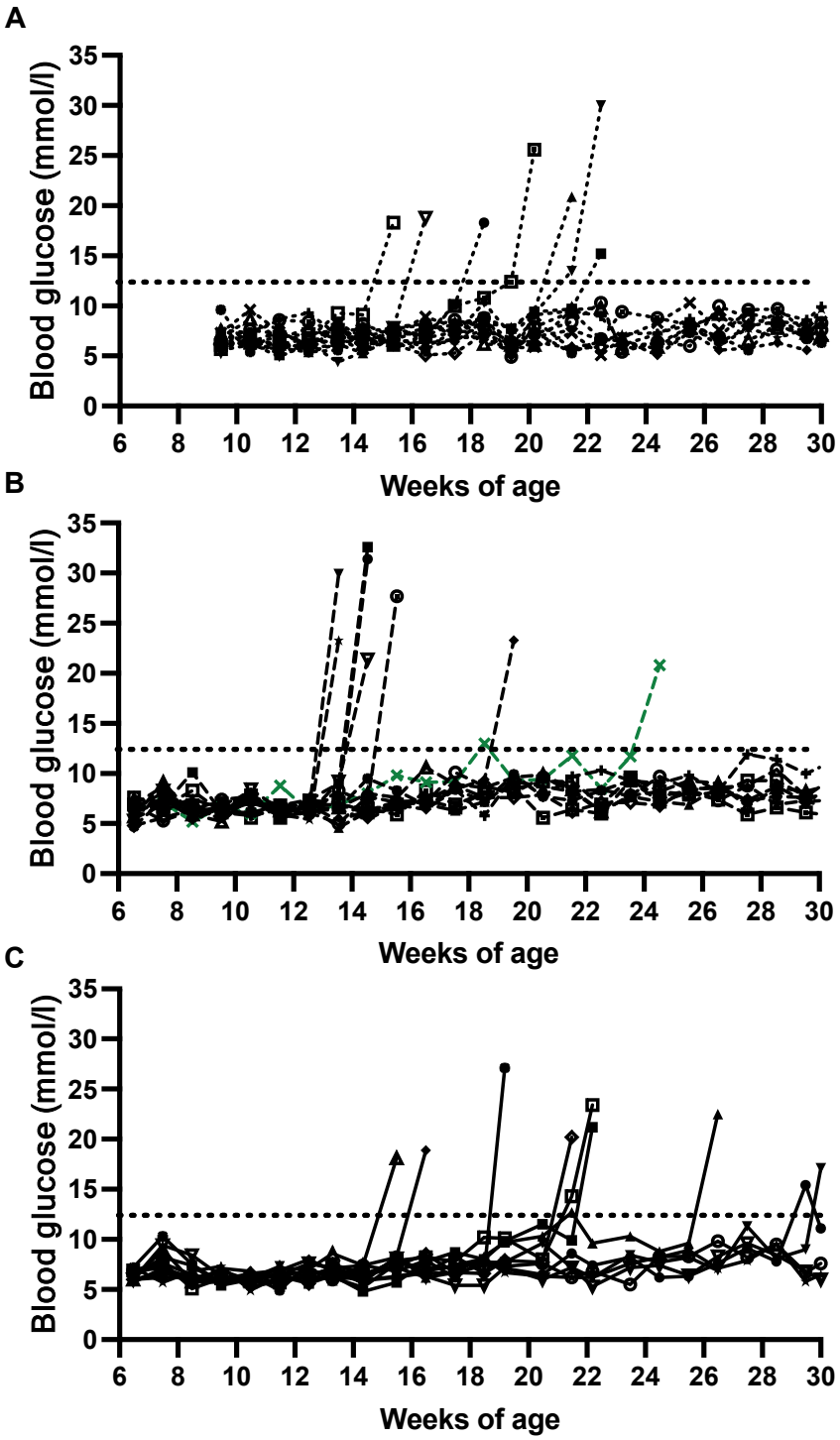
Supplementary Figure 1: CVB1-6 vaccine has no adverse effects on weight or blood glucose. Female NOD mice (5.1 - 6.3 weeks old) were mock vaccinated (buffer, n=13) or vaccinated with CVB1-6 vaccine (n=8) by interscapular (subcutaneous) injection on three occasions (on days 0, 14 and 28, n=3 or on days 0, 21 and 35, n=5). (a, b) Percentage weight change from day 0 in buffer treated (left) and CVB1-6 vaccinated mice (right). Shown are the mean values ± SD. The dotted line indicates the weight prior to the first vaccination on day 0. In (b) the weight data has been separated into new data (black lines) and data previously published in (1). (c) Blood glucose values for the buffer treated (left) and CVB1-6 vaccinated (right) mice from day 0. The dotted line indicates the diabetes threshold. The blue lines were previously published in (1).

For Peer Review Only

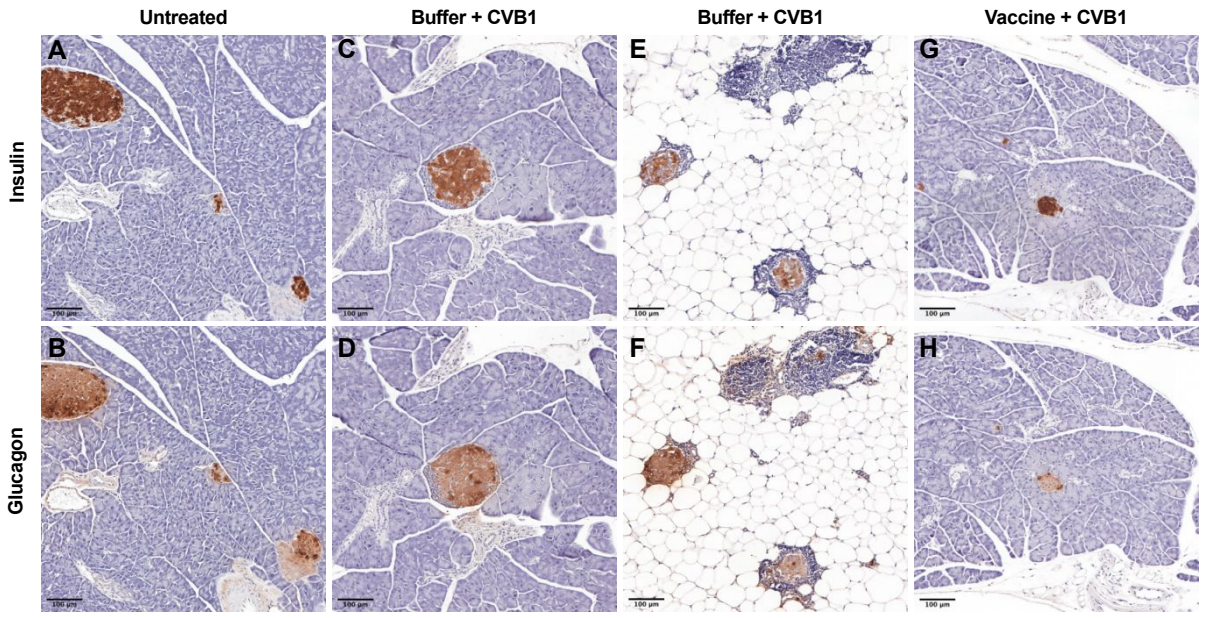


Supplementary Figure 2: CVB1-6 vaccine has no adverse effects on weight or blood glucose. Female NOD mice (4.9 - 7.1 weeks old) were left untreated (n=10), mock-vaccinated (n=15) or vaccinated (n=14) with CVB1-6 vaccine by interscapular (subcutaneous) injection on either two (days 0 and 21; n=6 for buffer, n=10 for CVB1-6 vaccine) or three (days 0, 21 and 35; n=9 for buffer, n=4 for CVB1-6 vaccine) occasions. (a) Percentage weight change from day 0 in untreated, ~~buffer treated~~mock-vaccinated (buffer) and CVB1-6 vaccinated mice. Shown are the mean values ± SD. The dotted line indicates the weight prior to the first vaccination on day 0. (b) Blood glucose values for the untreated (b), mock-vaccinated (buffer) ~~treated~~ (c) and CVB1-6 vaccinated (d) mice from day 0 post initial vaccination. The dotted line indicates the diabetes threshold.

For Peer Review Only



Supplementary Figure 3: CVB1 vaccine protects against CVB1 accelerated diabetes. Female NOD mice were left (a) untreated (dotted lines; n=16~~5~~; blood glucose levels monitored from 8 weeks of age), (b) mock-~~-~~vaccinated with vaccine buffer and infected with CVB1 virus (dashed lines; buffer + CVB1; n=16; 6.3 – 6.9 weeks old) or (c) vaccinated with CVB1 vaccine and infected with CVB1 virus (solid lines; vaccine + CVB1; n=12; 6.3 – 6.9 weeks old). Vaccinations (buffer or vaccine injections) were performed on days 0, 21 and 35 and the mice were infected with virus (10^7 PFU by i.p. injection, total volume 200µl) on day 42 (12.3 -12.9 weeks of age). (a-c) Blood glucose levels were monitored up to 30~~25~~ weeks of age. The dotted line indicates the diabetes threshold. In (b) the mouse in green was borderline diabetic until 25 weeks of age and excluded from some of the statistical analyses performed in Fig. 3e in the main article text.



Supplementary Figure 4: CVB1 vaccine prevents CVB1-mediated exocrine tissue destruction. Female NOD mice were left untreated (a, b; n=15; blood glucose levels monitored from 8 weeks of age), mock-vaccinated with vaccine buffer and infected with CVB1 virus (c-f; buffer + CVB1; n=16; 6.3 – 6.9 weeks old) or vaccinated with CVB1 vaccine and infected with CVB1 virus (g, h; vaccine + CVB1; n=12; 6.3 – 6.9 weeks old). Vaccinations (buffer or vaccine injections) were performed on days 0, 21 and 35 and the mice were infected with virus (10^7 PFU by i.p. injection, total volume 200µl) on day 42 (12.3 -12.9 weeks of age). Mice were followed until diabetes onset or 25 weeks of age and at the terminal timepoints pancreas was collected for histological analysis. (a-h) Representative images of pancreas histology from mice that did not develop diabetes by the terminal endpoint. Sequential sections were stained with insulin (a, c, e, g) or glucagon (b, d, f, h) and assessed by light microscopy. The images in c-f come from the same mouse and show a part of the exocrine tissue with healthy appearance (c, d) and another part with extensive fatty replacement of acinar cells by fat -(e, f). Scale bars are shown in the bottom left-hand corner of each image.

References

1. Stone VM, Hankaniemi MM, Laitinen OH, Sioofy-Khojine AB, Lin A, Diaz Lozano IM, Mazur MA, Marjomaki V, Lore K, Hyoty H, Hytonen VP, Flodstrom-Tullberg M: A hexavalent Coxsackievirus B vaccine is highly immunogenic and has a strong protective capacity in mice and nonhuman primates. Sci Adv 2020;6:eaaz2433