SUPPLEMENTAL MATERIAL

Kallewaard, et al. Tissue-specific deletion of the coxsackievirus and adenovirus receptor (CAR) protects mice from virus-induced pancreatitis and myocarditis

SUPPLEMENTAL METHODS

Immunostaining heart and pancreas

Tissues were frozen in OCT Tissue-Tek using liquid nitrogen and 7 μm sections were cut by cryosector. Specimens were fixed with ice-cold acetone, permeabilized with 1% Triton X-100, and blocked with 4% BSA in saponin buffer. Pancreas sections were stained with rabbit polyclonal anti-CAR (H-300, Santa Cruz Biotechnology; 1:250 dilution), followed by Goat anti-rabbit Alexa Fluor 488 (Invitrogen; 1:500 dilution). Heart sections were stained with affinity-purified rabbit polyclonal anti-CAR 1:50 dilution (Cohen et al., 2001) and mouse monoclonal anti-N-cadherin (3B9, Zymed) followed by goat anti-rabbit FITC (Jackson Immunoresearch) and goat anti-mouse IgG1 Alexa Fluor 594 (Invitrogen 1:500)

Immunoblot Analysis

Tissues from 6-week-old mice were homogenized in cold RIPA buffer [50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% Triton, 0.5% Deoxycholic Acid, 0.1% SDS, plus protease inhibitor cocktail (Roche)], incubated on ice for 20 min, sonicated, and then clarified by centrifugation. Lysate proteins were separated by electrophoresis in SDS-polyacrylamide gels, and blotted to nitrocellulose. CAR was detected with rabbit polyclonal anti-CAR (H-300, Santa Cruz Biotechnology; 1:2000 dilution) and horseradish peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare,

1:12,500 dilution). GAPDH was detected with anti-GAPDH antibody directly conjugated to horseradish peroxidase (Santa Cruz Biotechnology; 1:1000 dilution). Blots were developed with chemiluminescent substrates (Pierce).

Quantitative PCR to assess deletion of CAR exon 2 in DNA and RNA.

For quantification of CAR gene deletion, DNA was extracted from paraffin-embedded tissue scrolls using Qiagen DNA FFPE kit according to the manufacturer's instructions. Quantitative PCR was performed on genomic DNA using Brilliant SYBR Green QPCR Master Mix (Stratagene). Primers were designed to detect CAR exon 2 (deleted upon Cre-mediated recombination) and CAR exon 3. Reactions were performed using the SYBR Green (with dissociation curve) program on an Mx4000 Multiplex Quantitative PCR System (Stratagene). All reactions were performed in triplicate and Ct values were normalized using an internal rox control. The ratio of CAR exon 2 relative to the CAR exon 3 was calculated for each sample. To obtain the relative deletion efficiency for the whole organ of the knockouts we compared the ratio determined for the knockout animals to the ratio determined for the control animals.

Quantification of exon 2 mRNA was performed on RNA isolated by homogenization in Trizol followed by RNeasy extraction (Qiagen) from heart and pancreas samples. 1 μg of RNA per sample was reverse transcribed using oligo dT, Super-Script III reverse transcriptase, and accompanying reagents (Invitrogen). Exon 2 primers were used, as for DNA. All values were normalized to the HPRT housekeeping control, and then expression values for the KO animals were compared to those for controls.

Chemical Induction of Pancreatitis

To induce mild acute pancreatitis, Panc-KO and littermate controls were given eight hourly intraperitoneal injections of caerulein (Sigma, 50 g/kg body wt). As a control, Panc-KO and control animals were given saline injections and used as 0 hr control (n=3 per group). Mice were killed at 5 h (n=3 per group), 12 h (n=3 per group), and 24 h (n=2 per group) after the initiation of pancreatitis. To assess serum amylase levels, blood was obtained from Panc-KO mice and controls, placed on ice for 15 min, and centrifuged at 3,000 rpm and 4°C for 10 min. The supernatant was retained, and amylase levels were determined using the EnzChek ultra amylase assay (Invitrogen) as per the manufacturer's instructions.

Quantitative PCR to measure cytokine gene expression

Analysis of cytokine gene expression was performed on RNA from heart samples of four infected heart knockout mice and four infected controls 7 days post infection, as well as two non-infected heart knockout and two non-infected controls. RNA was reverse transcribed as for measurement of CAR mRNA (above) except that random hexamers were used for priming. All values were normalized to the HPRT housekeeping control, and then expression values for the infected animals were compared to those for uninfected animals of the same genotype (although values for uninfected knockout and controls were virtually identical).

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SUPPLEMENTAL TABLE T1

	fwd	rev
CAR floxed v. wt	AATGGTGCCCGAGGAACGAAC	GCTTGTTGTTTGGTTGGGTTT
Pdx-Cre	TTGAAACAAGTGCAGGTGTTCG	TTCCGGTTATTCAACTTGGACC
MHC-Cre	ATGACAGACAGATCCCTCCTATCTCC	ATAATCACTCGTTGCATCGAC
CAR exon 2	TTTCACCAGTGGTTTGAGCA	TGGTCTTCGGGACTGAGAGT
CAR exon 3	AAGTCTGGCGACGCATCTAT	GGGCTTTCTTCACTTTGCAC
IL-6	GTTCTCTGGGAAATCGTGGA	TTCTGCAAGTGCATCATCGT
IL-10	CCCAGAAATCAAGGAGCATT	TTTTCACAGGGGAGAAATCG
MCP-1	AGGTCCCTGTCATGCTTCTG	TTGCTGGTGAATGAGTAGCAG
IP-10	TCATCCTGCTGGGTCTGAGT	CCTATGGCCCTCATTCTCAC
IFN-β	CCCTATGGAGATGACGGAGA	CCCAGTGCTGGAGAAATTGT
IFN-γ	TCAAGTGGCATAGATGTGGAA	TCTGGCTCTGCAGGATTTTC
HPRT	GGCCAGACTTTGTTGGATTTG	TGCGCTCATCTTAGGCTTTGT

SUPPLEMENTAL FIGURE

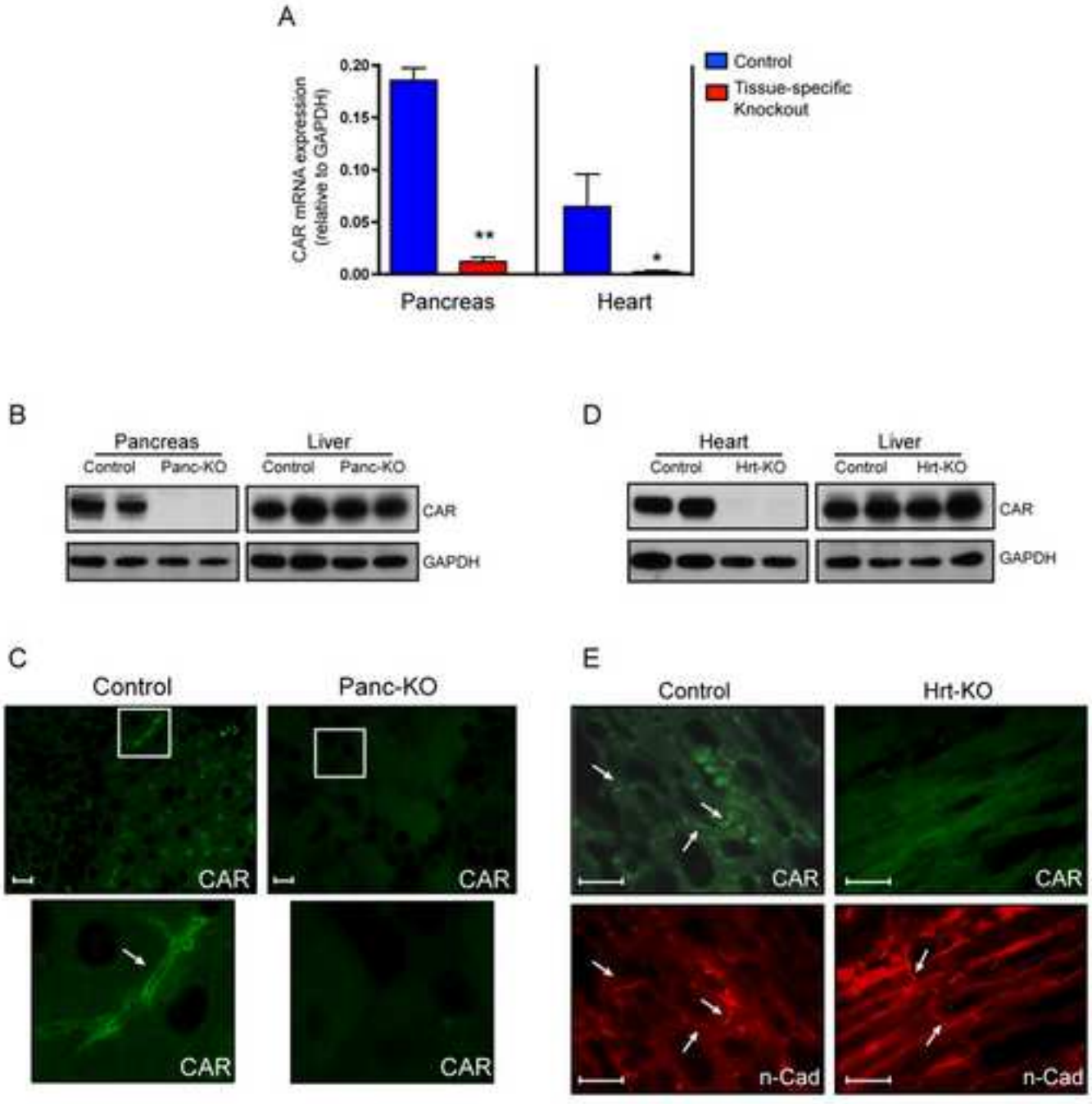
S1. Tissue-specific CAR deletion from the pancreas and heart. A) CAR mRNA levels determined by quantitative PCR, relative to HPRT control; error bars represent SEM (pancreas, 4 animals; heart, 3 animals; **, $p<0.005$; *, $p<0.05$). B) Immunoblot detection of CAR protein in the pancreas (left panel) and liver (right panel) of pancreas-specific CAR deletion mice (Panc-KO) and littermate control mice; 25 μg of protein was blotted and probed with anti-CAR antibody, then blots were stripped and reprobed with anti-GAPDH antibody to confirm equal loading. C) Indirect immunofluorescence staining for CAR on pancreas tissue sections from a Panc-KO mouse and a control mouse. In the control, CAR was expressed in the acinar and ductal cells, concentrated in areas of cell-to-cell contact, with little or no CAR staining evident within the islets. Other investigators have reported mouse CAR expression limited either to acinar cells (Mena et al., 2000) or to ducts (Raschperger et al., 2006), whereas others have reported it on islets (Drescher et al., 2004; Mukai et al., 2007); these differences may relate to the specificities of the reagents used. Upper panel, 40X magnification, Scale bars, 20 microns; white box depicts the area that is shown in lower panel at 100X magnification. The arrow indicates an area in which CAR staining is concentrated at acinar junctions. CAR deletion from the pancreas resulted in no gross histological abnormalities and Panc-KO mice showed normal regulation of blood glucose after oral glucose challenge (data not shown), suggesting normal function of the endocrine pancreas. D) Immunoblot detection of CAR protein in the heart (left panel) and liver (right panel) of heart-specific CAR deletion mice (Hrt-KO) and littermate control mice; 25 μg of protein was blotted and probed with anti-CAR antibody, then blots were stripped and reprobed with anti-

GAPDH antibody to confirm equal loading. E) Indirect immunofluorescence staining for CAR (upper panels) and the junctional marker N-cadherin (lower panels) on heart tissue sections from Hrt-KO and control mice. Arrows indicate staining at intercellular junctions (intercalated discs). Scale bars, 20 microns. Histologic examination showed no difference between Hrt-KO and control mice; we did not investigate whether Hrt-KO animals displayed subtle conduction abnormalities recently reported in adult mice with heart-specific CAR deletion (Lim et al., 2008; Lisewski et al., 2008).

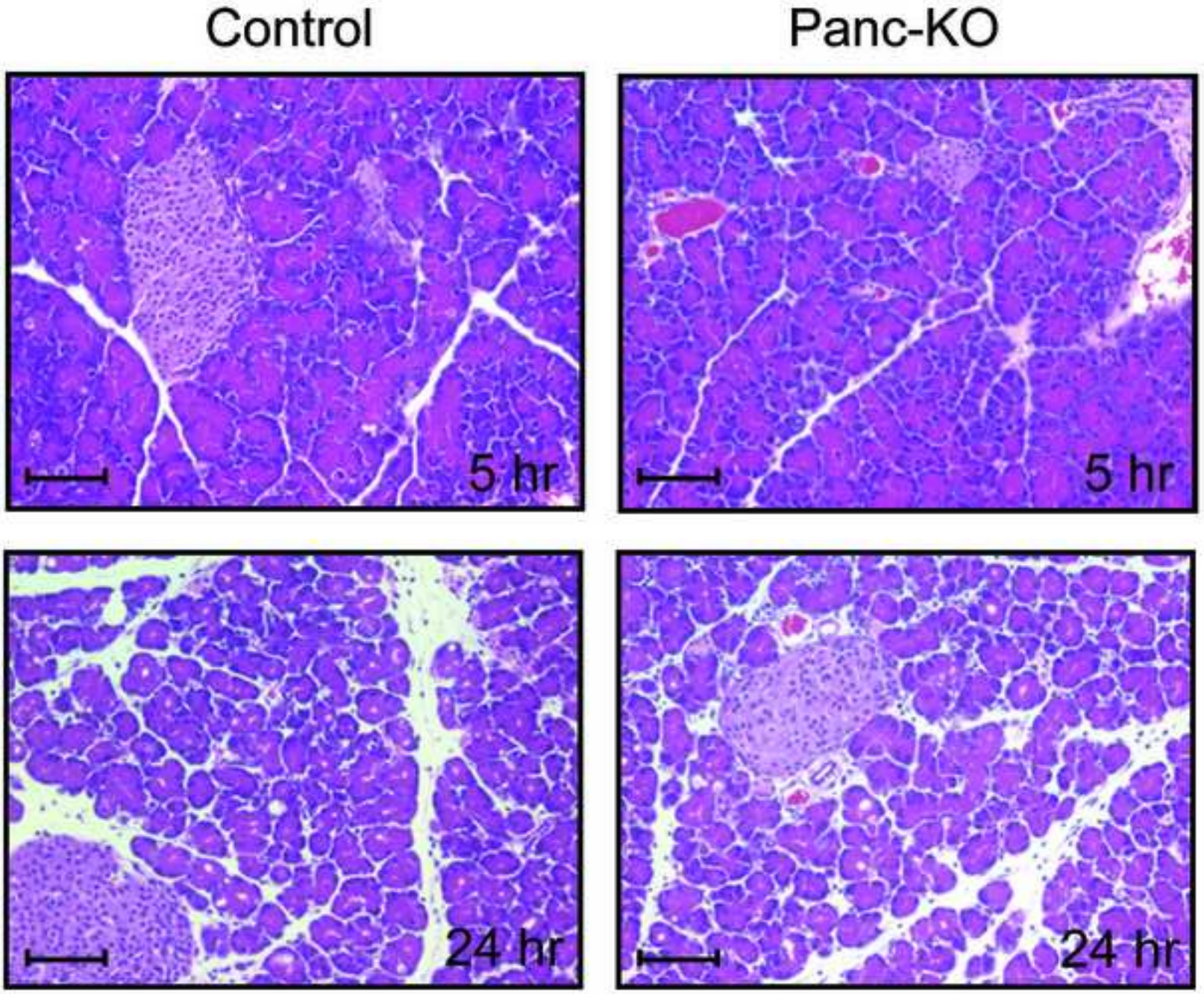
S2. Caerulein-induced pancreatitis. Mice were injected with caerulein as described in Experimental Procedures; pancreatic sections were taken 5 and 24 hr, and stained with H & E. Scale bars, 80 microns.

S3. Infection of double (panc/hrt) knockout mice. Panc- and Hrt-KO mice were crossed, and double panc/hrt KO progeny ($CAR^{F/F}$, MHC Cre^{+}, Pdx-Cre^{+}) were identified. A) Mice were infected with $1x10^5$ PFU/mouse of CVB3-H3, sacrificed on day 7, and tissues were removed for plaque assay in triplicate. The mean titer for each group is depicted as a line. The shaded regions represent the lower limits of viral detection; when no PFU were detected, samples were assigned the value of 3 PFU before normalization for tissue weight. Asterisks indicate a statistical difference between the KO and control groups (**, $p<0.005$). B) Mice were infected with $1x10^5$ PFU/mouse of CVB3-H3, and blood glucose levels were measured daily (**, $p<0.005$). C) H & E stain of pancreas (top) and heart (below) sections from double-KO CAR deletion mice and littermate controls. Scale bars, 80 microns.

[F] Supplemental Figure 1



[F] Supplemental Figure 2



[F] Supplemental Figure 3