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2 **Pancreatic nerve electrostimulation inhibits recent onset**
3 **autoimmune diabetes**

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22

1 **Electrical modulation of peripheral nerves has been proposed as a novel approach to treat**
2 **immune-mediated inflammatory diseases (IMIDs)¹. Type-1 diabetes (T1D) is an**
3 **autoimmune disease that results from the destruction of insulin-producing pancreatic β**
4 **cells by autoreactive T lymphocytes following their activation in pancreatic draining**
5 **lymph nodes (LNs)². The sympathetic nervous system inhibits inflammation via binding**
6 **of noradrenaline or adrenaline to β_2 -adrenergic receptors (ARs) expressed on immune**
7 **cells³. Since LNs are innervated by sympathetic nerves, we hypothesized that pancreatic**
8 **nerve electrical stimulation (PNES) may inhibit local inflammation and T1D progression.**
9 **Here we have developed a minimally invasive surgical procedure for long-term**
10 **implantation of micro-cuffs electrodes onto the nerve that projects to pancreatic draining**
11 **LNs. When applied to recently diabetic Non-Obese-Diabetic (NOD) mice, PNES inhibited**
12 **insulinitis and T1D progression with minimal bystander tissue effects by inhibiting the**
13 **migration of pathogenic effector T lymphocytes from pancreatic draining LNs to**
14 **pancreatic islets. Our data provide a rationale for the use of bio-electronic medicine as a**
15 **potential treatment modality for T1D.**

16 Little is known about the nerves that project to the pancreas in mice. Gross anatomy of
17 the pancreatic region showed that the superior mesenteric artery branching from the inferior
18 vena cava is associated with a 50 μm -diameter nerve-like structure (**Suppl. Fig. 1a**) and
19 supplied blood to the pancreas head and neck (**Suppl. Fig. 1b**). To confirm that this structure
20 was a nerve and to identify the innervated tissues, we placed a hook electrode onto it and a
21 recording electrode on visceral tissues. Evoked compound action potentials (CAPs) occurred
22 in pancreatic LNs and pancreas head but not in liver when electrostimulation was applied
23 (**Suppl. Fig. 2**). Pancreatic nerve-like structures expressed tyrosine hydroxylase (TH)
24 demonstrating the presence of catecholaminergic fibers (**Fig. 1a**). In agreement with the known
25 ability of the SNS to induce vasoconstriction, high frequency and amplitude (20 Hz, 1 mA)

1 electrical stimulation of the pancreatic nerve reduced pancreatic blood flow using laser speckle
2 perfusion imager (**Fig. 1b**). Light-sheet-based fluorescent imaging of TH-stained LNs showed
3 a high-density network of catecholaminergic fibers showing that the pancreatic nerve did not
4 only project to the pancreas itself, but also to the LNs that drained this gland (**Fig. 1c**). To
5 characterize the electrophysiological properties of the pancreatic nerve fibers, we used
6 fluorescent reporter tdTomato^{TH-Cre} transgenic mice carrying the tdTomato fluorescent protein
7 gene downstream of the tyrosine hydroxylase (TH) gene promoter. Red fluorescence axons
8 were readily detected in both the pancreatic nerve (**Suppl. Fig. 3a**) and the pancreatic LN
9 medulla zone (**Suppl. Fig. 3b**). To confirm that the pancreatic nerve projected to the pancreatic
10 LN, we placed these red fluorescent axons of the pancreatic nerve into a suction electrode and
11 recorded field action potentials (FAPs) with a microelectrode. FAPs were readily detected when
12 pancreatic nerve electrical stimulation (PNES) was above 400 μ A (**Fig. 1d**). FAPs were
13 inhibited by tetrodotoxin (TTX) confirming that they resulted from neuronal activity (**Suppl.**
14 **Fig. 4a**). Nerve conduction velocity was 0.5 ± 0.26 m/s at 25 °C (n = 4) suggesting that axons
15 were unmyelinated⁴.

16 Should PNES be considered as a therapeutic treatment, electrical parameters need be
17 optimized to minimize bystander tissue damage and short-term alterations in pancreatic blood
18 flow and glycemia, while allowing release of therapeutic levels of neurotransmitters. After
19 testing various electrical stimulation parameters, we found that a frequency of 10 Hz and an
20 amplitude of 450 μ A increased noradrenaline levels in pancreatic LNs (**Fig. 1e**) without altering
21 pancreatic blood flow (**Fig. 1b**) or glycemia (**Suppl. Fig. 5**). Furthermore, these parameters did
22 not induce exhaustion of axonal excitability (**Suppl. Fig. 4b, 4c**) therefore preserving
23 therapeutic efficacy.

24 We then investigated whether PNES could impact secretion of pro-inflammatory
25 cytokines, immune cell trafficking and antigen cross-priming in the pancreatic draining LNs of

1 non-diabetic prone mice. Because anesthetics interfere with immunity⁵, we developed a
2 minimally invasive surgical procedure for sustainable implantation of micro-cuffs electrodes
3 onto the pancreatic nerve allowing studies to be conducted on conscious animals. Compared to
4 sham-stimulated mice in which a cuff was implanted but electrical stimulation was not applied,
5 PNES mice exhibited higher numbers of T and B-lymphocytes in pancreatic draining but not
6 in non-draining LNs (**Fig. 2a**, **Suppl. Fig. 6a-c**). No alterations in the frequency of myeloid cell
7 types were observed **Suppl. Fig. 6d**). One session of PNES reduced LPS-induced pro-
8 inflammatory cytokine mRNA levels in pancreatic LNs but not in spleen (**Fig. 2b**). We next
9 assessed the impact of electrostimulation on the ability of dendritic cells (DC) to cross-prime
10 CD8⁺ T cells taking advantage of the RIP-OVA mice in which ovalbumin (OVA) is exclusively
11 expressed in β -islets. In this model, naïve OVA-specific CD8 T (OT-1) cells transferred in these
12 mice spontaneously proliferate as consequence of OVA antigen presentation by DC in
13 pancreatic lymph nodes. When applied over a 4-day period, PNES inhibited pancreatic auto-
14 antigen cross-priming as shown by reduced proliferation of adoptively transferred islet-specific
15 CD8⁺ T cells (**Fig. 2c**). In agreement with a critical role of noradrenaline, the effects of PNES
16 on both immune cell accumulation and LPS-induced pro-inflammatory cytokine mRNA
17 production were abolished in mice deficient in β_2 -AR (ADRB2^{ko}) (**Fig. 2a**, **2b**). Likewise,
18 administration of the β_1/β_2 -AR antagonist propranolol showed that the inhibitory effect of
19 PNES on auto-antigen cross-priming was β -AR-dependent (**Fig. 2c**).

20 We next investigated the impact of PNES on T1D progression in NOD mice that
21 spontaneously develop autoimmune diabetes between 3 and 6 months of age. Once diagnosed
22 with diabetes, NOD mice were implanted with a micro-cuff electrode onto the pancreatic nerve
23 and PNES was applied one day later three times a day for 6 weeks (**Fig. 3a**). Mice returned to
24 normoglycemia after surgery, a phenomenon that was likely due to the anti-inflammatory effect
25 of anesthetics^{6,7}. However, while glycemia started to increase again in sham stimulated mice

1 resulting in full-blown diabetes, it remained below 150 mg/ml in PNES mice (**Fig. 3b**). To
2 investigate the underlying mechanisms, we took advantage of a well-characterized synchronous
3 model of diabetes in which diabetogenic T cells from NOD mice are transferred into
4 immunodeficient syngenic NOD-SCID recipients⁸. While all (13 out of 13) sham-stimulated
5 mice developed T1D within 4 weeks after transfer, only 55% (7 out of 13) of PNES recipients
6 did ($p = 0.015$, **Fig. 3c**). This result showed that PNES inhibits T1D progression by acting on
7 pathogenic effector T cells. In another set of experiments, mice were sacrificed 2 weeks after
8 adoptive transfer and analyzed for both insulinitis and number of lymphocytes in pancreatic LNs.
9 PNES mice had 2.7-fold as many islets than sham stimulated mice (84.5 ± 20.3 versus $31.75 \pm$
10 3.6 , $p = 0.029$) (**Fig. 3d**). In addition, the proportion of non-infiltrated islets was higher in PNES
11 mice than in sham stimulated mice (55% versus 17.5%) (**Fig. 3d**). Furthermore, while PNES
12 and sham-stimulated mice exhibited similar number of lymphocytes in non-draining LNs,
13 PNES mice had 4- to 5-fold more lymphocytes in draining LNs ($29.0 \pm 12.0 \times 10^4$ versus $6.0 \pm$
14 1.5×10^4 per mouse, $p = 0.006$), a result similar to what we also observed in C57BL/6 mice
15 (**Fig. 3e**). Our data are compatible with a model in which PNES prevents T1D by inhibiting the
16 migration of pathogenic effector T lymphocytes from pancreatic draining LNs to pancreatic
17 islets.

18 We next investigated whether T1D inhibition could be achieved by on-demand PNES,
19 or alternatively required persistent stimulation. Once diagnosed with diabetes, NOD mice were
20 implanted with a micro-cuff electrode onto the pancreatic nerve and glycaemia was monitored
21 daily for up to two months. When glycaemia exceeded 200 mg/dl, 9 to 12 PNES sessions (450
22 μA , 10 Hz, 2 min. duration) were applied 8 hours apart. While all mice eventually became
23 hyperglycemic, glycaemia increased 2.25 times more slowly in PNES mice (**Fig. 3f, Suppl.**
24 **Fig. 7**). In another set of experiments, we sacrificed mice 2 weeks after the first PNES session.
25 PNES mice had 4.3-fold as many islets as sham stimulated mice (26.0 ± 1.5 versus 6.0 ± 1.0

1 per mouse, $p = 0.0006$) (**Fig. 3g**). Likewise, insulinitis was less severe in PNES mice as
2 demonstrated by the proportion of non-infiltrated islets (30.9% versus 15.8%).

3 Two clinical studies have shown that vagus nerve stimulation can be beneficial for
4 patients with Immune-Mediated Inflammatory Diseases (IMIDs), i.e. rheumatoid arthritis⁹ and
5 Crohn's Disease¹⁰. However, vagus nerve electrical stimulation lacked efficacy in some
6 patients, and had serious adverse effects, presumably because of its lower nerve fiber
7 specificity. Indeed, the vagus nerve contains both afferent and efferent fibers, and it regulates
8 the function of multiple organs. We have made the hypothesis that applying electrical
9 stimulation to an exclusively efferent nerve proximal to the target organ would be both more
10 efficient therapeutically and more specific. Here, we have identified a sympathetic nerve that
11 projects into pancreatic LNs. Selective electrostimulation of this nerve was sufficient to
12 completely inhibit T1D progression in both a spontaneous and an adoptive transfer models of
13 T1D with minimal bystander effects. We also found that PNES inhibited LPS-induced
14 production of inflammatory cytokines, antigen cross-presentation in pancreatic LN, and
15 resulted in increased numbers of lymphocytes in pancreatic draining LN. These results are in
16 agreement with previous studies showing an inhibitory role of β 2-AR signaling on secretion of
17 inflammatory cytokines by various myeloid cell types³, antigen cross-presentation by dendritic
18 cells¹¹ and lymphocyte egress from LN¹². Since T1D is dependent on antigen cross-
19 presentation, production of inflammatory cytokines and migration of autoreactive effector T
20 cells from pancreatic LNs to pancreatic islets², PNES could possibly prevent T1D progression
21 by interfering with all such pathways.

22 Bioelectronics medicine offers the possibility to apply on-demand treatment based on
23 recording of physiological parameters such as glycemia. While our pilot experiments showed
24 that on-demand PNES was not as efficient as continuous PNES at inhibiting T1D progression,
25 technological refinements such as optimized electrical stimulation parameters or

1 implementation of a closed-loop system are within reach and may greatly improve its
2 therapeutic efficacy. Thus, our study provides a rationale for the future use of bioelectronic
3 medicine in treatment of autoimmune diabetes.

1 **Figure Legends**

2 **Figure 1: Pancreatic nerve electrophysiological and functional characterization. (a)**

3 Confocal microscopy of a representative pancreatic nerve section stained for Tyrosine
4 Hydroxylase (TH, red) or NeuroFilament (NF, blue). Scale bar = 20 μm . **(b)** Impact of PNES
5 on surface pancreatic blood flow measured by laser speckle. Representative (of 5 mice) color-
6 coded images (left) and quantification (right) before, during and after PNES for 15 s.
7 Quantification was performed on the area delineated by the dotted line. **(c)**. A representative
8 image (of 3 mice) of maximal projection of a TH-stained LN, cleared and imaged by light-sheet
9 microscopy at low magnification. Scale bar = 500 μm . **(d)** Representative recordings of
10 fluorescent axon electrical activity in pancreatic LNs from tdTomato^{TH-Cre} mice following
11 PNES. Representative (of 4 experiments) FAPs (left) and peak amplitudes (right) as a function
12 of PNES intensity. **(e)** Noradrenaline content in pancreatic LNs following PNES (450 μA , 10
13 Hz, 2 min.) or sham stimulation (n = 10). Mean \pm S.E.M. **, p < 0.01.

14

15 **Figure 2: Impact of PNES on immune cell number, cytokine production and autoreactive**

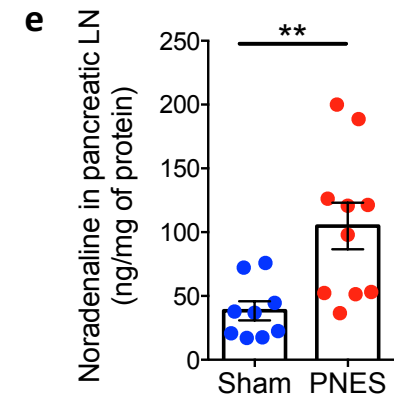
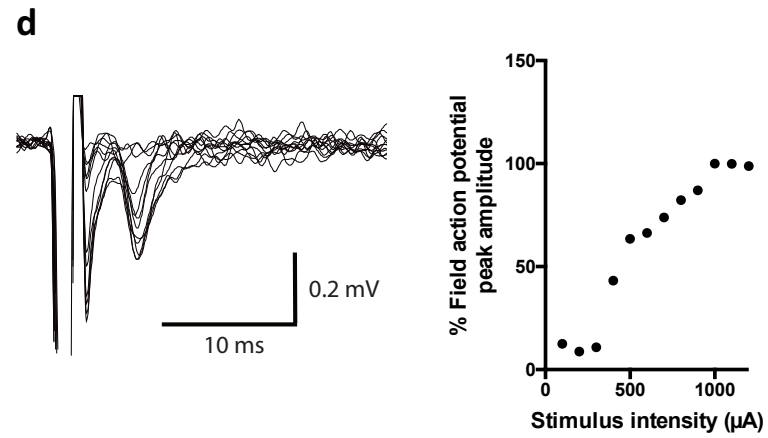
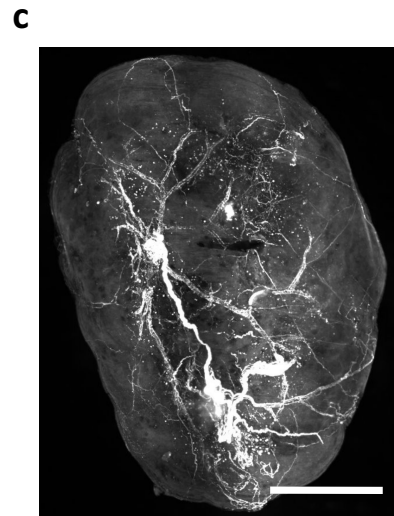
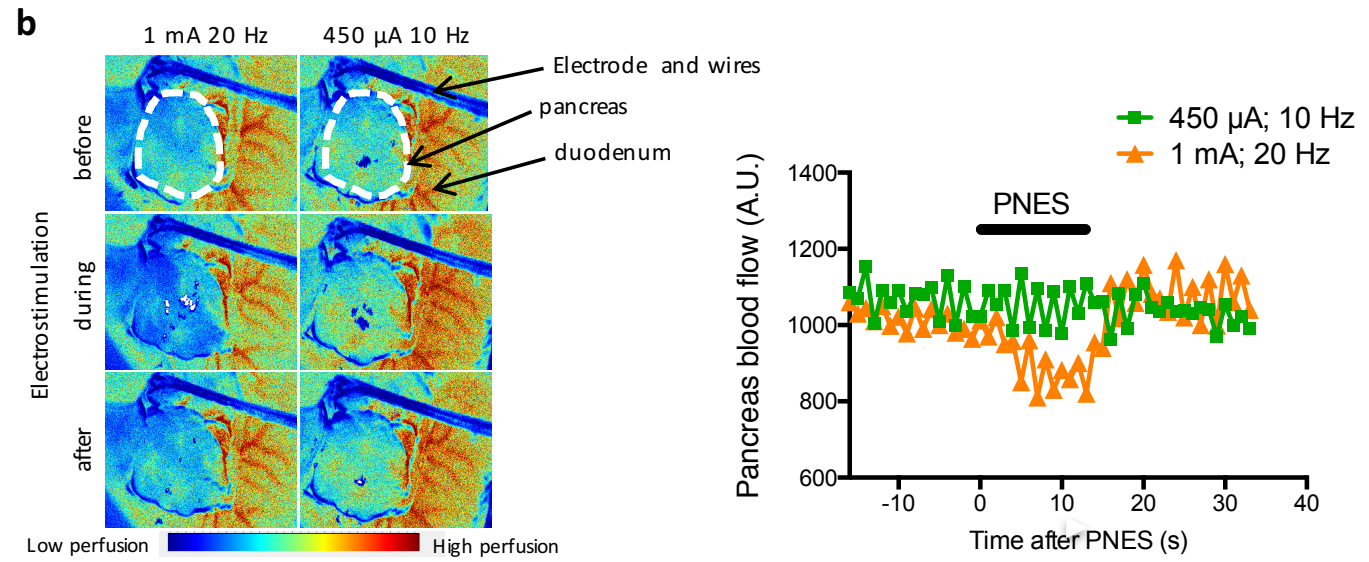
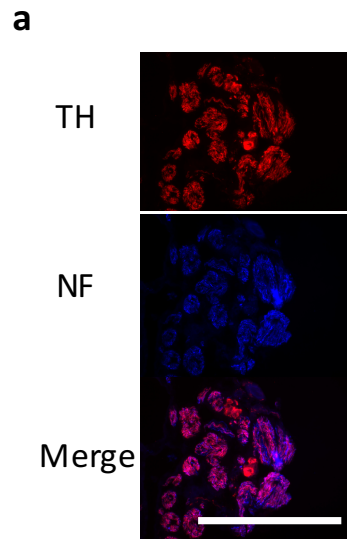
16 **T cell proliferation in pancreatic LNs. (a)** Impact of PNES (450 μA , 10 Hz, 2 min duration,
17 3 sessions 3 hours apart) on lymphocyte numbers in pancreatic LNs. Schematic representation
18 of the experimental protocol (left). Number of B cells, CD4⁺ and CD8⁺ T cells in wt (empty
19 bars) and ADRB2^{ko} (dashed bars) mice (right). Mean \pm S.E.M. of 3 experiments (n = 6-
20 10/group). **(b)** Impact of PNES (450 μA , 10 Hz, 2 min duration) on LPS-induced TNF- α , IL-6
21 and IL-1 β mRNA levels in spleen and pancreatic LNs. Schematic representation of the
22 experimental protocol (left). mRNA levels in the indicated tissues in wt (empty bars) and
23 ADRB2^{ko} (dashed bars) mice (right). Mean \pm S.E.M. of 3 experiments (n = 6-8/group). **(c)**
24 Impact of PNES (450 μA , 10 Hz, 2 min. duration, 3 sessions/day for 4 days) on autoreactive T
25 cell proliferation. Schematic representation of the experimental protocol (left). Representative

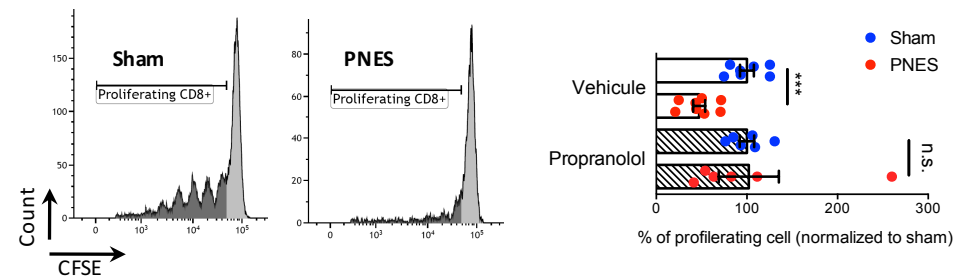
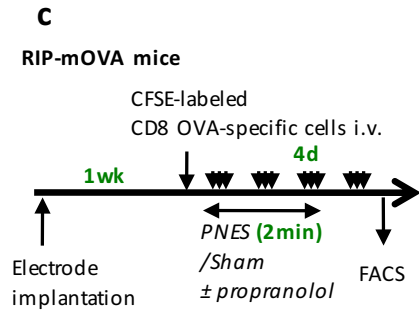
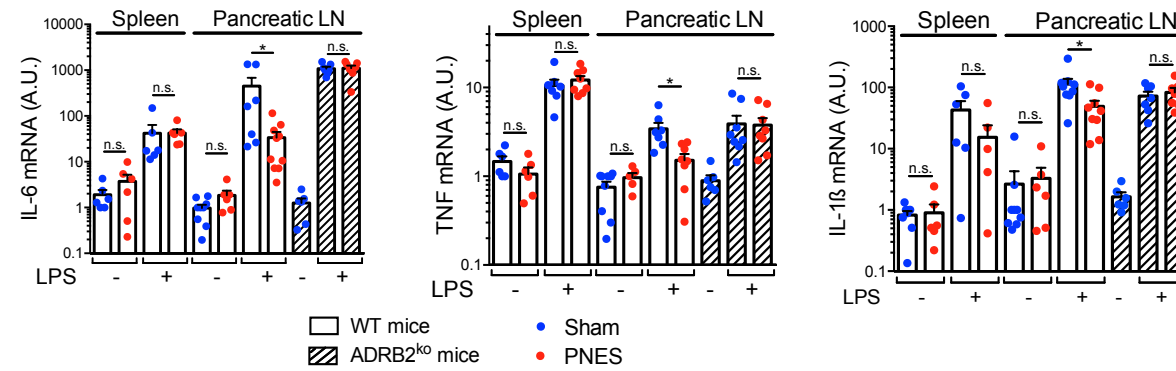
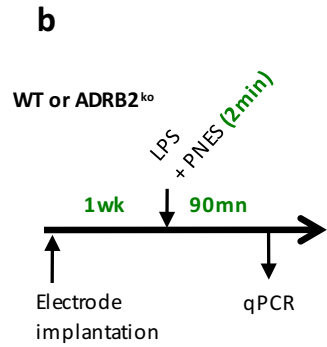
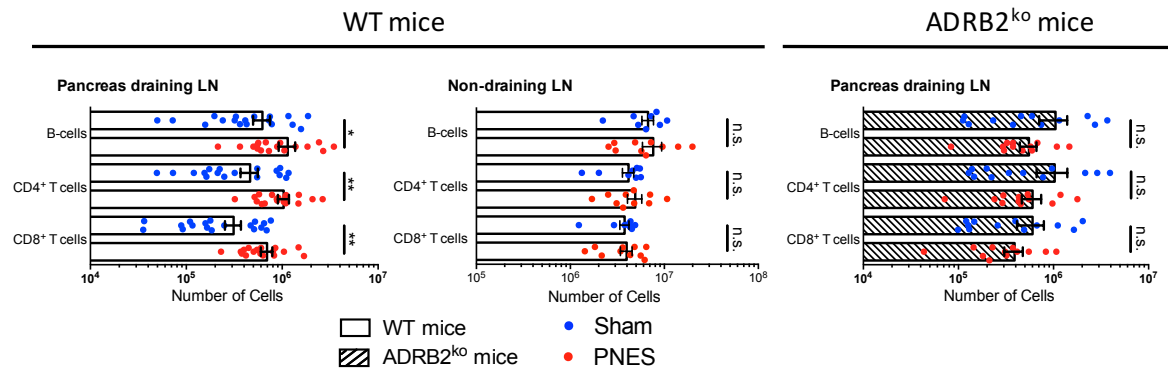
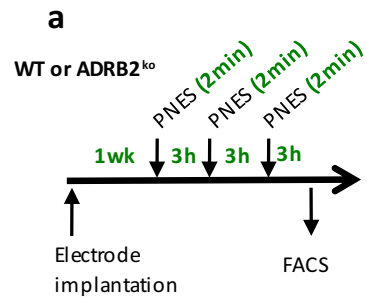
1 FACS profiles of CFSE-labeled CD8⁺ OVA-specific T cells following PNES and sham
2 stimulation in RIP-mOVA mice (middle panel). CD8⁺ OVA-specific T cell proliferation after
3 PNES (normalized to sham) in mice treated (dashed bars) or not (empty bars) with propranolol
4 (right panel). Mean ± S.E.M. of 3 experiments (n = 6-8/group). *, p < 0.05; **, p < 0.01.

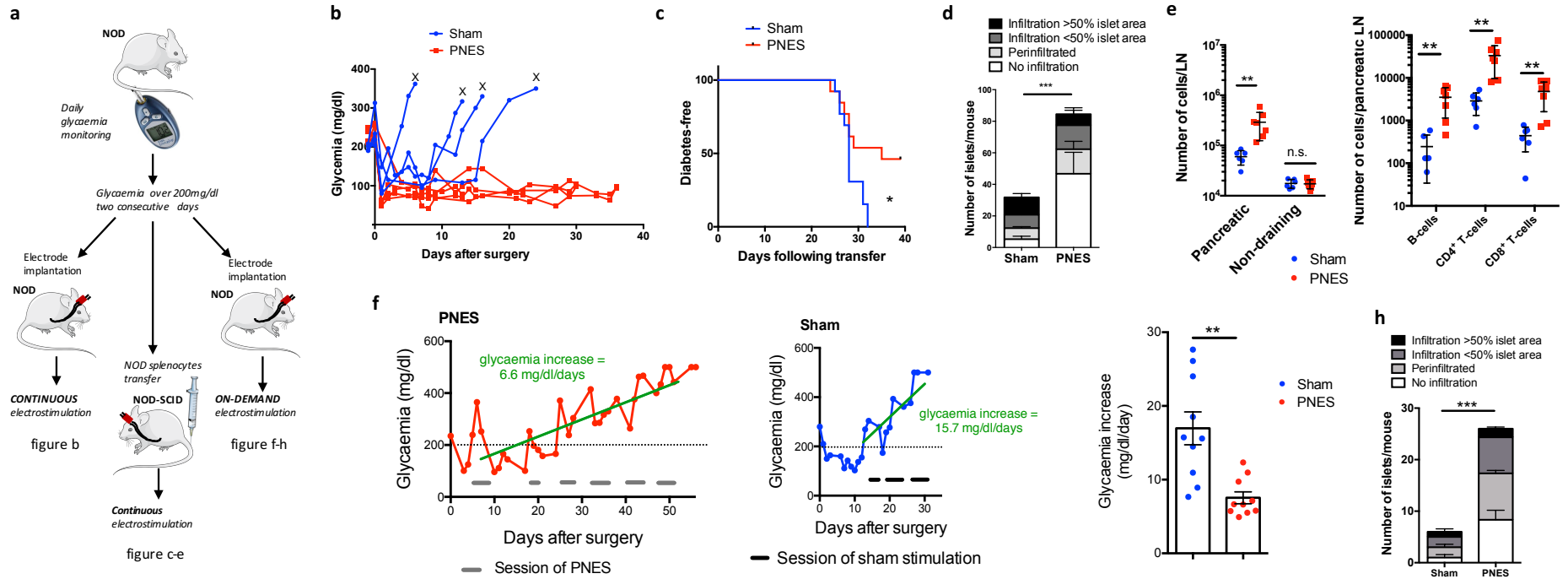
5

6 **Figure 3: Impact of PNES on glycaemia and insulinitis in NOD mice.** (a) Schematic
7 representation of the experimental protocol. Recently diabetic NOD mice (glycaemia over 200
8 mg/dl two consecutive days) were implanted onto the pancreatic nerve with micro-cuff
9 electrodes and PNES was applied either continuously after surgery (b) or on-demand when
10 glycaemia exceeded 200mg/dl (f-h). In other experiments, NOD-SCID recipient were
11 implanted onto the pancreatic nerve with micro-cuff electrodes, injected with 5 x 10⁶
12 splenocytes from recently diabetic NOD mice and PNES was applied continuously (c-e). (b)
13 Glycaemia following continuous PNES in individual mice (n = 4 / group). (c) Diabetes
14 incidence in NOD-SCID mice (n = 13 / group). (d) Number of islets per mouse and proportion
15 of islets exhibiting severe, mild and low immune cell infiltration two weeks after PNES. Mean
16 ± S.E.M. (n = 4 / group). (e) Total number of cells (left panel) and number of B, CD4⁺ and
17 CD8⁺ T cells (left panel) in pancreatic LNs two weeks after PNES. Mean ± S.E.M. (n = 5-6 /
18 group). (f) Glycaemia increase following on-demand PNES. Representative mice (left panel)
19 and mean glycaemia increase (right panel) following PNES or sham stimulation. Mean ±
20 S.E.M. (n = 10/group). (h) Number of islets per mouse and proportion of islets exhibiting
21 severe, mild and low immune cell infiltration two weeks following on-demand PNES. Mean ±
22 S.E.M. (n = 4 / group). *, p < 0.05; **, p < 0.01; ***, p < 0.001.

23







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2 work was funded by a collaborative research grant from GSK Bioelectronics R&D. This work
3 was also supported by the LABEX SIGNALIFE (#ANR-11-LABX-0028-01) and the FHU
4 Oncoage.

5
6 **Competing financial interest.** A patent is pending on pancreatic nerve electrostimulation

7

8

1 **Methods**

2 **Mice:** All experiments were performed with female C57BL/6, NOD, NOD-SCID mice
3 (Charles River), or RIP-mOVA¹³, OT-I¹⁴, ADRB2^{ko}¹⁵ and tdTomato^{TH-Cre}^{16, 17} mice
4 backcrossed onto the C57BL/6 background for at least 10 generations. Mice were housed on a
5 12 hours light/dark cycle (lights on/off at 7 am/7 pm) with food *ad libitum*. Mice were treated
6 in accordance with our local Animal Care and Use Committee guidelines.

7 **Electrodes and surgery:** For studies in anaesthetized animals, mice were anaesthetized by an
8 intraperitoneal injection of a mixture of ketamine (75 mg/kg) and xylazine (60 mg/kg) and a
9 hook electrode was placed under the pancreatic nerve. For studies in conscious animals, mice
10 were anaesthetized with isoflurane and the area around the right abdominal artery next to the
11 kidney was exposed (**Suppl Fig. 1a**). One mm length 100 µm-sling micro-cuff electrodes
12 (CorTec) were implanted onto the pancreatic nerve.

13 **Recordings of CAP and FAP:** After placing a hook electrode onto the pancreatic nerve and
14 artery, platinum-iridium recording electrodes (Phymep) were placed onto the pancreatic nerve,
15 pancreatic lymph node, pancreas tissue and liver for CAP recording using a wireless recording
16 system (W8, Multi-Channel Systems). Ground/Reference wires were placed into the nearby
17 tissue. For FAP, recordings were performed on explants from tdTomato^{TH-Cre} that were placed
18 in a recording chamber at room temperature (20-25°C) superfused with oxygenated artificial
19 cerebrospinal fluid (ACSF) under a microscope (Zeiss). The pancreatic nerve was introduced
20 into a suction-stimulating electrode stimulated with square pulses of 1 ms and intensities of 10-
21 1500 µA. Recordings were made using pipettes made from borosilicate glass capillary
22 (Hilgenberg) with resistance of 3–6 MW when filled with extracellular solution, placed near
23 red fluorescent axons. Signals were amplified using an Axopatch 200B (Axon Instruments),
24 digitized at 10kHz via an Digidata 3200 interface (Molecular devices) controlled by

1 pClamp10.0 software (Molecular Devices) and digitally filtered at 3KHz. CAP and FAP
2 recordings were performed in a Faraday cage.

3 **Electrostimulation:** For studies on anaesthetized mice, Master-8 (A.M.P.I.), PlexStim V2.3
4 (Plexon) and STG 4002 stimulator (Multichannel system) were used respectively for CAP,
5 pancreatic blood perfusion and FAP recording. For all studies in conscious animals, mice were
6 placed in individual cage and connected to a PlexStim V2.3 (Plexon) or MAPS (Axonic)
7 stimulator. Unless specified, the set-up of the electrostimulation were rectangular charged-
8 balanced biphasic pulses with 450 μ A pulse amplitude, 2 ms pulse width (positive and negative)
9 at 10 Hz frequency for 2 minutes. In propranolol-treated groups, mice were injected
10 intraperitoneally with a 5mg/kg dose, 30 min before electrostimulation.

11 **Noradrenaline levels:** Pancreatic LNs were harvested and snap-frozen in liquid nitrogen
12 immediately after electrostimulation. The organ was processed and noradrenaline was measured
13 by ELISA (DLD Diagnostika GmbH) via manufacturer recommendations.

14 **Flow Cytometry:** Single-cell suspensions were stained with anti-CD45 (clone 30F11), anti-
15 CD3 (17A2), anti-CD4 (RM4-5), anti-CD8 α (53-6.7), anti-CD19 (1D3). All antibodies are
16 from BD Biosciences. Dead cells were excluded using 7-AAD staining. Data were acquired on
17 SP6800 (Sony) flow cytometer and analyzed using Kaluza software.

18 **RT-PCR:** RNAs from pancreas were isolated *via* the manufacturer's instructions (miRNEasy
19 micro kit, Quiagen), reverse transcribed using QuantiTect Reverse transcription kit (Quiagen)
20 and amplicons were quantified SyberGreen Master Kit (Roche) and LightCycler 480 II
21 (Roche). mRNAs cytokine expression were normalized to GAPDH using LightCycler software
22 (Roche).

23 **In vivo cross-priming assay:** Cross-priming *in vivo* was assessed in Rip-mOVA mice. 10^7 of
24 CFSE-labeled OT-I CD8⁺ T cells were injected intravenously to these mice at day 0. Between

1 day 1 and day 4, animals were electrostimulated or not (450 μ A, 10 Hz, 2 minutes) 3 times a
2 day (3 hours apart). On day 4, mice were sacrificed, pancreatic lymph node removed and CD8⁺
3 proliferation was assessed by flow cytometry using SP6800 (Sony). Rip-mOVA mice were
4 treated with propranolol (5mg/kg ip) 30mn before each stimulation.

5 ***Adoptive cell transfers in NOD-SCID:*** Recipients were adult 6-week-old NOD-SCID mice.
6 Animals were injected intravenously with 5×10^6 splenocytes from overtly diabetic NOD mice.

7 ***Glycaemia follow-up:*** Glycaemia was monitored using a Free Style Papillon Vision (Abbott)
8 taking a blood drop (<10 μ l) from the tail. NOD and NOD-SCID mice were considered diabetic
9 when glycaemia were over 250mg/dl for two successive days. To determine the increase slope
10 of NOD mice linear regression to determine glycaemia including the first glycaemia time point
11 over 200mg/dl and the 2 glycaemia time point exceeding 499mg/dl.

12 ***Laser speckle imaging:*** After anaesthesia, the pancreas tissue was exposed and placed about
13 30 cm below the Moor-FLPI laser speckle perfusion imager (Moor instruments Ltd.). Then the
14 pancreatic blood perfusion images were saved and analyzed by the Image Review Program of
15 Moor-FLPI-V2.0 software. The round region of interest (ROI) with the same area in each LSP
16 image was selected for measuring the pancreatic blood perfusion.

17 ***Statistics:*** All findings shown have been reproduced in at least two independent experiments.
18 Data are presented depending on their scale and distribution with arithmetic mean and standard
19 deviation (mean \pm s.e.m.). Normality was assessed using a Kolmogorov–Smirnov test. To
20 compare independent measurements we used a t-test and Mann–Whitney U-test as appropriate.
21 To compare dependent measurements we used a paired t-test. To compare more than two groups
22 we used one-way ANOVA followed by Tukey’s post hoc test. Diabetes-free curve is visualized
23 by Kaplan–Meier curves and statistically compared by log-rank test. Statistical analysis was
24 performed using GraphPad Prism v.6. The p values <0.05 were considered statistically

1 significant.

2

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