Overexpression of Interleukin-1β in the Murine Pancreas Results in Chronic Pancreatitis

SUPPLEMENTARY MATERIALS

Supplementary Materials and Methods

Tissue Preparation

Pancreata were isolated and washed in cold DEPC water. Portions were cut and snap frozen in liquid nitrogen for protein and RNA extraction. Proteins were extracted using a tissue homogenizer and RIPA buffer + protein inhibitors (complete EDTA free, Roche Applied Science, Indianapolis, IN, USA) and protein quantification was performed using the Bradford method. For RNA isolation, frozen samples were ground using a pestle in liquid nitrogen and homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA was extracted according to manufacturer's instructions and cleaned using RNeasy columns (Qiagen, Valencia, CA, USA). The remaining tissue was fixed overnight in 10% neutral buffered formalin before transfer to 70% ethanol. Specimens were then processed and embedded in paraffin for histologic analysis.

Histology, Immunohistochemistry, Immunofluorescence, Microscopy

Tissue sections (5 μm) were stained with H&E for morphologic analysis Masson's trichrome or Sirius red staining was performed to evaluate the extent of fibrosis.

For immunohistochemical staining, slides were deparaffinized in xylene and endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide in methanol for visualization using the peroxidase reaction. Alternatively, for visualization with the alkaline phosphatase reaction, slides were incubated with 20% acetic acid in methanol for 2 min. Antigen retrieval was performed by boiling the slides in citrate buffer (10mM pH 6.0) in a water bath for 20 min., except for cytokeratin 19 staining, for which incubation with pepsin solution (37°C) for 15 minutes was used for antigen retrieval (Abcam, Cambridge, MA, USA, # ab8194). Slides were rinsed in PBS Tween 0.05% and blocked for 30 min. with 2% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) - PBS. Primary antibodies and biotinylated secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA) were diluted in 2% bovine serum albumin - PBS and incubated for one hour each at room temperature. Subsequently, slides were incubated with alkaline phosphatase or peroxidase conjugated streptavidin (Dako North America, Inc., Carpinteria, CA, USA) and either VectorRed substrate (Vector Laboratories, Burlingame, CA, USA, #SK 5100) or 3,3'-diaminobenzidine (Sigma-Aldrich) as chromogens, respectively. Slides were counterstained with haemtoxylin and mounted for viewing. Primary antibodies used were: BrdU (Abcam, #ab6326, 1/300), α-smooth muscle actin (Dako North America, Inc, M0851, 1/200), desmin (Lab Vision, Fremont, CA, USA, #RB-9014, 1/200), amylase (Sigma-Aldrich, #A8273, 1/500), and cytokeratin 19 (Clone Troma-III developed by Rolf Kelmer was obtained from Development Studies Hybridoma Bank developed under the auspices of the NICHD and

maintained by the University of Iowa, Department of Biological Sciences, 1/25). For primary mouse monoclonal antibodies the ARK kit (Dako North America, Inc) was used according to the manufacturer's instructions.

For immunofluorescence staining, slides were prepared as described above but endogenous peroxidase or alkaline phosphatase blocking was not performed. FITC or Texas Red conjugated secondary antibodies (Jackson Immunoresearch) were utilized. Counterstaining was performed with DAPI (Invitrogen) and slides were mounted in Vectashield mounting medium (Vector Laboratories).

Bright field and fluorescence images were acquired using an Eclipse TU2000-U microscope (Nikon, Melville, NY, USA) connected to a cooled color CCD camera (RTKE Diagnostic Instruments, Sterling Heights, MI, USA) using SPOT software. Confocal fluorescence microscopy was performed using an LSM 510 NLO multiphoton confocal microscope (Zeiss, Thornwood, NY, USA). FITC was excited with a 488 nm argon laser and Texas Red was excited with a 543 nm helium-neon laser. DAPI was imaged using two-photon excitation by a Coherent Mira titanium-sapphire laser (Santa Clara, CA, USA) tuned to 800 nm.

Proliferation and Apoptosis Quantification

Proliferation index was assessed from six non overlapping 400X magnification fields by counting BrdU positive and negative acinar cells. Apoptosis quantification was performed the same way, using the TUNEL assay (ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit, Chemicon, Billerica, MA, USA) according to the manufacturer's instructions.

cDNA Synthesis and Polymerase Chain Reaction

Reverse transcription of mRNA was performed using Superscript First-Strand Synthesis System III (Invitrogen) after digestion with DNase I (Promega, San Luis Obispo, CA, USA) according to the manufacturers' instructions. PCR was performed on cDNA with primer pairs listed in supplementary table 1. Amplification program was as follows: 94°C 2 min., [94°C 30sec.; annealing –see supplementary table 1 for temperatures – 30sec.; extension 72°C 30sec.]x35 but for GAPDH (x30), final extension 72°C-7min..

Interleukins Quantification

To quantify human interleukin-1β in pancreatic tissue, ELISA assays were performed with the Quantikine® Human IL-1β immunoassay (R&D systems, Minneapolis, MN, USA, #DLB50) according to the manufacturer's instructions. Bone marrow myeloid cells secretion of Interleukin 6 was quantified in vitro using the Mouse IL-6 Elisa kit (R&D systems, Minneapolis, MN, USA, #DY406) 24 hours after stimulation with 10 ng/mL human interleukin-1β (BD Biosciences , # 354042).

Flow Cytometry

Small pancreatic samples were harvested and kept on ice before being minced with a blade and digested at 37°C, on a rotating wheel for 25 minutes, with collagenase P 0.05% (Roche Applied Science), pronase 0.02% (Roche Applied Science), and DNAse 0.1% (Roche Applied Science) in Hank's Balanced Salt Solution (Invitrogen)

complemented with bovine serum albumin 0.1% (Sigma) and HEPES 20 mM (Invitrogen). Cells were washed with Hank's Balanced Salt Solution supplemented with 10% fetal bovine serum (Invitrogen) and subsequently with PBS containing 0.5% bovine serum albumin and 1mM EDTA. Immunolabelling was performed after incubation with rat serum (eBioscience, San Diego, CA, USA) using PE-conjugated CD3 and Gr1, and FITC-conjugated CD11b and CD19 (eBioscience). Cells were stained with DAPI to assess viability. Flow cytometry analysis was performed using an LSRII (Becton Dickinson, San Jose, CA, USA) after gating out dead cells.

Magnetic Resonance Imaging

Magnetic Resonance Imaging was performed with a 9.4T magnet and a Bruker Avance 400 spectrometer equipped with a Bruker micro2.5 imaging gradient set with maximum gradient strength of 150 guass/cm. A linear polarized RF birdcage coil insert 3.8 cm in outer diameter, was inserted into the gradient set. There was a 3 cm diameter space in the coil for the animal holder which provided 2.5cm diameter for the animal to be secured for imaging. Two tuning and matching capacitors allowed the RF coil to be tuned to the lamour frequency of 400 MHz. Prior to scanning, mice were anesthetized with 2% isoflourane and a PE-10 size catheter was inserted into the peritoneal cavity. A syringe filled with .7cc of 50 μM gadodiamide (Omniscan) was inserted to the distal side of the catheter. The Magnetic Resonance Imaging scanning protocol consisted of first adjusting the position of the animal in the scanner, with triplanar MSME scout scans, so that the stomach was in the center of the scanning volume (2.1 cm x 2.1 cm x 1.5 cm). Then, shimming was performed and spectrometer parameters were adjusted (5 minutes). A T2 weighted fast spin echo scan was then performed (25 minutes). This was followed by a pre-contrast T1 weighted spin echo scan (15 minutes). The contrast agent gadodiamide was then administered. A series of 3 T1 weighted scans were then performed (each 15 minutes). T2 scans employed echo times of 29.4 ms and repetition times of 1462 ms. T1 scans employed echo times of 7.6 ms and repetition times of 278 ms.

Glucose Tolerance Test

Blood glucose levels were measured, after an overnight fast (baseline) and then 20min., 40min., 60min., and 120 min. after intra-peritoneal injection of glucose 2 mg/g body weight. Blood glucose level was measured using a OneTouch glucometer (LifeScan, Inc., Milpitas, CA, USA).

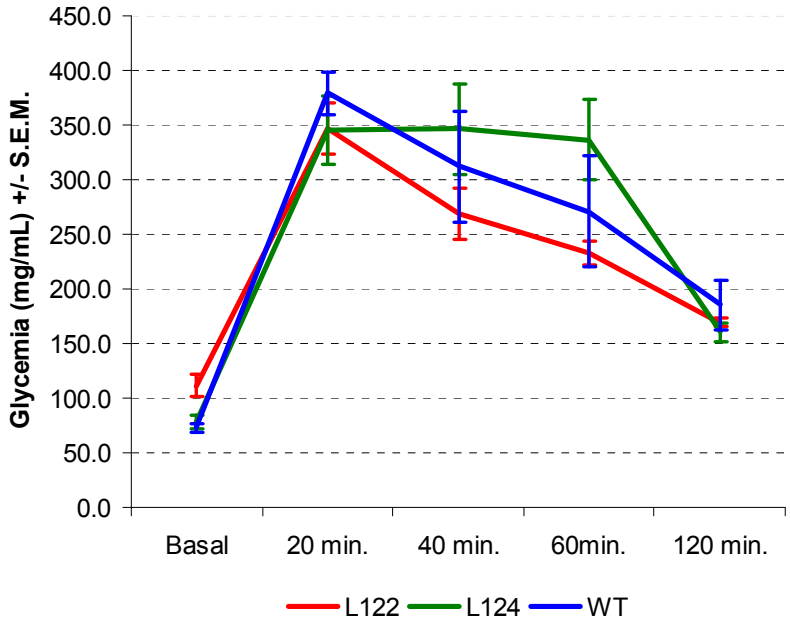
Research of Fat Malabsorption

Fat malabsorption was researched by performing Oil Red O staining on stool smears. Groups of 4 mice were fed for three consecutive days with a diet containing 45% fat (Research diet, Inc., New Brunswick, NJ, USA). Stools were then collected over two 6-hour periods on two consecutive days, homogenized in water (10μL/mg stool), and centrifuged for 5 min. at 200g. Five μL of supernatant was applied on a gelatin-coated glass slide and mixed with 5 μL of freshly diluted and filtered Oil Red O. Slides were dried and mounted for viewing.

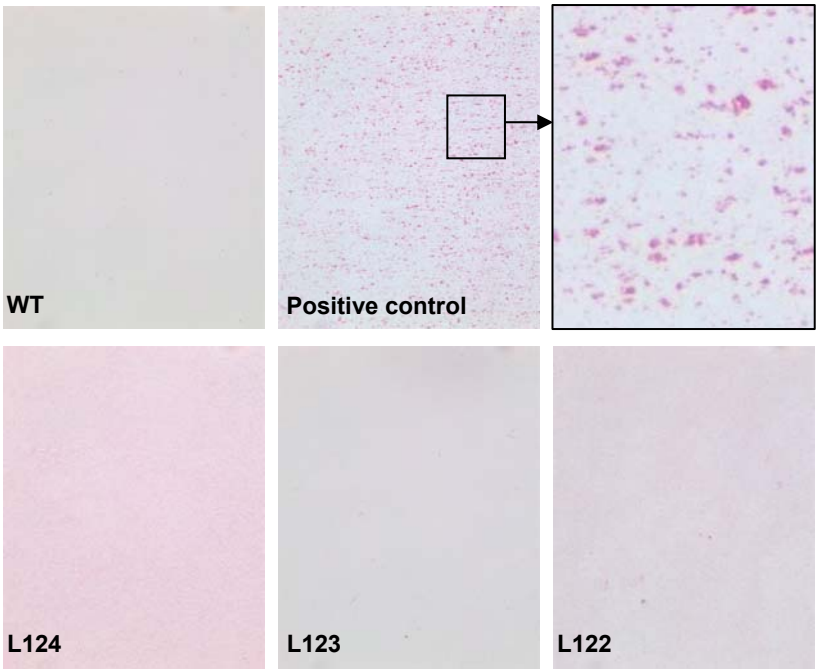
Supplementary table 1: PCR primers

Primer name	sequence	Length	Annealing Temperature	Comment
mCOX2 F	ggcccttcctcccgtagcaga	449	60	
mCOX2 R	tcatcagaccaggcaccagaccaa			
mTGFβ1 F	acaattcctggcgttacctt	172	50	
mTGFβ1 R	tggagtttgttatctttgctgtca			
mTNFα F	cccaaggcgccacatctcc	575	60	
mTNFα R	ggggcaggggctcttgacg			
mCXCL1 F	ggccccactgcacccaaacc	186	55	
mCXCL1 R	tgttgtcagaagccagcgttcacc			
mSDF1 F	aaaccagtcagcctgagctacc	164	55	
mSDF1 R	aatttcgggtcaatgcacactt			
mCXCR4 F	atggaaccgatcagtgtgag	217	55	
mCXCR4 R	tccttagcttcttctggtaacc			
mTIMP1 F	ccacaatccaacgagaccacc	335	50	
mTIMP1 R	gggatagataaacagggaaacact			
mMMP2 F	ccgggagcgcaacgatgga	495	60	
mMMP2 R	gagaaaagcgcagcggagtgacg			
mMMP7 F	ctttgatgggccagggaacact	144	55	
mMMP7 R	aattcatgggtggcagcaaaca			
mMMP9 F	cgccggcgttcagggagatg	474	55	
mMMP9 R	aagacgaaggggaagacgcacagc			
mGAPDH F	tcaccaccatggagaaggc	168	55	
mGAPDH R	gctaagcagttcgtggtgca			
mIL1 F	ggagaaccaagcaacgacaaaata	211	55	
mIL1 R	tggggaactctgcagactcaaac			
IL1RA- sshIL1β F	ggcctccgcagtcacctaatca	415	57	Genotyping primer
IL1RA- sshIL1β R	tggggaactgggcagactcaaa			
Ela prom - sshIL1β F	acctgtgcttttccctgccttcta	917	57	Genotyping primer
Ela prom - sshIL1β R	ttgttgctccatatcctgtccctg			
sshIL1β polyA F	aatctgtacctgtcctgcgtgttg	476	57	Genotyping primer
sshIL1β polyA R	tcccatatgtccttccgagtgaga			
P53 F	agcctgcctagcttcctcagg	330 (mutated)	57	Genotyping primer
P53 R	cttggagacatagccacactg	290 (wt)		

Supplementary Figures



Supplementary Figure 1: Glucose tolerance test in elastase sshIL-1β mice with low (L124) or high (L122) level of interleukin-1β expression, and in wild type littermates. Results are expressed as means (n=5), error bars are S.E.M.



Supplementary Figure 2: Oil Red O staining of stool smears after 48-hour high fat diet in elastase sshIL-1β mice from the three different founder lines (L122-3-4) and wild type littermates (WT). Positive control is ground high fat diet.