Published in final edited form as:

Transplantation. 2013 April 27; 95(8): 1000–1007. doi:10.1097/TP.0b013e318286483a.

Up-Regulation of IL-1 β , IL-6, and CCL-2 by a Novel Mouse Model of Pancreatic Ischemia-Reperfusion Injury^{1, 2, 3}

Keri E. Lunsford, M.D., Ph.D.⁴, Brandon Jackson Baird, B.S.⁵, Gregory D. Sempowski, Ph.D.^{5,6}, Diana M. Cardona, M.D.⁶, Zhiguo Li, Ph.D.⁷, Kent J. Weinhold, Ph.D.⁴, Debra L. Sudan, M.D.⁴, and Todd V. Brennan, M.D., M.S.^{4,8}

⁴Department of Surgery, Duke University Medical Center, Durham, NC, USA

⁵Department of Medicine, Duke University Medical Center, Durham, NC, USA

⁶Department of Pathology, Duke University Medical Center, Durham, NC, USA

⁷Department of Biostatistics and Bioinformatics, Duke University Medical Center, Durham, NC, USA

Abstract

Little is known about the immunologic events surrounding pancreatic ischemia-reperfusion injury (IRI) due to a lack of established experimental models. The purpose of the current study was to develop a mouse model for pancreatic IRI to serve as a basis for immunologic characterization of pancreatic organ damage at transplantation.

Methods—Reversible ischemia was surgically induced by vascular isolation of the distal pancreas for 0, 10, 20, or 30 minutes. Mice receiving laparotomy without clamping served as sham-operated controls. Following reperfusion, mice were serially assayed for biochemical and histologic evidence of inflammation, proinflammatory cytokine and chemokine production, and inflammatory gene up-regulation.

Results—Following induction of pancreatic IRI, serum amylase and LDH peaked at 6 hours and returned to baseline by 120 hours following injury in all groups. Mice undergoing 30 minutes of IRI demonstrated greatest biochemical evidence of inflammation. Histologic scoring similarly demonstrated marked inflammation in mice subjected to 30 minutes IRI compared to controls. Serum cytokine/chemokine analysis demonstrated significant up-regulation of G-CSF, IFN- γ , TNF- α , IL-2, IL-1 β , IL-6, CCL-2, CCL-5, CXCL-1, and MIP-2. Similar up-regulation of *ccl2*,

¹Disclosure: No conflict of interest to disclose

²This work was presented in part at the American Transplant Congress 2012 in Boston, MA, the American Society of Transplantation Annual Scientific Exchange 2011 in San Antonio, TX, and the American Society of Transplant Surgeons 2012 Winter Symposium in Miami, FL.

³Authorship: Lunsford KE – Participated in research design, writing of the paper, performance of the research, and data analysis; Baird BJ – Participated in writing of the paper, performance of the research, and data analysis; Sempowski GD – Participated in research design, writing of the paper, data analysis, and contributed new reagents and analytic tools; Cardona DM – Participated in writing of the paper, performance of the research, and data analysis; Li Z – Participated in writing of the paper and data analysis; Weinhold KJ – Participated in research design and contributed new reagents and analytic tools; Sudan DL – Participated in writing of the paper; Brennan TV – Participated in research design, writing of the paper, and data analysis.

⁸Please address correspondence to: Todd V. Brennan, MD, MS, Assistant Professor of Surgery, Division of Transplantation, Department of Surgery, Duke University Medical Center, DUMC 3512, Durham, NC 27710, phone: 919-684-8245, fax: 919-684-8716, todd.brennan@dm.duke.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

il1b, *il6*, *fos*, *hspa1a*, *hspd1*, and *cd14* gene expression was detected by real-time PCR analysis of pancreatic tissue.

Conclusions—This novel model of distal pancreatic IRI in the mouse demonstrates time-limited pancreatic inflammation and injury by histologic and biochemical indices. Inflammation may be, in part, a result of the immunologic effects of IL-1 β , IL-6, and CCL-2. This model provides a method by which immunologic mechanisms of pancreatic IRI can be elucidated.

Keywords

ischemia-reperfusion injury; pancreas; mouse

INTRODUCTION

Currently, pancreas transplantation offers the most efficacious long-term cure for insulin-dependent diabetes mellitus. Since the first vascularized pancreas transplant in 1966 (1), pancreas transplantation outcomes have improved significantly. From 2000 to 2005, pancreas transplants had one year and five year graft survival of 80.1% and 50.6% (2). However, long-term pancreas outcomes are significantly worse compared to other whole organ allografts such as liver, kidney, and heart. Previous studies have demonstrated that different tissues elicit organ-specific allogeneic immune response (3–5). One possible reason for this disparity may be increased susceptibility of a tissue to ischemia-reperfusion injury (IRI) or activation of particular innate immune pathways by different tissues. Thus, in order to advance our understanding of immune responses elicited by particular tissues, knowledge of the immune pathways activated as a result of ischemic damage may prove critical.

IRI represents the initial stimulus for organ damage following transplantation. Not only are tissues themselves susceptible to warm and cold ischemic injury prior to transplantation, but the damaged cells subsequently serve as potent stimuli for the innate immune system. Stimulation of innate immune pathways may then lead to the activation and maturation of alloreactive T and B cells, further potentiating allograft damage (reviewed in (6)). In addition to parenchymal damage, IRI may ultimately stimulate acute rejection of the transplanted organ.

In contrast to the well-characterized role of IRI in allograft outcomes for liver and kidney transplantation, little is known about IRI of the pancreas. Much of this disparity results from a relative paucity of established animal models to study the events surrounding ischemic tissue insults. In the present study, we sought to (1) establish a reproducible mouse model for pancreatic IRI, (2) characterize the histologic and biochemical damage associated with injury, and (3) evaluate the immunologic markers of ischemic damage to the pancreas.

RESULTS

Establishment of a novel mouse model for pancreatic IRI

Few murine models evaluating pancreatic IRI have previously been described. The mouse pancreas is supplied by the celiac axis (superior pancreaticoduodenal artery and lienopancreatic artery) and the superior mesenteric artery (7). Total pancreatic vascular isolation was first attempted. C57BL/6 mice underwent laparotomy with occlusion of the superior pancreaticoduodenal artery, inferior pancreaticoduodenal artery, and lienopancreatic artery for 10 minutes (n=3). Visible ischemic changes were observed in both the pancreas and the duodenum. Following reperfusion, all of animals expired within 90 minutes (range 60–90 minutes). On necropsy, persistent patchy duodenal necrosis was observed.

Given the high mortality associated with total pancreatic ischemia, distal pancreatic vascular isolation was next performed. The distal mouse pancreas is supplied by the lienopancreatic artery with collaterals from the lienal artery (Fig. 1A). To induce selective distal pancreatic ischemia, the lienopancreatic and lienal vessels were clamped. Experimental mice underwent 10 minutes of ischemia followed by reperfusion (n=3). Vascular clamping was associated with initial blanching of the distal pancreas followed by gross erythema and induration as ischemia progressed (Fig. 1B). Visible ischemic changes reversed following reperfusion. All mice recovered with 100% survival at one-week. These results identify distal pancreatic vascular isolation as a feasible model for IRI evaluation.

Distal pancreatic IRI results reversible chemical pancreatic damage

In order to determine the optimal duration of IRI, C57BL/6 mice underwent 10 minutes (n=3), 20 minutes (n=3), or 30 minutes (n=7) of distal pancreatic ischemia. Control mice underwent clamp application with immediate removal (0 min, n=7) or sham laparotomy (n=3). Serum was serially assayed for amylase for 1 week following IRI. Amylase concentrations peaked at 6 hours following IRI and returned baseline by 5 days (Fig. 2A). Amylase increased by a mean of 11.9 ± 1.8 -fold after 10 min IRI, 15.3 ± 1.3 -fold after 20 min IRI, and 19.3 ± 1.6 -fold after 30 min IRI. Serum was similarly assayed for lactate dehydrogenase (LDH) following pancreatic IRI. LDH peaked 6 hours following IRI and normalized by 24–48 hours in all groups (Fig. 2B). Peak LDH levels increased by a mean of 1.1 ± 0.1 -fold after 10 minutes IRI, 1.5 ± 0.3 -fold after 20 minutes IRI, and 3.5 ± 1.0 -fold after 30 minutes IRI.

Before applying the t-test to compare the peak serum amylase between different times of IRI, we assessed the normality of the data. We first fit a one-way ANOVA model to the data using time of IRI as the single factor, and then we draw the normal Q-Q plot of the residuals. Results in Fig. 2C show that the normality assumption is reasonable since the points in the normal Q-Q plot are approximately around a straight line. The results of our analysis using t-test show that peak serum amylase was significantly increased in mice undergoing 10 min, 20 min, and 30 min of IRI compared to both 0 min ($p=0.04$, $p=0.01$, and $p<0.005$, respectively) and sham-operated controls ($p<0.005$ for all). Peak serum amylase was not significantly different between 0 min IRI and sham controls. We further fit a linear model to the data, and the results show that there is a strong linear increasing trend of the peak serum amylase when time of IRI increases. The coefficient of time of IRI in this model is estimated to be 547.9 with a p-value $<10^{-10}$. The fitting of the linear model to the data is shown in Fig. 2D.

Similar to the amylase data, we assess the normality assumption of the LDH data before performing the t-test. Results show that after we discard an outlier, which is a data point in the 30 min IRI group, the normality assumption seems to be reasonable (Fig. 2C). Results of t-tests show that peak LDH was significantly increased after 30 minutes IRI in comparison to sham ($p=0.05$) and 0 minute IRI ($p=0.006$). Peak serum LDH was not significantly different between 0 minute IRI and sham controls. Together, these data confirm significant chemical pancreatic inflammatory damage induced by distal pancreatic IRI. These effects were most notable in mice undergoing 30 minutes of IRI; thus, 30 minutes IRI mice were utilized for further studies. The data also suggest an obvious increasing trend of LDH when time of IRI increases. Hence we further fit a linear model to the data using time of IRI as the covariate. The coefficient of the time of IRI in this model is estimated to be 4.48 with a p-value less than 10^{-4} . The fitting of the model is shown in Fig. 2D.

In order to evaluate the endocrine effects of distal pancreatic IRI, serum from C57BL/6 mice undergoing 30 minutes (n=3) or control mice undergoing 0 minutes (n=3) of IRI was serially assayed for glucose during the first 48 hours following reperfusion. All serum glucose

values remained less than 200 mg/dL (range 120–192 mg/dL) regardless of the time point following reperfusion. There was no significant difference in serum glucose between 30-minute IRI or control (0-minute) IRI mice at any time point. Thus, no effects of IRI on endocrine function were detectable.

Distal pancreatic IRI results in reversible pancreatic inflammatory infiltration

Animals subjected to 30 minutes of IRI were serially sacrificed and the distal pancreas was histologically evaluated (n=3). Serial sections were stained with hematoxylin and eosin and systematically evaluated by a single pathologist. Morphologic examination revealed significant pancreatic necrosis, neutrophilic and lymphocytic inflammation, epithelial vacuolization, and apoptosis following 30 minutes of IRI (Fig. 3A) in comparison to control tissue (Fig. 3B). Histologic inflammation was most notable 24–72 hours after IRI, with only rare pancreatic necrosis noted by 144 hours after IRI (Fig. 3C). These findings suggest that the pancreatic inflammation induced by pancreatic IRI is reversible.

Distal pancreatic IRI up-regulates inflammatory cytokines IL-1 β and IL-6 and chemokine CCL-2

Following 30 minutes of distal pancreatic IRI, animals were sacrificed at 6 (n=8) or 24 (n=7) hours, and serum was collected for multiplex chemokines and cytokines analysis. Serum from naïve C57BL/6 mice (n=3), sham-operated mice at 6 (n=3) or 24 (n=3) hours, and 0 minutes IRI at 6 (n=3) or 24 (n=6) hours served as controls. Serum cytokines GM-CSF, IL-1 α , IL-7, IL-9, IL-10, IL-15, and IL-17 were less than the minimum detectable concentration, and were thus excluded from analysis. Leukocyte growth factors GM-CSF was significantly up-regulated by pancreatic IRI (Fig. 4A). In addition, pancreatic IRI induced early up-regulation of pro-inflammatory cytokines IL-2, IL-6, and TNF- α at 6 hours. By 24 hours following injury, up-regulation of IL-1 β and IFN- γ were additionally noted (Fig. 4A). In contrast, IL-4, -5, -12, and -13 were not significantly increased by pancreatic IRI (not shown). Serum chemokine CXCL10 (IP-10) was less than the minimum detectable concentration and was thus excluded from analysis. Among chemokines, distal pancreatic IRI significantly up-regulated CCL-2, CCL-5, and CXCL-2 (Fig. 4B), but not CCL-3 or CCL-4, or CXCL-10 at 6 and 24 hours after reperfusion. In addition, early up-regulation of CXCL-1 at 6 hours but not 24 hours after reperfusion was observed.

Distal pancreatic IRI activates specific inflammatory genes

Following 30 minutes distal pancreatic IRI, experimental animals were sacrificed at 24 hours after reperfusion (n=4). The distal pancreas was harvested and total RNA was isolated and evaluated by commercially available quantitative RT PCR array analysis. Pancreata from sham-operated C57BL/6 mice (n=3) and 0 min PIRI mice (n=3) were isolated for comparison, and pancreata from naïve C57BL/6 mice (n=3) served as controls. A PCR-array profile specific to innate immunity and early signal transduction pathways was utilized based on the significant histologic infiltration of innate immune effectors as well as the serum cytokine profile following IRI. Only 7 of 84 experimental genes had biologically significant up-regulation following 30 minutes of pancreatic IRI in comparison to naïve controls. These include *ccl-2*, *cd14*, *fos*, *hspa1a*, *hspd1*, *il1b*, and *il6* (Fig. 5). An additional 8 genes (*map2k4*, *nfkbl1*, *nfkbia*, *nfkbl1*, *peli1*, *rela*, *ripk2*, and *traf6*) demonstrated significant expression variation in comparison RNA isolated from naïve pancreata but not sham or 0 minute IRI controls.

DISCUSSION

At present, pancreatic allografts have an increased risk for graft loss compared with other solid organs, such as liver and kidney allografts (8). Prior animal models have demonstrated

tissue or organ-specific predilection for activation of particular pathways of allogeneic rejection (3–5). Increased sensitivity to IRI may, in part, be responsible for this clinical disparity. IRI may contribute to this by direct damage to the allograft, by activating vascular endothelium and increasing the likelihood of graft thrombosis, or by providing the inflammatory milieu for activation of aggressive immune responses.

Despite the likely importance of IRI for pancreas transplantation, few studies have addressed immune events surrounding this initial inflammatory insult. This is primarily a result of limited experimental models for detailed immunologic study. Prior pancreatic ischemia models have utilized rats, given their larger size. Both whole pancreas (9) and distal pancreatic (10) IRI models have previously been described in the rat as models of acute pancreatitis. However, mouse models allow for larger range of immunologic reagents, transgenic and knockout strains, and a wider body of literature for comparison with IRI in other organ systems. Thus, the purpose of the present study was to establish a reproducible mouse model for pancreatic IRI characterized both histologically and biochemically in terms of inflammatory damage associated with injury. This model would serve as a frame-work for evaluation of immunologic markers of pancreatic ischemic damage.

Initial feasibility experiments focused on translating previously established total and partial pancreatic IRI rat models into a smaller mouse model. Whole pancreas Ischemia resulted in ischemia to both the pancreas and the duodenum. The later was not well tolerated by mice and the model was, thus, abandoned. In contrast, mice subjected to vascular isolation of the distal pancreas demonstrated both short- and long-term survival. There was ~10% mortality associated with initial procedures primarily secondary to technical complications, and incidence decreased as proficiency improved.

Evaluation of different durations of IRI demonstrated that 30 minutes of ischemia resulted in time-limited and reversible pancreatic inflammatory process. Longer durations of ischemia resulted signs of acute distress in recipients, requiring early sacrifice. Murine models of pancreatitis elicit increases in serum amylase and LDH (11). The present model similarly demonstrated chemical pancreatitis that peaked 6 hours following reperfusion and normalized by 120 hours. The degree and time course of inflammation detected by elevations in amylase and LDH was paralleled in the increased expression of proinflammatory chemokines and cytokines following IRI. This implicates the proinflammatory milieu in induction of the observed chemical pancreatitis. Because some pancreatic injury can occur due to dissection, some controls underwent pancreatic dissection and clamping with immediate release. Degree of injury was significantly greater due to ischemic damage than due to dissection alone. Thus, IRI rather than mechanical pancreatic injury is primarily responsible for the observed inflammatory damage.

Histologically, pancreatic IRI was associated with pancreatic necrosis as well as lymphocytic and neutrophilic infiltration. Previous pancreatic IRI models noted endothelial activation and infiltration of leukocytes as early as 30 minutes after reperfusion (9). Along with inflammatory cell infiltration, multiple pro-inflammatory cytokines were up-regulated. Most notably, IL-1 β , IL-6, and CCL-2 were up-regulated both in serum and in tissue. Additional pro-inflammatory cytokines were detectable in serum, including IL-2, TNF- α and IFN- γ . These cytokines are well known to have critical roles in T cell activation and differentiation, suggesting a mechanism by which IRI potentiates allograft rejection.

In particular, CCL-2 potentiates early inflammation in both the liver and pancreas. CCL-2 levels correlate linearly with transaminitis and may correlate with early acute allograft rejection following orthotopic liver transplantation (12). In the cerulean-induced pancreatitis, CCL-2 is specifically elevated in pancreatic tissue. Furthermore, blockade

protects against pancreatic inflammation in that CCL-2 deficient mice demonstrate blunted pancreatitis (13). Thus, CCL-2 blockade may limit inflammatory cytokine-induced damage of transplanted pancreata by decreasing neutrophil infiltration and IL-6.

RT-PCR array analysis showed HMGB1 mRNA significantly increased secondary to pancreatic IRI. HMGB1 increase is associated with IRI in several organs. In particular, neutralizing antibody against HMGB1 protected kidneys from IRI, decreasing serum creatinine, neutrophil infiltrate, and tubular apoptosis (14). This neutralizing antibody also lowers IL-6, TNF- α , and CCL-2. In contrast, recombinant HMGB1 exacerbates renal damage (15). These pathways may similarly provide a target for abrogating pancreatic IRI.

Several intracellular proteins are also up-regulated by pancreatic IRI. *C-fos*, which regulates cellular apoptosis, has previously been associated with intestinal IRI (16). Combined with *c-jun*, *c-fos* is a component of transcription factor AP-1, which regulates a variety of pro-inflammatory factors. AP-1 is up-regulated following pancreatic ischemic damage in humans (17). RT-PCR further identified up-regulation of *cd14*. CD14 is a pattern-recognition receptor associated with various TLRs expressed in abundance on macrophages and monocytes (18). Soluble CD14 (sCD14) is up-regulated in sepsis (19). In addition, sCD14 is increased secondary to ischemia induced by aortic supraceliac cross-clamping (20), but not infra-renal cross-clamping, implicating pancreas, liver, and kidneys in the release of sCD14 (21). Evaluation of these pathways in pancreatic IRI may identify potential protective therapeutic targets.

In summary, this study establishes for the first time an *in situ* mouse model for pancreatic IRI by distal pancreatic vascular isolation. This model reversibly establishes ischemic damage to the pancreas. Reperfusion is associated with significant pancreatic parenchyma injury and production of pro-inflammatory chemokines and cytokines. This model provides the framework by which immunologic mechanisms of pancreatic IRI may be elucidated.

MATERIALS AND METHODS

Experimental Animals

Animals received humane care according to the *Guide for the Care and Use of Laboratory Animals* (22). Study protocols received approval by the Duke University Institutional Animal Care and Use Committee. C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) at 6–8 weeks of age. Mice were bred and maintained at the Cancer Center Isolation Facility (Duke University).

Induction of distal pancreatic IRI

Mice were pre-medicated with Ketoprofen (5 mg/kg SubQ) and were anesthetized with continuous inhaled isoflurane. Mice were atraumatically restrained in a supine position on a warming blanket. The abdomen was clipped, sterilely prepped and draped. A three-centimeter midline laparotomy incision was made. The duodenum and first portion of jejunum were reflected to expose the pancreas. Using minimal dissection, the lienopancreatic and lienal vessels were exposed. Heparinized saline (0.5 mL of 10 U/mL) was instilled intraperitoneally, and lienopancreatic and lienal vessels were occluded with microvascular clamps, resulting in vascular isolation of the distal 40% of the pancreas. Complete ischemia was visually verified. Ischemia was maintained for 0–30 minutes, after which the clamps were removed. For 0 minutes of ischemia, dissection was performed and clamps were placed and then immediately removed. Following reperfusion, fascia and muscle was re-approximated with 4-0 Vicryl, and skin was closed using 4-0 Prolene. Bupivacaine (0.25%) was topically applied during closure. Mice were placed under warming lights and carefully monitored postoperatively. Additional fluids were provided by

intraperitoneal injection of warm ringers solution (40–80 mL/kg/24 hours) as needed. Sham mice underwent laparotomy, intraperitoneal heparin installation, and abdominal closure only. Experimental mice were subjected to distal pancreatic ischemia by selective clamping of the lino-pancreatic and lienal vessels were clamped for 10 minutes (n=3), 20 minutes (n=3), or 30 minutes (n=7) followed by reperfusion. Control mice underwent clamp application with immediate removal (0 min, n=7) or sham laparotomy (n=3).

Evaluation of experimental animals

Blood was serially collected via jugular puncture prior to operation, at 1, 3, 6, 12, and 24 hours, and daily thereafter. Mice were serially sacrificed at 1, 6, 24, 48, 72, and 144 hours following procedure. At sacrifice, anesthetized mice underwent bilateral thoracotomy, and blood and organs were harvested. Tissue not immediately utilized was formalin-fixed, preservation in RNAlater (Qiagen, Frederick, MD), or snap frozen.

Biochemical monitoring

Serum was serially monitored for glucose, amylase and LDH. Serum glucose was serially assayed on an Abaxis Vet Scan by the Duke Veterinary Diagnostic Laboratory (Durham, NC). Other samples were plated in triplicate on 96-well plates. Serum amylase was measured using EnzChek® Ultra Amylase Assay (Molecular Probes, Grand Island, NY) as fluorescent emission of starch digestion products at 512 nm. Serum LDH levels were monitored using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) as absorption at 490 nm. Assays were performed according to manufacturer instructions. Samples were detected on a SpectraMax M2 Microplate Reader and analyzed utilizing SoftMax® Pro Microplate Data Acquisition & Analysis Software (Molecular Devices, Sunnyvale, California).

Histologic analysis

Following formalin fixation, pancreatic tissue was processed, paraffin-embedded, and stained with hematoxylin and eosin by the Duke University Veterinary Diagnostic Labs (Durham, NC). De-identified 5 µm thick sections were evaluated and graded in a semi-quantitative fashion by a single pathologist. The degree of pancreatic edema, necrosis, neutrophilic infiltration, lymphocytic infiltration, epithelial apoptosis, and vascular thrombosis were each given a score between 0-4 in which 0= no injury, 0.5= focal/rare, 1= mild, 2= moderate, 3= marked and 4= massive. Total pathologic grade was obtained by the sum of each score. The grading system was created based on modifications of a previously published scoring system (10).

Serum cytokine/chemokine analysis

Serum cytokine/chemokines were detected utilizing the Milliplex® MAP Mouse Cytokine / Chemokine Magnetic Bead Panel (Millipore Corporation, Billerica, MA) according to manufacturer instructions. Briefly, serum was plated in duplicate on 96-well plates. Following overnight incubation with magnetic beads, samples were washed and incubated at room temperature with detection antibodies. Samples were detected following addition of streptavidin-phycoerythrin on a Millipore MAGPIX and analyzed with xPONENT 4.1 Software (Millipore Corporation, Billerica, MA). Manufacturer reported minimum detectable concentrations were G-CSF=2.0 pg/mL, GM-CSF=10.1 pg/mL, IFN-γ =2.2 pg/mL, IL-1α =3.9 pg/mL, IL-1β=2.8 pg/mL, IL-2=1.1 pg/mL, IL-4=0.4 pg/mL, IL-5=1.3 pg/mL, IL-6=1.4 pg/mL, IL-7=1.2 pg/mL, IL-9=28.8 pg/mL, IL-10=2.8 pg/mL, IL-12p40=3.9 pg/mL, IL-12p70=3.2 pg/mL, IL-13=7.3 pg/mL, IL-15=12.3 pg/mL, IL-17=0.3 pg/mL, IP-10=1.3 pg/mL, KC=1.4 pg/mL, MCP-1=4.8 pg/mL, MIP-1α =8.2 pg/mL, MIP-1β=14.3 pg/mL, MIP-2=25.9 pg/mL, RANTES=1.0 pg/mL, and TNF-α=1.2 pg/mL.

Extraction of total RNA

Total RNA was extracted from pancreatic tissue stored in RNAlater (Qiagen, Frederick, MD) utilizing RNAeasy Mini Plus Kit (Qiagen, Frederick, MD) according to manufacturer's instruction. Briefly, 30 mg of pancreatic tissue was homogenized in RLT Buffer in gentleMACS™ M Tubes using the gentleMACS™ Tissue Dissociator (Miltenyi Biotec, Auburn, CA). Lysates were transferred to gDNA Eliminator Spin Column (Qiagen, Frederick, MD) and further purified on RNAeasy elution columns. Total RNA was eluted in water and quantified by measuring absorbance at 260nm and 280nm.

Real-time (RT) PCR analysis

RT-PCR analysis was performed using the Mouse Toll-Like Receptor Signaling Pathway PCR Array (SABiosciences, Frederick, MD) according to manufacturer instructions. Briefly, cDNA was synthesized from total RNA with the RT-First Strand Kit (SABiosciences, Frederick, MD). The cDNA was diluted 1:10 and added a 96-well PCR plate. RT-PCR analysis was performed on a Bio-Rad CFX96 thermocycler (Bio-Rad, Hercules, CA) and analyzed on SABiosciences PCR Array Data Analysis software (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>). Statistical significance was defined as relatively high (>30) or low (<30) average threshold cycles compared to controls.

Statistical Analysis

Statistical calculations were performed using SPSS (SPSS version 11.5 for Windows, Chicago, IL) using the Student's *t* test. *P*<0.05 was considered significant.

Acknowledgments

This work was supported in part by grants from the American Society of Transplant Surgeons (B. J. B.), the American Society of Transplantation (T. V. B.), and the American Association for the Study of Liver Diseases (T. V. B.). The authors wish to thank Sara Sparks and Robyn Medinas for technical assistance, and John Yi, Ph.D. and Melissa Ventevogel for expert assistance with real time PCR analysis.

ABBREVIATIONS

IRI	Ischemia-reperfusion injury
LDH	Lactate dehydrogenase
HMGB1	High-mobility group protein B1
sCD14	Soluble CD14
TLR	Toll-like receptor

REFERENCES

1. Largiader F, Lyons GW, Hidalgo F, Dietzman RH, Lillehei RC. Orthotopic allotransplantation of the pancreas. American journal of surgery. 1967; 113(1):70. [PubMed: 5334434]
2. Gruessner AC, Sutherland DE. Pancreas transplant outcomes for United States (US) cases as reported to the United Network for Organ Sharing (UNOS) and the International Pancreas Transplant Registry (IPTR). Clinical Transplants. 2008:45. [PubMed: 19708445]
3. Lunsford KE, Gao D, Eiring AM, Wang Y, Frankel WL, Bumgardner GL. Evidence for tissue-directed immune responses: analysis of CD4- and CD8-dependent alloimmunity. Transplantation. 2004; 78(8):1125. [PubMed: 15502708]
4. Lunsford KE, Jayashankar K, Eiring AM, et al. Alloreactive (CD4-Independent) CD8+ T cells jeopardize long-term survival of intrahepatic islet allografts. Am J Transplant. 2008; 8(6):1113. [PubMed: 18522544]

5. Gao D, Li J, Orosz C, Bumgardner GL. Tissue specific variation in host responses to alloantigenic stimuli. *Transplantation*. 2000; 69(8):s358.
6. Brennan TV, Lunsford KE, Kuo PC. Innate pathways of immune activation in transplantation. *J Transplant*. 2010 2010.
7. Cook, MJ. The anatomy of the laboratory mouse. London ; New York: Academic Press; 1965. 143p p.
8. 2006 Annual Report of the U.S. Organ Procurement and Transplantation Network and the Scientific Registry of Transplant Recipients: Transplant Data 1996–2005. Rockville, MD: Department of Health and Human Services, Health Resources and Services Administration, Healthcare Systems Bureau, Division of Transplantation; Richmond, VA: United Network for Organ Sharing; Ann Arbor, MI: University Renal Research and Education Association;
9. Hoffmann TF, Leiderer R, Waldner H, Arbogast S, Messmer K. Ischemia reperfusion of the pancreas: a new in vivo model for acute pancreatitis in rats. *Research in experimental medicine Zeitschrift für die gesamte experimentelle Medizin einschliesslich experimenteller Chirurgie*. 1995; 195(3):125. [PubMed: 8570908]
10. Obermaier R, Benz S, Kortmann B, Benthues A, Ansorge N, Hopt UT. Ischemia/reperfusion-induced pancreatitis in rats: a new model of complete normothermic in situ ischemia of a pancreatic tail-segment. *Clinical and experimental medicine*. 2001; 1(1):51. [PubMed: 11467402]
11. Hartwig W, Schimmel E, Hackert T, et al. A novel animal model of severe pancreatitis in mice and its differences to the rat. *Surgery*. 2008; 144(3):394. [PubMed: 18707038]
12. Moench C, Uhrig A, Lohse AW, Otto G. The role of monocyte chemoattractant protein-1 in orthotopic liver transplantation. *Transplantation Proceedings*. 2003; 35(4):1452. [PubMed: 12826189]
13. Frossard JL, Lenglet S, Montecucco F, et al. Role of CCL-2, CCR-2 and CCR-4 in cerulein-induced acute pancreatitis and pancreatitis-associated lung injury. *Journal of Clinical Pathology*. 2011; 64(5):387. [PubMed: 21345872]
14. Wu H, Ma J, Wang P, et al. HMGB1 contributes to kidney ischemia reperfusion injury. *Journal of the American Society of Nephrology : JASN*. 2010; 21(11):1878. [PubMed: 20847143]
15. Katsargyris A, Klonaris C, Alexandrou A, Giakoustidis AE, Vasileiou I, Theocharis S. Toll-like receptors in liver ischemia reperfusion injury: a novel target for therapeutic modulation? Expert opinion on therapeutic targets. 2009; 13(4):427. [PubMed: 19335065]
16. Shima Y, Tajiri T, Taguchi T, Suita S. Increased expression of c-fos and c-jun in the rat small intestinal epithelium after ischemia-reperfusion injury: a possible correlation with the proliferation or apoptosis of intestinal epithelial cells. *Journal Of Pediatric Surgery*. 2006; 41(4):830. [PubMed: 16567203]
17. Benz S, Lobler M, Obermaier R, et al. New possible target genes in pancreatic ischemia/reperfusion-injury identified by microarray analysis. *Transplantation Proceedings*. 2002; 34(6):2369. [PubMed: 12270441]
18. Peri F, Piazza M. Therapeutic targeting of innate immunity with Toll-like receptor 4 (TLR4) antagonists. *Biotechnology advances*. 2012; 30(1):251. [PubMed: 21664961]
19. Jersmann HP. Time to abandon dogma: CD14 is expressed by non-myeloid lineage cells. *Immunology and cell biology*. 2005; 83(5):462. [PubMed: 16174094]
20. Hafez HM, Berwanger CS, McColl A, et al. Myocardial injury in major aortic surgery. *Journal of vascular surgery : official publication, the Society for Vascular Surgery [and] International Society for Cardiovascular Surgery, North American Chapter*. 2000; 31(4):742.
21. Tsoulfas G, Takahashi Y, Ganster RW, et al. Activation of the lipopolysaccharide signaling pathway in hepatic transplantation preservation injury. *Transplantation*. 2002; 74(1):7. [PubMed: 12134092]
22. Institute of Laboratory Animal Resources. Guide for the care and use of laboratory animals. 7 ed. Washington, DC: National Academy Press; 1996. p. 125

Figure 1. Model for distal pancreatic IRI

(A) The distal mouse pancreas receives its vascular supply from the lieno-pancreatic artery with collaterals from the lienal artery. (B) The lieno-pancreatic and lienal artery were clamped to induce distal pancreatic ischemia for 0, 10, 20, or 30 minutes followed by reperfusion. Vascular clamping resulted in initial blanching followed by gross erythema and induration of the pancreas as time progressed. Visible ischemic changes were isolated to the distal 40% of the pancreas.

Figure 2. Chemical evidence of pancreatic injury following distal pancreatic IRI

Distal pancreatic ischemia was performed for 0 (n=7), 10 (n=3), 20 (n=3), or 30 (n=7) minutes followed by reperfusion. Recipient serum of serially assayed for (A) amylase or (B) LDH. Naïve mice and mice receiving sham laparotomy (n=3) served as controls. (A) Serial assay for serum amylase (mean±SD) following distal pancreatic IRI demonstrated that amylase levels peak 6 hours after reperfusion and returned to baseline by 5 days. At 6 hours, peak amylase levels were significantly increased in comparison to 0 minute IRI following 10 minutes (p=0.04), 20 minutes (p=0.01), and 30 minutes (p<0.005). Peak serum amylase at 6 hours was also significantly increased in comparison to sham-operated controls following 10, 20, or 30 minutes (p<0.005 for all) but not 0 minutes of IRI. (B) Peak LDH values from 6 hours following reperfusion are shown (mean±SD). Peak LDH was significantly increased after 30 minutes IRI, but not 10 or 20 minutes IRI in comparison to sham (p=0.05) and 0 minute IRI (p=0.006). (C) Normal Q-Q plots of the residuals in one-way ANOVA models

for the amylase and LDH data. (D) Fitting of linear models to the amylase and LDH data, respectively.

Figure 3. Histologic evaluation of pancreatic injury following distal pancreatic IRI

Distal pancreatic ischemia was performed for 0 or 30 min followed by reperfusion. Mice were sacrificed at serial time points following IRI (1, 6, 24, 48, 72, and 144 hours after procedure). Pancreata were formalin-fixed and paraffin embedded, de-identified serial sections were stained with hematoxylin and eosin (n=3) and evaluated by a single pathologist. Pancreata obtained from naïve mice and mice undergoing sham laparotomies served as controls. (A) Morphologic examination at 24 hours following 30 min of IRI revealed significant pancreatic necrosis, associated neutrophilic and lymphocytic inflammation, and mild epithelial vacuolization and apoptosis (H&E, 100×). (B) In contrast, at 24 hours following 0 minutes of IRI, morphologic examination demonstrated mild edema and mild to moderate neutrophilic and lymphocytic inflammation (H&E, 100×). (C) The degree of pancreatic injury was assessed by evaluating the extent of parenchymal edema, necrosis, neutrophilic infiltration, lymphocytic infiltration, epithelial apoptosis, and vascular thrombosis and assigning a 0-4 score for each parameter [0= no injury, 0.5= focal/rare, 1= mild, 2= moderate, 3= marked and 4= massive]. Columns demonstrate the total pathologic grade, which was obtained by the sum of the scores.

Figure 4. Serum cytokine and chemokine production following distal pancreatic IRI
 Distal pancreatic ischemia was performed for 0 or 30 min followed by reperfusion.
 Recipient serum of serially assayed for by Luminex for presence of chemokines and

cytokines at 6 hours (PIRI-0 6h, n=3 and PIRI-30 6h, n=8) and 24 hours (PIRI-0 24h, n=6 and PIRI-30 24h n=7) after IRI. Naïve mice (n=3) and mice receiving sham laparotomy (Sham 6h, n=3 and Sham 24h, n=3) served as controls. (A) At 6 hours following IRI, cytokines G-CSF (p=0.01), IL-2 (p=0.03), IL-6 (p=0.05), and TNF- α (p<0.001) were significantly up-regulated by pancreatic IRI in comparison to sham controls. At 24 hours following IRI, cytokines G-CSF (p<0.001), IFN- γ (p=0.003), IL-1 β (p=0.01), IL-2 (p=0.006), IL-6 (p=0.05), and TNF- α (0.002) were significantly up-regulated in comparison to sham controls. (B) At 6 hours following IRI, serum chemokines CCL-2 (MCP-1, p=0.02), CCL5 (RANTES, p=0.01), CXCL1 (KC, p=0.01), and CXCL2 (MIP-2, p=0.03) were significantly up-regulated in comparison to sham controls. At 24 hours following IRI, serum chemokines CCL-2 (MCP-1, p=0.01), CCL5 (RANTES, p=0.005), and CXCL2 (MIP-2, p=0.02) were significantly up-regulated in comparison to sham controls. Columns represent mean $\pm$ SD for each group. *p<0.05 in comparison to sham controls.

Figure 5. Regulation of pro-inflammatory gene expression following distal pancreatic IRI
 Distal pancreatic ischemia was performed for 0 minutes (PIRI-0 24h, n=3) or 30 minutes (PIRI-30 24h, n=4) followed by reperfusion. Pancreata were harvested at 24 hours following reperfusion and total RNA was isolated. Real-time PCR arrays were then performed. Mice receiving sham laparotomy (n=3) served as controls. Regulation of gene expression was calculated in comparison to expression naïve control pancreata (n=3). Expression of genes *ccl-2* (p=0.04), *cd14* (p=0.05), *fos* (p<0.001), *hspa1a* (p=0.05), *hspd1* (p=0.05), *il1b* (p=0.04), and *il6* (p=0.04) were significantly up-regulated in the pancreata of mice subjected to 30 minutes of IRI, but not in mice subjected to 0 minutes of IRI or sham laparotomy. Columns represent mean $\pm$ 95% confidence interval for each group. *p<0.05 in comparison to sham controls. †p<0.001 in comparison to naive controls.