Selective Inhibition of Soluble TNF Using Xpro1595 Relieves Pain and Attenuates Cerulein-induced Pancreatic Pathology in Mice, With a Possible Link to Central Pain Processing

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Title: Selective inhibition of soluble TNF using XPro1595 relieves pain and attenuates cerulein-induced pancreatic pathology in mice, with a possible link to central pain processing

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Running Title: XPro1595 improves pancreatitis disease outcomes

ABSTRACT

Treatment of acute pancreatitis remains a challenge, with therapy focused on supportive care and treatment of the inciting etiology. Individuals with pancreatitis may experience severe upper abdominal pain, although pain mechanisms in patients with pancreatitis are incompletely understood and likely multifactorial, with possible pain processing occurring in the central nervous system, a process known to be associated with the upregulation of inflammatory cytokines. Inflammation plays a prominent role in the induction of acute pancreatitis, with the inflammatory cytokine tumor necrosis factor (TNF) both exacerbating cell death, pro-inflammatory signaling and edema, as well as promoting reparative and restorative mechanisms. This duality of function can be explained by different forms of the TNF ligand preferentially activating different receptor subtypes, whereby the uncleaved transmembrane form of the ligand (tmTNF) preferentially activates TNFR2 promoting restorative functions, but once cleaved the soluble form of TNF (solTNF) preferentially activates TNFR1 promoting detrimental pathology. For this reason, the traditional TNF inhibitors that inhibit both TNFR1 and TNFR2 have shown modest success in patients, but come with numerous side-effects, including immunological dysfunction and heart failure. Therefore, we sought to assess the effect of a novel selective inhibitor of solTNF (XPro1595) on pancreatic pathology and associated neuropathic pain in a mouse model of acute pancreatitis, and observe its effect on an area of the brain (the hippocampus) known to play a role in neuropathic pain processing. XPro1595 administration began after the initial peak in serum amylase to maximize clinical relevance. Administration of XPro1595 prevented pancreatic immune cell infiltration, that subsequently prevented tissue disruption and acinar cell death. These improvements in pathology were associated with a significant reduction in mechanical hypersensitivity (neuropathic pain). XPro1595 treatment also prevented an increase in hippocampal astrocyte reactivity, that may be associated with the prevention of neuropathic pain in this mouse model. Overall, we observed that selectively inhibiting solTNF using XPro1595 improved the pathophysiological and neurological sequelae of cerulein-induced pancreatitis in mice, which provides support of its use in patients with pancreatitis. This is the first study of its kind to identify a possible connection between hippocampal and pancreatic pathology and warrants further investigation.

KEY WORDS

Acute Pancreatitis, Cerulein, Mice, Inflammation, cytokines, TNF, TNFR1, Neuropathic pain

INTRODUCTION

Pancreatitis is a leading cause for gastrointestinal disease-related hospital admissions, and is primarily an inflammatory condition of the pancreas that can either be acute or chronic, lasting many years. Although individuals with pancreatitis can progress to have quite severe complications, the majority of patients endure only mild bouts of the disease with symptoms including upper abdominal epigastric pain, nausea and vomiting (1, 2). Acute pancreatitis can be broadly classified into interstitial edematous pancreatitis with peripancreatic fat stranding and fluid accumulation, or necrotizing pancreatitis that as the name suggests promotes necrosis of pancreatic parenchyma and/or peripancreatic tissue (2). These differences in disease pathologies has driven the creation of a plethora of existing pre-clinical animal models, with no single animal model displaying all aspects of acute or chronic pancreatitis. None-the-less these models frequently promote a systemic inflammatory response by either non-invasive (administration of toxins, transgenic mice or diet based) or invasive means (vessel/duct blockage or perfusion) (3).

Studies in patients and animals have identified the disease course and severity of pancreatitis is mostly governed by inflammatory cells that drive local and systemic immune responses, of which a major contributor is the upregulation of the inflammatory cytokine tumor necrosis factor (TNF). TNF production promotes the induction of inflammatory genes, acinar cell death and recruitment of immune cells (4-8), which prompted investigation of traditional TNF inhibitory therapies to prevent or reduce these pathologies and associated symptoms. Early studies in rodents modulating TNF ligand and receptor activity using TNF receptor fusion proteins or anti-TNF antibodies (e.g., Etanercept, Infliximab and Adalimumab) showed promise with reduced pancreatic pathologies such as edema, inflammation, necrosis and vacuolization (9-12), and similar positive outcomes were seen in patients when TNF inhibitors were administered to treat

other cooccurring conditions (13, 14). Unfortunately, the abundance of side-effects in these traditional TNF inhibitors (including immunological dysfunction and even the induction of pancreatitis itself (15-19)), combined with their apparent inability to reduce mortality in early studies performed on sepsis patients (20, 21) dampened enthusiasm for their further use in patients with pancreatitis. More than 2 decades on however, additional meta-analysis' of patients with sepsis have revealed an overall improvement in survival rates (22, 23), when studies are sufficiently powered, and this may have encouraged the examination of these traditional TNF inhibitors in patients with pancreatitis (24, 25). None-the-less, these traditional inhibitors still promote severe side-effects, and their use should be cautioned in patients.

The discrepancy between these paradoxical outcomes in rodents and humans warrants further investigation, but is likely due to the differences in TNF receptor subtype functions, that have complicated the TNF field until recently. TNF is first produced as a transmembrane protein (tmTNF) that preferentially activates TNF receptor 2 (TNFR2: CD120b or p75/p80) (26), but once TNF is cleaved from the cell membrane to exist in a soluble form (solTNF) it preferentially activates TNF receptor 1 (TNFR1: CD120a or p55/p60) (26). Although the two receptors can trigger some common signaling pathways (27), TNFR2 activation generally promotes beneficial outcomes such as cell survival, induction of neurogenesis, and promotion of CNS autoimmunity (28-30), while TNFR1 activity generally promotes detrimental outcomes such as cell death, aberrant neuronal plasticity, and exacerbation of the existing inflammatory response (28, 31, 32). Therefore, being able to selectively block the activity of solTNF/TNFR1, while sparing the activity of tmTNF/TNFR2 activity, would likely prove beneficial to patients, although traditional TNF inhibitors are unable to distinguish between the different TNF ligand or receptor subtypes. For this reason, a novel 'second generation' TNF inhibitor was developed that selectively inhibits only the soluble form of TNF (solTNF: XPro1595), with no known side-effects, and has been proven to be safe and well tolerated in patients (33), with an ability to reduce neuroinflammation in the brains of Alzheimer Disease patients (34). Indeed, within the pancreas TNFR1 activity is known to exacerbate cell death, inflammation and edema

(10), while TNFR2 promotes pancreatic regeneration (35, 36). Therefore, assessing the outcomes selectively inhibiting solTNF using XPro1595 in rodents with pancreatitis is highly clinically relevant.

Replicating the symptoms of acute pancreatitis in rodents, especially induction of visceral pain is challenging, as pain is a subjective experience and therefore difficult to assess in animals. None-the-less, animal models are useful to identify the possible underlying mechanisms of pain necessary to create therapeutic interventions. Many pre-clinical acute and chronic models of pancreatitis promote increased sensitivity of the pancreas to electrical stimulation, as well as promoting the induction of neuropathic pain (detection of a stimulus not normally detectable) in regions both local (abdomen) and distant (hindpaw) to the site of inflammatory origin (37-44), suggesting activation of both normal pain pathways and central sensitization. Alterations to peripheral A δ and C fiber activity promote the induction of pain (45, 46), while peripheral targets such as ion channels regulating neural sensitization (47, 48), and release of chemical mediators of inflammation such as pro-inflammatory cytokines (e.g. TNF/TNFR1) modulating these effects (47, 49-51). Central plasticity also overlays these pain pathways, involving central brain structures such as the hippocampus whereby reductions in synaptic protein expression can reduce LTP (52-54), leading to loss of input to cortical pain sensing regions (e.g somatosensory and prefrontal cortices) (55, 56). Importantly, established rodent models of neuropathic pain identify hippocampal TNF/TNFR1 activity regulates the peripheral hypersensitivity (52, 57, 58). Therefore, the systemic inflammatory response in individuals with pancreatitis that upregulates TNF/TNFR1 activity may play a substantial role in both the induction of pancreatic pathology and neuropathic pain.

For these reasons, we sought to investigate the effects of using the novel non-opioid ‘second-generation’ biologic XPro1595 to selectively bind and neutralize solTNF in a mouse model of pancreatitis. XPro1595 has a 17-hour half-life, can cross the blood-brain-barrier, has been successfully used in numerous pre-clinical inflammatory disease models (59-63), and is safe and well-tolerated in both cancer and Alzheimer

Disease patients (33, 34, 64, 65). The use of XPro1595 therefore has a high potential for translation to the clinic with the promise to improve outcomes in inflammatory pancreatic conditions.

METHODS

Animals

Male C57Bl/6J mice aged 2 to 4 months were used for the current study. Animals were housed in a 12-hour light/dark cycle with food and water ad libitum. Procedures related to animal use were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee (in accordance with NIH care and use of laboratory animals).

Acute Pancreatitis Model

On a single day, mice received 8 intraperitoneal injections of cerulein (50 µg/kg; Sigma) or vehicle (0.9 % saline), over 7 hours, spaced an hour apart each. Injections were alternated between the left and right sides of the abdomen to minimize any possible irritation from multiple needle injuries. The next morning (approximately 18 hours after the last cerulein injection) mice received a subcutaneous injection of either XPro1595 (10 mg/kg, INmuneBio) or vehicle (0.1M PBS).

Blood Collection and Amylase Analysis

Mice were anaesthetized with ketamine (75 mg/kg) and xylazine (14 mg/kg) by intraperitoneal injection, cardiac blood was collected into 1.5 ml polypropylene tubes and incubated on ice for 15 minutes, prior to centrifugation at 2000 rpm for 10 minutes. The resultant supernatant was removed and stored at -80 °C. On the day of analysis, serum was defrosted on ice, and approximately 40 µl loaded into a VETSCAN 'Comprehensive Diagnostic' rotor (Abaxis Inc.), prior to being analyzed in a Vetscan VS2 (Abaxis Inc.).

Histological Preparation, Staining and Immunohistochemistry

Mice were anaesthetized with ketamine (75 mg/kg) and xylazine (14 mg/kg) by intraperitoneal injection, prior to undergoing transcardial perfusion using approximately 15 ml PBS, followed by approximately 4 % paraformaldehyde (Sigma). Tissue was dissected, stored in 4 % paraformaldehyde for 2 hours, cryoprotected in 20 % sucrose in PBS for 48 hours, and then quickly frozen in OCT over isopentane on dry ice, and stored at -80 °C. Serial frozen coronal sections were cut 40 µm thick through the pancreas and hippocampus. Some slides underwent hematoxylin and eosin (H&E) staining to assess pancreas inflammatory infiltrates and tissue integrity. Other slides underwent immunohistochemical analysis to further assess inflammatory state. Slides containing sections of either pancreas or brain were permeabilized with 0.2 % triton X-100 (Sigma) in 2 % fish gel in PBS solution and immunohistochemically labelled with the primary antibody (1:2000 rabbit anti-GFAP, Dako; 1:2000 rabbit anti-IBA-1, Wako) overnight at 4 °C. Sections were washed 3 times in PBS, incubated in fluorescent secondary antibodies (1:500, Molecular Probes) for 30 min at room temperature, washed an additional 3 times in PBS, and coverslipped in Prolong Gold Antifade mounting medium containing DAPI (ThermoFisher). Sections were photographed (pancreas at 10x and 40x; brain at 40x) with equal exposure on an Olympus CK-2 inverted microscope, connected to a 3MP Amscope digital camera (MU300-CK) with Amscope Software version 3.2, prior to analysis using NIH ImageJ version 1.52a.

Histological Analysis

To assess pancreatic immune cell infiltration 2 and 7 days post-induction of acute pancreatitis, photographs of H&E stained pancreatic sections were semi-quantitated on an arbitrary scale within each field of view: 0 = no inflammatory cells present; 1 = some inflammatory cells present; 2 = many inflammatory cells present. At 7 days, within each H&E photograph we also semi-quantitated pancreatic tissue integrity (spaces between acinar cell clusters: 0 = normal pathology; 1 = some spaces evident; 2 = large amount of space evident appearing similar to a ‘cracking’ effect), acinar cell atrophy (0 = normal pathology; 1 = acinar atrophy present but not immediately apparent; 2 = acinar atrophy prevalent throughout the tissue), and integrity of intralobular duct (degree of invasion of spaces normally occupied by vessels within the large

pancreatic lobules: 0 = normal pathology, 1 = some invasion present but not immediately apparent, 2 = large amount of invasion present). Values for each photograph were averaged per section, per animal, and then per group. To assess the extent of circulating macrophage infiltration within the pancreas, the immunohistochemistry images were quantitated for the level of IBA-1. Images were imported into ImageJ, converted to gray scale and thresholded, and the area fraction of pixels positive for IBA-1 was quantitated. For each photograph, IBA-1 expression level (arbitrary units) were measured in fractional areas to give an average IBA-1 intensity. Values for each photograph were averaged per section, per animal, and then per group. The same protocol was applied to brain sections containing hippocampus at 7 days post-induction to quantitate astrocyte reactivity using the GFAP antibody.

Mechanical von Frey Hindpaw Neuropathic Pain

In a dimly lit room, a 10" x 19" extension window screen (Thermwell) was fully extended and placed atop 2 polystyrene boxes, with a desk lamp placed behind and just under the height of the screen, angled towards the investigator. Four mice at a time were placed on top of the screen, with a 600 ml glass beaker (Pyrex) placed over the top of each mouse to prevent escape. A disposable underpad was draped over the beakers to minimize any light and/or movement stimulation. After 15 minutes acclimatization under the beaker, hindpaw hypersensitivity was assessed by holding the von Frey filament (Bioseb) handle under the screen, and slowly raising the end of the filament up through the screen to press against the under-side of the mouse's hindpaw walking pad until a slight bend was observed in the fiber. Continued advancement/bending of the filament does not necessarily produce more force of application. The investigator tested the lightest filament first, and sequentially tested up through the filament sizes until a positive result was established. A positive result was the mouse noticing 3 out of 5 consecutive tests for each filament, defined as the mouse withdrawing its foot, licking or shaking its foot, or rapidly moving its body away from the stimulus. Once a positive result was established for each mouse, the testing was concluded for that mouse for that day. The testing occurred as rapidly as possible to reduce restraint

distress, although it was noticed that mice would often fall asleep during testing, which required gentle tapping from underneath the screen to wake up the animal.

Statistical Analysis

All data were assessed for homogeneity of variance, after which statistical analysis was performed. Histological differences were assessed using the Student's t-test, and behavioral differences (intra- and inter-group analysis) were assessed using two-way repeated measures analysis of variance with Student-Newman-Keuls method post hoc in SigmaPlot 13.0 where significance was <0.05. Data in figures are expressed as mean $\pm$ standard error of the mean.

RESULTS

Cerulein administration induces a temporary increase in serum amylase levels in mice

Increased expression of serum amylase activity in patients supports the diagnosis of acute pancreatitis. Therefore, we wanted to confirm whether 8 intraperitoneal injections of cerulein each hour over the course of 7 hours increases serum amylase levels. Mice received 8 injections of either cerulein (50 mg/kg) or vehicle, and a terminal blood draw was performed 6 hours after the last cerulein injection. Biochemical analysis of the animal's serum using a VetscanVS2 Comprehensive Diagnostic Rotor identified cerulein administration led to over a 6-fold increase in serum amylase levels, compared to vehicle-treated mice (Figure 1). Next, to identify the time course of serum amylase expression, serum amylase levels were also assessed 18 hours after the last cerulein injection on the prior day. At this later timepoint, biochemical analysis reveals amylase levels were no longer significantly different to vehicle-treated (i.e. non-pancreatitis) animals, suggesting the cerulein treatment protocol promotes a strong but transient increase in serum amylase levels.

Selective inhibition of solTNF prevents immune cell infiltration into the pancreas following cerulein-induction of acute pancreatitis

To identify the possible role of solTNF/TNFR1 activity in the disease course of pancreatitis we used cerulein to induce acute pancreatitis in mice, treated the next day with either XPro1595 or vehicle S.C., and allowed the mice to survive a further 24 hours (48 hours after cerulein administration). The mice were transcardially perfused and the pancreas removed and processed for H&E staining. The number of inflammatory cells present was semi-quantitated on an arbitrary scale. Observations by an investigator blinded to the treatment regime, identified that the vehicle-treated acute pancreatitis mice had a significant influx of inflammatory cells 48 hours after induction, as observed by H&E (score = 1.8 ± 0.03) (Figure 2A,B&G). In comparison, the XPro1595-treated acute pancreatitis mice had a significant reduction in the number of inflammatory cells present 48 hours after induction (score = 0.5 ± 0.4) (Figure 2D,E&G). By 7 days post-induction, inflammatory infiltrates were absent in both XPro1595- and vehicle-treated acute pancreatitis groups (score = 0) (Figure 2C,F&G), suggesting the inflammatory response within the pancreas resolves itself within a week, in accordance with other published rodent studies (66-68). To confirm the resolution of an inflammatory response in the same 7-day tissue, we semi-quantitated the level of ionizing binding adaptor protein 1 (IBA-1), as a marker of circulating macrophages within pancreatic islets (Figure 2H-M). ImageJ quantitation of high-resolution pancreatic images revealed no differences in IBA-1 expression at 7 days between vehicle- or XPro1595 treated mice (Figure 2N).

Selective inhibition of solTNF prevents adverse pancreatic pathology 7 days following cerulein-induction of acute pancreatitis

We next wanted to identify whether an early alteration in pancreatic inflammatory infiltrates had other effects on pancreas pathology at a later timepoint. For this, 7 days after the induction of acute pancreatitis we semi-quantitated pancreatic tissue integrity ('cracking' effect within small acinar clusters). We identified that administering cerulein reduces tissue integrity within small cell clusters (Figure 3A&C), while XPro1595 treatment prevented this effect (significantly less than vehicle-treated pancreatitis mice, and not different to non-pancreatitis mice) (Figure 3B&C). We also semi-quantitated acinar cell atrophy. We observed that vehicle-treated cerulein-induced acute pancreatitis promotes acinar atrophy by day 7

(Figure 3D&F), compared to vehicle-treated non-pancreatitis mice (Figure 3F). In contrast, the XPro1595-treated pancreatitis group did not display acinar atrophy (significantly less than vehicle-treated pancreatitis mice, and not different to non-pancreatitis mice) (Figure 3E&F). We further assessed the integrity of the intralobular duct between large pancreatic lobules. Cerulein-induced pancreatitis promoted significant disruption of pathology between the large pancreatic lobules, whereby acinar clusters within lobules often invaded these spaces (Figure 3G&I). This effect was not observed in the XPro1595-treated pancreatitis group (not significantly different to non-pancreatitis mouse group) (Figure 3H&I).

Selective inhibition of solTNF using XPro1595 attenuates cerulein-induced neuropathic pain

Acute pancreatitis often promotes severe and constant pain in the upper abdomen, which can extend around to the back, and last for a few days. In accordance with this, many pre-clinical rodent models display both pancreatic and referred neuropathic pain in both the abdomen and hindpaws (37-44). To assess the role of solTNF in the induction of pancreatitis-induced neuropathic pain, we measured the level of hindpaw mechanical hypersensitivity at 3, 5 and 7 days post-induction, in mice treated with and without XPro1595. Vehicle-treated non-pancreatitis mice (i.e. vehicle S.C. and I.P.) were assessed to establish baseline sensitivity response, set at 100%. We observed the hindpaw sensitivity of XPro1595-treated non-pancreatitis mice (i.e. XPro1595 S.C., and vehicle I.P.) was not different from vehicle-treated non-pancreatitis mice over the testing period (Figure 4), suggesting XPro1595 does not regulate hindpaw hypersensitivity under non-pathogenic conditions. Next, we assessed the hindpaw sensitivity of mice with cerulein-induced acute pancreatitis. We identified that vehicle-treated acute pancreatitis mice (i.e. Vehicle S.C., and cerulein I.P.) displayed persistent hindpaw hypersensitivity, beginning from the first day of testing (day 3) until the last (day 7). In contrast, the hindpaw hypersensitivity of XPro1595-treated pancreatitis mice (XPro1595 S.C., and cerulein I.P.), was not significantly different to baseline control mice at any time point tested, and was significantly better than vehicle-treated pancreatitis mice on days 5 and 7.

Effect of inhibiting solTNF using XPro1595 on hippocampal astrocyte reactivity after induction of pancreatitis in mice

In recent years there has been strong support for the involvement of the hippocampal inflammatory response in the development of peripheral pain. Specifically, rodent models show hippocampal TNF/TNFR1 activity regulates hindpaw neuropathic pain (52, 58). Since acute pancreatitis promotes a systemic inflammatory response in both humans and rodents (69, 70), including TNF upregulation within the hippocampus (71), we sought to determine whether our model of pancreatitis promotes an inflammatory state within the hippocampus that could play a role in the development of peripheral hindpaw mechanical hypersensitivity. We semi-quantitated hippocampal GFAP expression, as a marker of astroglial reactivity 7 days after cerulean administration. We observed that cerulein-induced acute pancreatitis increased the tendency to astrocyte reactivity (GFAP expression) in the hippocampal CA1 region 7 days post-induction, compared to non-pancreatitis mice (Figure 5A,B&E), which was not apparent in the XPro1595-treated pancreatitis group (Figure 5C,D&E).

DISCUSSION

The onset of acute pancreatitis causes abdominal tenderness and pain, as well as nausea and vomiting that coincides with a spike in the blood pancreatic enzymes amylase and lipase (72), and an inflammatory response (69). Many causes of acute pancreatitis have been identified including pancreatic duct obstruction, alcoholism and a genetic mutation, with management including removal of any obstruction/s, nutritional regulation (including pancreatic enzyme supplementation and hydration), and pain management (73). Pharmacologic interventions have targeted the inhibition of proteolytic enzymes using broad spectrum anti-protease inhibitors that showed variable outcomes in animals if delivered before disease onset (74, 75). Unfortunately, these inhibitors failed to show any effect in patients, possibly due to administration at a time point after peak enzymatic activity, which may be unavoidable given the short peak in enzyme activity in patients (76, 77). Another pharmacological direction is to regulate the immune response, which also displays different phases of pro- and anti-inflammation, but which may represent a more clinically relevant

timepoint. Animal studies inhibiting pro-inflammatory mediators including IL-6, and ICAM, or bolstering anti-inflammatory mediators such as IL-10 have also shown variable successes (78-82), but enthusiasm for their use in patients has diminished due to limited benefits observed in regulating the inflammatory response (83-86). Notably however, early studies in rodents using TNF inhibitors showed promise with improved pancreatic pathology (9-12), with positive outcomes also seen in patients (13, 14), although their abundance of side-effects combined with their inability to reduce mortality in early studies on sepsis patients (20, 21) dampened enthusiasm for their further use in patients with pancreatitis. More than 2 decades later however, additional meta-analysis' reveal an overall improvement in survival rates in patients with sepsis when studies are sufficiently powered (22, 23). That combined with the development of a novel selective inhibitor of solTNF (XPro1595) with no known side-effects, that has proven to be safe and well tolerated (33), with an ability to reduce neuroinflammation in patients (34), warrants additional investigation. Indeed, our pre-clinical studies identify that selectively inhibiting solTNF by administering XPro1595 in a clinically relevant window reduces the course of the disease by limiting pancreatic inflammatory infiltrates. This early reduction in inflammatory severity prevents subsequent pancreatic pathological damage, despite this study being performed in a rodent model of pancreatitis (cerulein induction) that is known to resolve the inflammatory response within 7 days.

One of our important findings in these studies is the improvement of neuropathic pain "referred pain" over the course of the study in the XPro1595-treated mice, even after the resolution of the inflammatory response in all groups (Day 7). This is important because it suggests that the early inflammatory response is a significant contributor to the development of pancreatitis-induced pain, which can be alleviated by selectively inhibiting solTNF. Several lines of evidence suggest TNF plays a key role in regulating pain. First, TNF can regulate signaling along the traditional pain pathways at the level of the nociceptors (87, 88), dorsal root ganglion (50, 89), thalamus (90), and somatosensory cortex (90). In support of this, administering infliximab to block TNF in patients with rheumatoid arthritis reduced pain-induced CNS activity (90). Second, strong evidence supports of role of TNF throughout the limbic system to regulate

319 pain (52-54, 90, 91), possibly by incorporating emotional memories of pain. One region of the limbic
320 system that has gained a lot of attention is the hippocampus whereby hippocampal TNF/TNFR1 activity
321 regulates the severity of neuropathic pain (52-54, 91). Indeed, in our mouse model we observed a strong
322 tendency for upregulation of GFAP in the hippocampus 7 days after the induction of acute pancreatitis
323 suggesting that in our model hippocampal TNF/TNFR1 activity could be contributing to the induction of
324 neuropathic pain since reactive astrocytes are known synthesizes of excess TNF (92, 93). However, the
325 subcutaneous administration of XPro1595, while clinically relevant, prevents determination of the
326 molecular mechanisms involved.

CONCLUSION

Excess levels of the inflammatory cytokine TNF plays a prominent role in many inflammatory disease pathologies, including the induction of pancreatitis. Attempts to use TNF receptor fusion proteins or monoclonal antibodies to regulate this cytokines function have shown some successes clinically, but these have been fraught with complications due to their numerous adverse side-effects, including drug-induced acute pancreatitis. Our data provide support for the clinical use of a novel second generation TNF inhibitor XPro1595 that selectively inhibits only the detrimental soluble form of TNF to prevent the disease sequelae, while sparing the beneficial transmembrane form of TNF to allow reparative cellular mechanisms to remain.

ABBREVIATIONS

LTP	long term potentiation
solTNF	soluble form of tumor necrosis factor
tmTNF	transmembrane form of tumor necrosis factor
TNFR1	tumor necrosis factor receptor 1
TNFR2	tumor necrosis factor receptor 2

DECLARATIONS

Ethics approval and consent to participate

All experiments were performed under approval of the VCU Institutional Animal Care and Use Committee.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

RR and MD performed the experiments and tabulated the data. KJD designed, planned, funded, analyzed and interpreted the data. All authors read and approved the final manuscript.

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FIGURE LEGENDS

Figure 1: Cerulein administration promotes induction of pancreatitis. Acute pancreatitis was induced by 8 intraperitoneal injections of cerulein (50 mg/kg) or vehicle to mice, spaced an hour apart each, and serum amylase concentrations were measured 6 and 18 hours after the last cerulein injection. Cerulein-treated mice displayed a temporary spike in serum amylase concentration at 6 hours, that had reduced back to baseline levels by the next morning (n=3 per group). NC = non-cerulein, C = cerulein, **p<0.01, ***p<0.001.

Figure 2: XPro1595 treatment attenuates cerulein-induced pancreatic inflammatory infiltrates.

Photomicrographs illustrate hematoxylin and eosin stained sections of mouse pancreas in non-cerulein (A&D) and cerulein (B,C,E&F) mice, 2 days after cerulein/vehicle administration. Vehicle-treated non-cerulein mice had an absence of inflammatory infiltrates (A) (n=5), but cerulein treatment promoted an

influx of inflammatory cells within 2 days (**B**) (n=3), which had resolved by 7 days (**C&G**) (n=4). XPro1595 treatment significantly reduced the immune cell infiltration at 2 days (**E**) (n=3), that was even further reduced by 7 days (**F**) (n=4). A week following pancreatitis induction the circulating macrophage population was also minimal in cerulein-treated mice, independent of vehicle or XPro1595 treatment (n=4 each). C = cerulein, V = vehicle, XP = XPro1595, **p<0.01, scale bars = 100 μ m.

Figure 3: Selective inhibition of solTNF in mice with acute pancreatitis prevents pancreatic tissue degradation at 7 days after induction. Photomicrographs illustrate H&E stained pancreatic sections from mice with acute pancreatitis (cerulein treated) with either vehicle- (**A**) (n=4) or XPro1595-treatment (**B**) (n=4), where quantification shows vehicle-treated pancreatitis mice have significant deterioration of tissue integrity (**C**), compared to pancreatitis mice treated with XPro1595 (n=4), or non-pancreatitis mice (n=3-4). In accordance, the vehicle-treated pancreatitis mice also display an exacerbated level of pancreatic acinar cell atrophy (**D**) that is not observed in the XPro1595 treated pancreatitis group (**E&F**). A week after induction of pancreatitis the intralobular ducts in the vehicle-treated group appeared to be invaded by groups of acini spreading apart (**G**), although this was less prominent in the XPro1595-treated group (**H&I**). NC = non-cerulein, C = cerulein, V = vehicle, XP = XPro1595, *p<0.05, **p<0.01, ***p<0.001, scale bar in A&B = 500 μ m, scalebar in D&E = 25 μ m, scalebar in G&H = 500 μ m.

Figure 4: Soluble TNF activity reduces hindpaw mechanical hyper-sensitivity following induction of acute pancreatitis in mice. Graph shows mechanical hypersensitivity of the hindpaw when touched by von Frey filaments, as a percentage of pre-cerulein baseline data for each mouse. No differences were observed in hypersensitivity of non-pancreatitis mice between baseline and on days 3, 5 and 7, independent of treatment (n=6-8). In contrast, vehicle-treated pancreatitis mice displayed significantly more hypersensitivity than non-pancreatitis control mice at each time point assessed during the first week (n=9). Treating mice with XPro1595 prevented this hypersensitivity at all time-points tested (n=9), and this group displayed significantly less hypersensitivity than the vehicle-treated pancreatitis group on the

640 last 2 days of testing. NC = non-cerulein, C = cerulein, V = vehicle, XP = XPro1595, *p<0.05,
641 ***p<0.001.

642

643 **Figure 5: Hippocampal GFAP expression is more pronounced in cerulein-treated mice without**
644 **XPro1595 administration.** CA1 hippocampal GFAP expression was semi-quantitated 7 days following
645 induction of pancreatitis in mice. Non-pancreatitis mouse groups, independent of vehicle or XPro1595
646 treatment, did not display reactive astrogliosis (**A&C**) (n=3-4). In contrast, the vehicle-treated
647 pancreatitis tissue (**B**) had numerous reactive astrocytes and when quantitated displayed a tendency for
648 increased GFAP expression (n=4), compared to the non-pancreatitis groups, but was not statistically
649 significantly different (**E**). XPro1595 treated pancreatitis mice prevented the induction of reactive
650 astrocytes (**D**) (n=4). NC = non-cerulein, C = cerulein, V = vehicle, XP = XPro1595, scalebar = 100 µm.

Figures

Figure 1

Cerulein administration promotes induction of pancreatitis. Acute pancreatitis was induced by 8 intraperitoneal injections of cerulein (50 mg/kg) or vehicle to mice, spaced an hour apart each, and serum amylase concentrations were measured 6 and 18 hours after the last cerulein injection. Cerulein-treated mice displayed a temporary spike in serum amylase concentration at 6 hours, that had reduced back to baseline levels by the next morning (n=3 per group). NC = non-cerulein, C = cerulein, **p<0.01, ***p<0.001.

Figure 2

XPro1595 treatment attenuates cerulein-induced pancreatic inflammatory infiltrates. Photomicrographs illustrate hematoxylin and eosin stained sections of mouse pancreas in non-cerulein (A&D) and cerulein (B,C,E&F) mice, 2 days after cerulein/vehicle administration. Vehicle-treated non-cerulein mice had an absence of inflammatory infiltrates (A) (n=5), but cerulein treatment promoted an influx of inflammatory cells within 2 days (B) (n=3), which had resolved by 7 days (C&G) (n=4). XPro1595 treatment significantly reduced the immune cell infiltration at 2 days (E) (n=3), that was even further reduced by 7 days (F) (n=4). A week following pancreatitis induction the circulating macrophage population was also minimal in cerulein-treated mice, independent of vehicle or XPro1595 treatment (n=4 each). C = cerulein, V = vehicle, XP = XPro1595, **p<0.01, scale bars = 100 μ m.

Figure 3

Selective inhibition of solTNF in mice with acute pancreatitis prevents pancreatic tissue degradation at 7 days after induction. Photomicrographs illustrate H&E stained pancreatic sections from mice with acute pancreatitis (cerulein treated) with either vehicle- (A) (n=4) or XPro1595-treatment (B) (n=4), where quantification shows vehicle-treated pancreatitis mice have significant deterioration of tissue integrity (C), compared to pancreatitis mice treated with XPro1595 (n=4), or non-pancreatitis mice (n=3-4). In accordance, the vehicle-treated pancreatitis mice also display an exacerbated level of pancreatic acinar cell atrophy (D) that is not observed in the XPro1595 treated pancreatitis group (E&F). A week after induction of pancreatitis the intralobular ducts in the vehicle-treated group appeared to be invaded by groups of acini spreading apart (G), although this was less prominent in the XPro1595-treated group (H&I). NC = non-cerulein, C = cerulein, V = vehicle, XP = XPro1595, *p<0.05, **p<0.01, 629 ***p<0.001, scale bar in A&B = 500 μ m, scalebar in D&E = 25 μ m, scalebar in G&H = 500 μ m.

Hindpaw Neuropathic Pain

Figure 4

Soluble TNF activity reduces hindpaw mechanical hyper-sensitivity following induction of acute pancreatitis in mice. Graph shows mechanical hypersensitivity of the hindpaw when touched by von Frey filaments, as a percentage of pre-cerulein baseline data for each mouse. No differences were observed in hypersensitivity of non-pancreatitis mice between baseline and on days 3, 5 and 7, independent of treatment (n=6-8). In contrast, vehicle-treated pancreatitis mice displayed significantly more hypersensitivity than non-pancreatitis control mice at each time point assessed during the first week (n=9). Treating mice with XPro1595 prevented this hypersensitivity at all time-points tested (n=9), and this group displayed significantly less hypersensitivity than the vehicle-treated pancreatitis group on the last 2 days of testing. NC = non-cerulein, C = cerulein, V = vehicle, XP = XPro1595, *p<0.05, 640 ***p<0.001.

Figure 5

Hippocampal GFAP expression is more pronounced in cerulein-treated mice without XPro1595 administration. CA1 hippocampal GFAP expression was semi-quantitated 7 days following induction of pancreatitis in mice. Non-pancreatitis mouse groups, independent of vehicle or XPro1595 treatment, did not display reactive astrogliosis (A&C) (n=3-4). In contrast, the vehicle-treated pancreatitis tissue (B) had numerous reactive astrocytes and when quantitated displayed a tendency for increased GFAP expression

(n=4), compared to the non-pancreatitis groups, but was not statistically significantly different (E). XPro1595 treated pancreatitis mice prevented the induction of reactive astrocytes (D) (n=4). NC = non-cerulein, C = cerulein, V = vehicle, XP = XPro1595, scalebar = 100 μ m.