

Stat2 loss disrupts damage signalling and is protective in acute pancreatitis

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Abstract

The severity of sterile inflammation, as seen in acute pancreatitis, is determined by damage-sensing receptors, signalling cascades and cytokine production. Stat2 is a type I interferon signalling mediator that also has interferon-independent roles in murine lipopolysaccharide-induced NF-κB-mediated sepsis. However, its role in sterile inflammation is unknown. We hypothesised that Stat2 determines the severity of non-infective inflammation in the pancreas. Wild type (WT) and Stat2^{-/-} mice were injected i.p. with caerulein or L-arginine. Specific cytokine-blocking antibodies were used in some experiments. Pancreata and blood were harvested 1 and 24 h after the final dose of caerulein and up to 96 h post L-arginine. Whole-tissue phosphoproteomic changes were assessed using label-free mass spectrometry. Tissue-specific Stat2 effects were studied in WT/Stat2^{-/-} bone marrow chimera and using Cre-lox recombination to delete Stat2 in pancreatic and duodenal homeobox 1 (*Pdx1*)-expressing cells. Stat2^{-/-} mice were protected from caerulein- and L-arginine-induced pancreatitis. Protection was independent of type I interferon signalling. Stat2^{-/-} mice had lower cytokine levels, including TNF-α and IL-10, and reduced NF-κB nuclear localisation in pancreatic tissue compared with WT. Inhibition of TNF-α improved (inhibition of IL-10 worsened) caerulein-induced pancreatitis in WT but not Stat2^{-/-} mice. Phosphoproteomics showed downregulation of MAPK mediators but accumulation of Ser412-phosphorylated Tak1. Stat2 deletion in *Pdx1*-expressing acinar cells (Stat2^{flox/Pdx1-cre}) reduced pancreatic TNF-α expression, but not histological injury or serum amylase. WT/Stat2^{-/-} bone marrow chimera mice were protected from pancreatitis irrespective of host or recipient genotype. Stat2 loss results in disrupted signalling in pancreatitis, upstream of NF-κB in non-acinar and/or bone marrow-derived cells.

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Introduction

The response to tissue damage involves signalling pathways such as Toll-like receptors, MAPK and transcription factors such as NF-κB [1]. Transcription factor activation initiates inflammatory and healing processes by producing pro- and anti-inflammatory cytokines that include TNF-α, ILs and IFNs, which also serve to amplify the inflammatory response through their own signalling pathways. Stat2 is a mediator of type I IFN signalling, but it is increasingly evident that Stat2 has a key role in mediating inflammatory responses beyond this pathway [2,3]. We have previously demonstrated an IFN-independent role for Stat2, but not other Stat

proteins, in the response to bacteria- and virus-derived molecular patterns and in a mouse model of sepsis [4]. However, its role in non-infective inflammation, which is a characteristic of acute pancreatitis (AP), has received scant attention to date.

AP is a clinically significant example of damage-induced or 'sterile' inflammation. It is a potentially life-threatening, highly unpredictable and increasingly common inflammatory disease [5,6] with no specific treatment. Production and activity of inflammatory cytokines are central to the pathogenesis of AP [7], but a number of challenges exist to studying AP in humans, especially in the early, initiating stages of the disease. These include the inaccessibility of pancreatic tissue and a variable time interval between the onset of

symptoms and presentation to hospital. Mouse models can be used to address both of these challenges, but no single model recapitulates all the features of AP seen in man. Injection with supraphysiological concentrations of caerulein activates intracellular signalling and metabolic pathways that generate toxic mediators that cause a rapid mild pancreatitis. Injection with L-arginine causes a much more severe inflammation and can recapitulate later or secondary events in human pancreatitis.

To date, studies of type I IFN in the pathogenesis of pancreatitis have focused on known canonical functions of the type I IFN receptor (Ifnar) and signalling [8], but not Stat2. In the current study, we tested the hypothesis that Stat2-mediated signalling plays a role in AP as a model of non-infective tissue inflammation.

We found that Stat2 loss is protective against pancreatic injury in two independent models of AP in mice. Stat2 loss was associated with reduced expression of inflammatory cytokines, and inhibiting cytokines can modulate the severity of pancreatitis in wild type (WT) but not *Stat2*^{-/-} mice. We used bone marrow chimera and acinar cell-specific deletion of Stat2 to show that, despite the importance of acinar cells in cytokine production, non-acinar stromal and/or bone marrow-derived cells determine the severity of tissue inflammation. This gives us novel insight into the role of Stat2 in mediating the severity of inflammation.

Materials and methods

Experimental models of AP

Generation of *Stat2*^{-/-} mice was described previously [9]. Mice were housed in a conventional facility in individually ventilated cages with free access to food and water. All animal studies were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the UK Home Office and Local Ethics Committee at Queen Mary, University of London. Patients were not involved in the design of this study.

Pancreatitis was induced in male and female mice aged 6–9 weeks by hourly i.p. injections of 50 or 100 µg/kg caerulein (Sigma-Aldrich, St Louis, MO, USA) in PBS for 4 or 7 h and samples were collected at 1 h (hyperacute) and 24 h (acute) after the last injection, respectively. L-arginine-induced pancreatitis was induced by two i.p. injections of 4 g/kg L-arginine (Sigma-Aldrich) in PBS, 1 h apart.

For some experiments, mice were injected i.p. with 100 µg anti-TNF-α (Ultra-LEAF™ purified anti-mouse TNF-α antibody, clone MP6-XT22; Biolegend, San Diego, CA, USA) or 200 µg i.p. anti-IL-10 (LEAF™ purified anti-mouse IL-10 antibody, clone JES5-16E3; Biolegend) 1 h before caerulein treatment. For IFNAR1 blocking experiments, mice were injected with 500 µg i.p. anti-mouse IFNAR1 low endotoxin functional formulation (clone MAR1-5A3; Universal Biologicals, Cambridge, UK) 24 h before caerulein. For TNF-α

stimulation, mice received i.p. injections of 2 µg TNF-α (Peprotech, London, UK) 24 h before caerulein injections. Control mice were injected with PBS alone.

Mice were euthanised at the end of the experiment by terminal inhalational anaesthesia and cervical dislocation. The abdominal cavity was incised, and the pancreas dissected and removed. Tissues were snap frozen for protein and RNA extraction or fixed immediately in 10% neutral-buffered formalin and embedded in paraffin blocks for H&E and immunohistochemistry. Blood samples were taken, and serum was stored in aliquots at -80 °C until use. Serum amylase levels were measured using a Cobas analyser (Roche, Basel, Switzerland). Tissues were dried following incubation at 56 °C for 72 h.

Tissue oedema in the hyperacute experiment was calculated by subtracting the dry weight from the initial wet weight, by determining the pancreatic oedema index [10] and by assessing Evans Blue density. Mice were injected i.v. 30 min before caerulein treatment with 1% w/v Evans Blue (Sigma-Aldrich). At the end of the experiment, animals were sacrificed, and tissues were harvested and incubated in 10% formamide at 56 °C for 24 h. The amount of accumulated Evans Blue in the tissue supernatant was quantified by spectroscopy at 570 nm.

Bone marrow chimeric mouse production

Femurs and tibias were harvested from donor mice. The ends of each bone were removed and bone marrow cells flushed out with PBS/0.2% FBS (Life Technologies, Carlsbad, CA, USA). Erythrocytes were removed by hypotonic lysis and cells were washed and counted using Trypan Blue exclusion. Bone marrow cells were clarified through a 70 µm cell strainer, resuspended at 5 million cells/ml and kept on ice until injection into recipient mice 2 h later. Recipient mice were X-ray irradiated using a RadSource RS-2000 (Brentwood, TN, USA) and 2 × 5 Gy doses approximately 4 h apart; donor cells were injected into recipients via the tail vein (1 million cells/mouse) 1 h after the second irradiation. Mice were maintained on antibiotic-supplemented drinking water (enrofloxacin, 0.02 mg/ml; Bayer, Leverkusen, Germany) for 3 days prior to irradiation and up to 2 weeks after cell transfer. Mice were used for experiments 6 weeks after cell injection.

Serum cytokine ELISA

Serum was analysed for TNF-α, IFN-γ, IL-1β, IL-6, IL-10, IL-17A using a multiplex (six-plex) ELISA (BD Biosciences, San Diego, CA, USA) and for IL-1B, IL-6 and IL-10 using the mouse magnetic luminex assay (R&D Systems, Minneapolis, MI, USA). Serum was used as a 1:4 dilution in PBS for the analysis.

RT-qPCR

Tissue samples were sonicated on ice in Trizol (Thermo Fisher Scientific, Waltham, MA, USA) and total RNA was isolated as recommended by the manufacturer.

RNA concentrations were measured spectrophotometrically (NanoDrop 2000, Thermo Fisher) and 3 µg was used for each cDNA preparation. cDNA was reverse transcribed using the Superscript IV protocol (Invitrogen, Carlsbad, CA, USA) and qPCR was performed using the rotor-gene SYBR green PCR kit (Qiagen, Hilden, Germany) (see supplementary material, Table S1B for gene names and primer sequences used). Cycle threshold (ct) values were determined for each sample and the ct value difference (Δ ct) was used to calculate the factor of differential expression ($2^{\Delta\text{ct}}$). Correction for cDNA input deviations was performed using *Actb*.

Protein immunoblots

Whole cell extracts were prepared by homogenising tissues in lysis buffer (Tris 50 mM, NaCl 150 mM, EDTA 2 mM, 1% NP-40) containing Halt™ protease and phosphatase inhibitor cocktail (Thermo Fisher). Tissue samples were homogenised on ice then incubated with vigorous shaking at 4 °C for 30 min. Cells were then vortexed and centrifuged for 10 min at $13\,600 \times g$ at 4 °C. Cytoplasmic and nuclear extracts were prepared. Frozen tissues were homogenised in hypotonic buffer A (HEPES 10 mM, pH 7.5, KCl 10 mM, EGTA 0.1 mM, EDTA 0.1 mM, pH 8.0, 1 mM DTT) containing protease/phosphatase inhibitor cocktail. Cells were kept on ice for 10 min, 0.3% NP-40 was added and then vortexed briefly and spun down for 30 s at 4 °C. Supernatant (cytoplasmic fraction) was removed and stored on ice for protein quantification. The pellet was washed again with buffer A, and the hypertonic, high-salt buffer C was added (HEPES 20 mM, pH 7.5, NaCl 0.4 M, EDTA 1 mM, EGTA 1 mM, glycerol 20%) together with the protease/phosphatase inhibitor cocktail. After centrifugation (10 min at $13\,600 \times g$, 4 °C), the supernatant was collected and referred to as the nuclear fraction.

Protein concentration was measured using a Pierce BCA assay as recommended by the supplier (Thermo Fisher). Aliquots of equal concentration were mixed in 4× sample buffer consisting of 277.8 mM Tris-HCl, pH 6.8, 44.4% (v/v) glycerol, 4.4% lithium dodecyl sulfate (LDS), 0.02% bromophenol blue (Bio-Rad, Hercules, CA, USA) with additional 50 mM DTT, boiled at 95 °C for 5 min and stored at −80 °C. See supplementary material, Table S1A for the antibodies used.

Histology

Sections of formalin-fixed paraffin-embedded tissues were stained with H&E. Two pathologists, blind to the treatment group, scored digitised images of the whole specimen for oedema (0, absent or rare; 1, oedema in the interlobular space; 2, oedema in the intralobular space; and 3, the isolated-island shape of pancreatic acinus), inflammation (0, absent; 1, mild; 2, moderate; and 3, severe) and parenchymal necrosis (0, absent; 1, focal [$<5\%$]; 2, and/or sublobular [$<20\%$]; 3, and/or lobular [$>20\%$]). Parenchymal haemorrhage was not seen. The first pathologist was asked to re-score the slides in a

blinded manner and a second pathologist, also blinded, scored the slides independently using the same scoring system and intra- and interobserver kappa scores calculated. Images were taken on a NanoZoomer 2.0HT (Hamamatsu, Shizuoka, Japan) scanner using NDP.scan 3.2.12 software (Hamamatsu) and displayed at 20× magnification.

Immunofluorescence microscopy

Pancreas cell suspensions were prepared as described [11]. Isolated acinar cells were plated on to chamber slides (Nunc, Thermo Fisher) precoated with 50 µg/ml type 1 collagen (Thermo Fisher) in 0.02 M acetic acid, 0.2 µm-filtered and incubated overnight at 37 °C 5% CO₂. Cells were treated with 10 ng/ml TNF-α (Pepro- tech) or 1 nM cholecystokinin (CCK; Cambridge Biosciences, Cambridge UK) for the indicated time points. Cells were fixed in cold paraformaldehyde (PFA) (4% PFA in PBS) for 20 min at room temperature. Cells were permeabilised in 0.3% Triton ×100 in PBS for 10 min and blocked in 5% BSA/PBS for 30 min at room temperature. Cells were incubated with anti-p65 (clone D14E12) XP® rabbit mAb (Cell Signaling Technology, London, UK) primary antibody (1:200) in 2% BSA/PBS/0.05% Tween-20 at 4 °C overnight. See supplementary material, Table S1 for the antibodies used. Cells were washed three times with wash buffer (PBS/0.05% Tween-20) and stained with goat anti-rabbit Alexa Fluor 488-conjugated antibody 1:500 (Thermo Fisher) in 2% BSA/PBS/0.05% Tween-20 for 1 h at room temperature. Slides were washed twice in wash buffer and once in PBS and dehydrated in 70–100% ethanol series prior to mounting in VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories, Peterborough, UK). Confocal images were acquired on a Zeiss LSM 710 upright scanning confocal microscope using a 63× oil objective, numerical aperture 1.4. Z-stacks were acquired at 1 µm intervals 20 µm above and below the midline of the cell cluster. Images were analysed using ImageJ (NIH, Bethesda, MD, USA) and IMARIS (Bitplane, Zurich, Switzerland) software. Localisation of indirectly labelled p65 in the nuclei was quantified by preparing a three-dimensional threshold image in DAPI and measuring the mean intensity values for the green channel in each nucleus.

Phosphoproteomic analysis

Pancreata were crushed, vortexed and sonicated in ice-cold 8 M urea (in 20 mM HEPES pH 8.0) supplemented with Na₃VO₄, NaF, Na₂P₂H₂O₇ (sodium pyrophosphate) and beta-glycerol phosphate. Lysates were reduced, alkylated and digested overnight. Following desalting by solid-phase extraction, 350 µg total protein was phosphoenriched using TiO₂ beads prior to phosphoproteomic analysis by liquid chromatography-mass spectrometry as described previously [12].

Statistical analysis

All serum amylase, ELISA, IMARIS and RT-qPCR data were analysed using Prism software (GraphPad Inc., San Diego, CA, USA). Data are presented as mean $\pm$ SEM. Raw mass spectrometry data were analysed using Proteome Discoverer 1.4. The Mascot search engine (version 2.2.06) was used to automate peptide identification against the SwissProt database. Fragment tolerance was set at 0.8 Da. Mass spectrometry quantitative normalised total spectra for phosphorylation modifications were analysed using Scaffold Viewer 4.7.2 (Proteome Software, Portland, OR, USA). Pathway analysis was performed using PANTHER gene enrichment software (<http://pantherdb.org>).

Results

Stat2^{-/-} mice are protected from AP

To assess the effect of Stat2 on tissue inflammation, AP was induced in WT and *Stat2*^{-/-} mice by giving hourly i.p. injections of caerulein for 4 or 7 h. Tissue and blood samples were harvested 1 h (which we defined as hyperacute) and 24 h (which we defined as acute) after the final injection (see supplementary material, Figure 1A). *Stat2*^{-/-} mice had significantly lower serum levels of amylase compared with WT following hyperacute injury (Figure 1A, 3808 IU/ml versus 8001 IU/ml, $p < 0.0001$). At this time point, pancreatic tissue damage was mild, but there was significantly less pathology (in particular oedema) in *Stat2*^{-/-} mice compared with WT (Figure 1B, see supplementary material, Figure S1B–D). In the acute model (seven hourly injections of caerulein and harvesting after 24 h), injury was more evident histologically and *Stat2*^{-/-} mice had less pancreatic necrosis and interlobular oedema compared with WT (Figure 1B), although amylase levels had returned to baseline in both genotypes at this time point (not shown).

We did not find any differences in the expression of the receptors that are activated by caerulein (CCK-AR or CCK-BR) between WT and *Stat2*^{-/-} pancreatic extracts that could account for the difference in severity of injury observed (see supplementary material, Figure S1E). Total Stat2 levels in whole cell extracts from whole WT pancreata were unaffected by caerulein treatment (see supplementary material, Figure S1F). We excluded the possibility that loss of Stat2 protects from hyperacute caerulein-induced pancreatitis through a type I IFN-mediated signalling loop by pretreating animals with 500 μ g/i.p. Ifnar-1 blocking antibody. Although the antibody effectively inhibited IFN- α -mediated Mx1 production in splenocytes (Figure 1E), it had no significant impact on amylase levels in *Stat2*^{-/-} mice and did not protect WT mice from caerulein-induced pancreatitis (Figure 1F).

Stat2 loss was protective in a second, more severe model of pancreatitis induced by L-arginine injection. *Stat2*^{-/-} mice had lower levels of serum amylase

compared with WT mice at 24 and 72 h following L-arginine treatment (8768 IU/ml versus 4829 IU/ml, $p < 0.05$, Figure 1E). L-arginine caused marked pancreatic necrosis, oedema, tissue destruction and inflammatory cell infiltration over 96 h in WT mice, which was much less evident in *Stat2*^{-/-} mice at all time points (Figure 1F, see supplementary material, Figure S1G, H). Intraobserver kappa score for histology scoring = 0.845 and interobserver kappa = 0.746.

Stat2 mediates AP upstream of cytokine production and activity

We did not observe clinical signs of systemic inflammation nor significant increases in serum concentrations of cytokines in hyperacute or acute caerulein-treated models (see supplementary material, Figure S2). However, elevated serum IL-6, IL-1 β and IL-10 were detected at later time points in WT L-arginine-treated mice (72 h), and these levels were significantly lower in *Stat2*^{-/-} mice (Figure 2A).

AP led to increased expression of inflammatory genes in pancreatic tissue. Consistent with protection from injury, expression of inflammatory genes encoding, TNF- α and IL-6, was lower in *Stat2*^{-/-} pancreas compared with WT (Figure 2B) in caerulein- and L-arginine-induced pancreatitis (Figure 2C). By contrast, levels for the anti-inflammatory protein IL-10 were higher in PBS-treated control *Stat2*^{-/-} mice compared with WT and although treatment with caerulein increased the expression of IL-10 in WT mice, there was no further increase from baseline in *Stat2*^{-/-} mice. Therefore, we tested the hypothesis that inhibition of TNF- α in mice would protect against AP, whereas inhibition of IL-10 would worsen tissue injury in WT mice. In order to study early inflammatory and not secondary events, we focused on the hyperacute caerulein model. Pre-administration of 100 μ g i.p. TNF- α blocking antibody resulted in significant reduction in serum amylase (6483 IU/ml WT versus 4442 IU/ml WT + anti-TNF- α , $p < 0.05$) and histological injury in WT but not *Stat2*^{-/-} mice (Figure 3A–C). Pretreatment with recombinant TNF- α (2 μ g i.p.) did not significantly increase serum amylase in caerulein-treated mice of either genotype (see supplementary material, Figure S3). Pretreatment with 200 μ g i.p. IL-10 blocking antibody resulted in increased serum amylase (1997 IU/ml WT versus 2873 IU/ml WT + anti-IL-10, $p < 0.05$) and histological injury in WT mice, but did not have an effect in *Stat2*^{-/-} mice (Figure 3D–F), suggesting that Stat2 acts upstream of the production of cytokines such as TNF- α or IL-10.

The NF- κ B family of transcription factors mediates cytokine release following tissue injury. To model the activation of NF- κ B *in vitro*, we stimulated primary acinar cells from WT or *Stat2*^{-/-} mice with CCK-8, a synthetic octapeptide analogue of caerulein [13,14]. We found a rapid and statistically significant shift from cytoplasmic to nuclear localisation of NF- κ Bp65 at 5 and 15 min in WT cells, whereas in *Stat2*^{-/-} cells there was little evidence of the oscillation of NF- κ Bp65

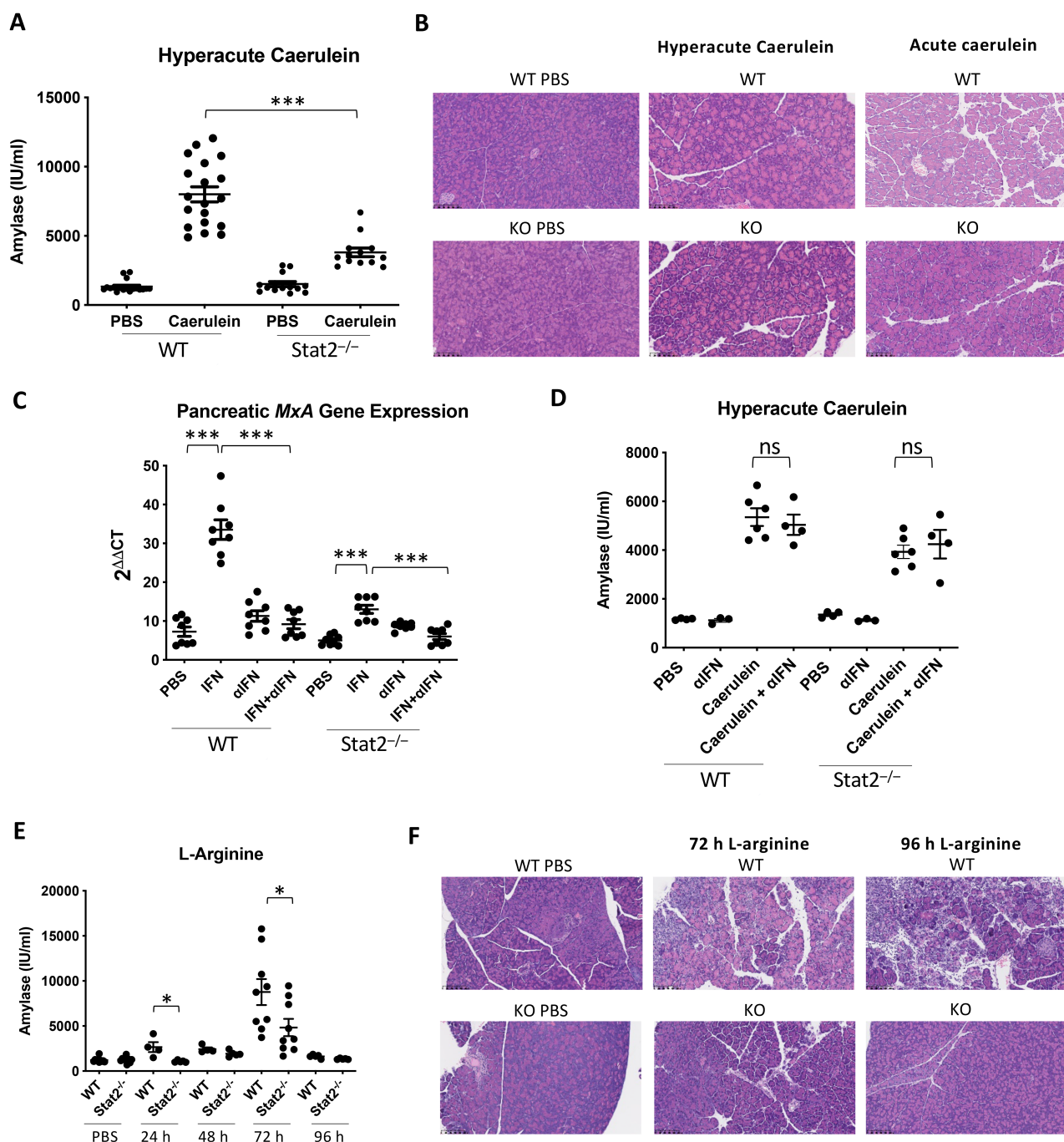


Figure 1. Stat2 loss reduces the severity of experimental pancreatitis in two models. (A) Serum concentration of amylase and (B) representative H&E-stained histology following injection with caerulein at the time points indicated (hyperacute = 1 h after four hourly injections and acute = 24 h after seven hourly injections). Effect of Ifnar1 blocking antibody (IFN- α) on (C) IFN- α -induced (IFN) *Mx1* gene expression and (D) caerulein-induced serum amylase expression. (E) Serum concentration of amylase and (F) histology following injection with L-arginine at the time points indicated. WT, wild type, KO, Stat2^{-/-}, *** $p < 0.001$, * $p < 0.05$, ns, not significant.

(Figure 4A,B). Thus, in pancreas tissue, Stat2 influences the cellular localisation of NF- κ Bp65 and, hence, cytokine production.

Loss of Stat2 impairs signalling in damage pathways

To determine whether Stat2 loss had a more global impact on intracellular signalling that leads to cytokine production, we used label-free tandem mass spectrometry to study Stat2- and caerulein-dependent

phosphoprotein abundance within the pancreas. This allowed us to examine the signalling defects that accompany Stat2 loss and that are associated with impaired NF- κ B localisation. Total protein was extracted from WT and Stat2^{-/-} pancreata following treatment with either PBS or caerulein (hyperacute model, $n = 4$ in each group) and processed for whole phosphoproteomic analysis as described previously [12]. We detected 611 proteins with peptide sequence concordance threshold 95% and protein threshold 99.9% (used to confirm the

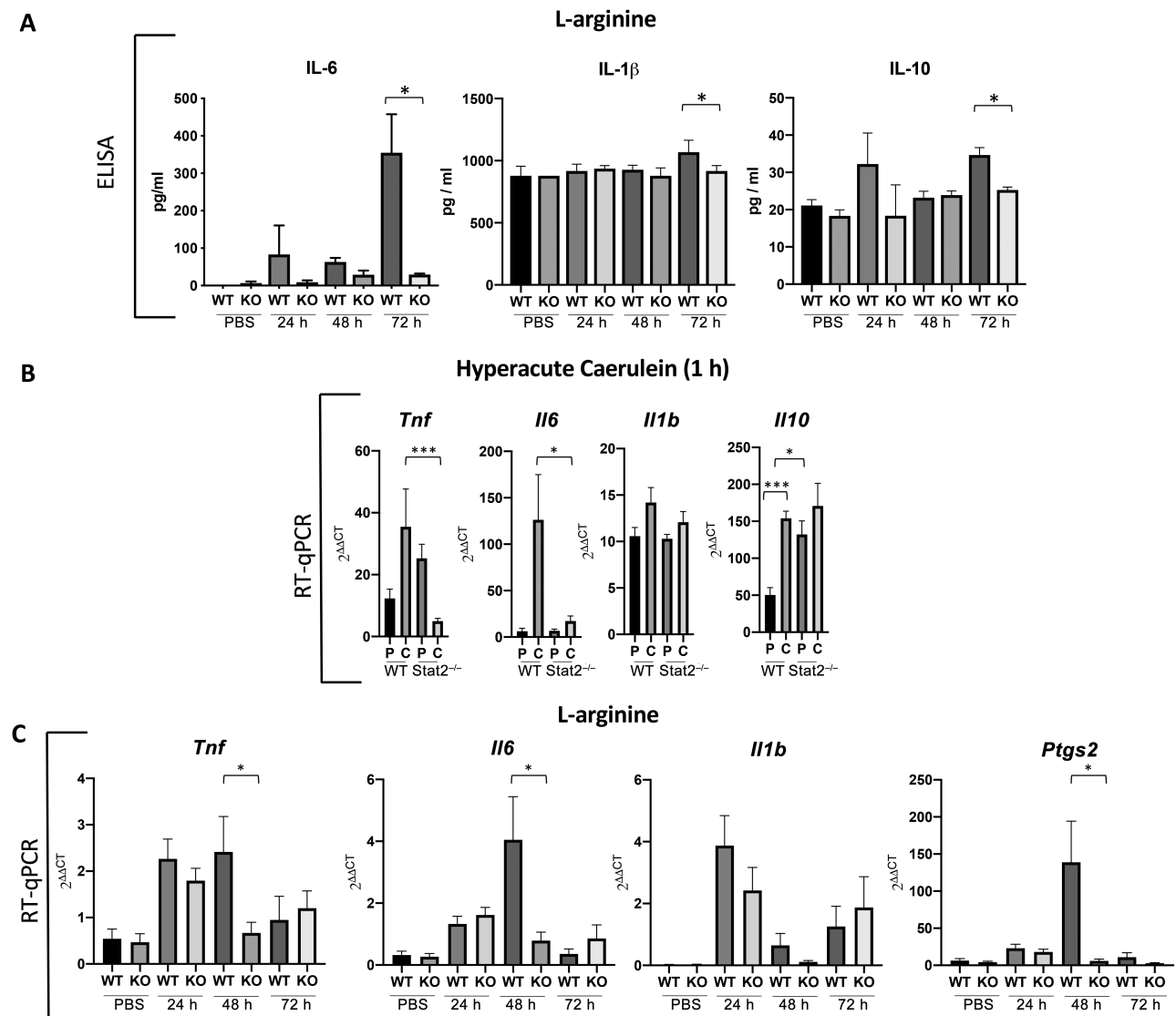


Figure 2. The effect of Stat2 loss on cytokine production. (A) Serum cytokine concentrations in WT and *Stat2*^{-/-} (KO) mice following treatment with PBS or L-arginine. RNA expression of genes shown in WT and *Stat2*^{-/-} (KO) whole pancreata following treatment with PBS (P) or caerulein (C) in (B) hyperacute (1 h after four hourly injections) caerulein- or (C) L-arginine-induced pancreatitis at the time points indicated. **p* < 0.05.

presence and identity of a protein). Based on the expression level of these proteins, 15 of 16 samples clustered according to the experimental condition in an unsupervised hierarchical clustering algorithm (Figure 5A and see supplementary material, Figure S4). Overall, the total number of phosphoproteins identified was comparable in each of the experimental conditions (Figure 5B).

In order to maximise signalling pathway discovery, we used predetermined thresholds for differences of interest at abundance ratios of <0.75 and >1.33. We identified 348 unique phosphoproteins that were differently expressed in our experimental conditions (see supplementary material, Table S2). The expression of 257 phosphoproteins was altered in WT pancreas as a result of caerulein treatment. In total, 175 phosphoproteins were altered in caerulein-treated *Stat2*^{-/-} versus WT pancreas, of which 128 were detected in the inflamed

but not control samples (66 upregulated and 62 downregulated in *Stat2*^{-/-}). We conducted a GO Panther analysis of all 175 phosphoproteins that were differentially expressed between WT and *Stat2*^{-/-} in unstimulated and in stimulated tissue. This highlighted pathways involved in signalling, and the response to injury, including the CCK receptor signalling pathway (*n* = 12 phosphoproteins, *p* = 1.1×10^{-6} , Table 1). Five of six significantly identified pathways contain at least one MAPK, including MAP2K2 (MEK2), the principal activator of extracellular signal related kinases (ERK1 and ERK2 [15]). The activating phosphorylation of MEK2 at serine S226 was more abundant in inflamed WT pancreata compared with *Stat2*^{-/-} in the phosphoproteome analysis by mass spectrometry (Figure 5C). Using immunoblotting, we found that the expression ERK1/2 pT202/Y204, a downstream target of MEK2, was also

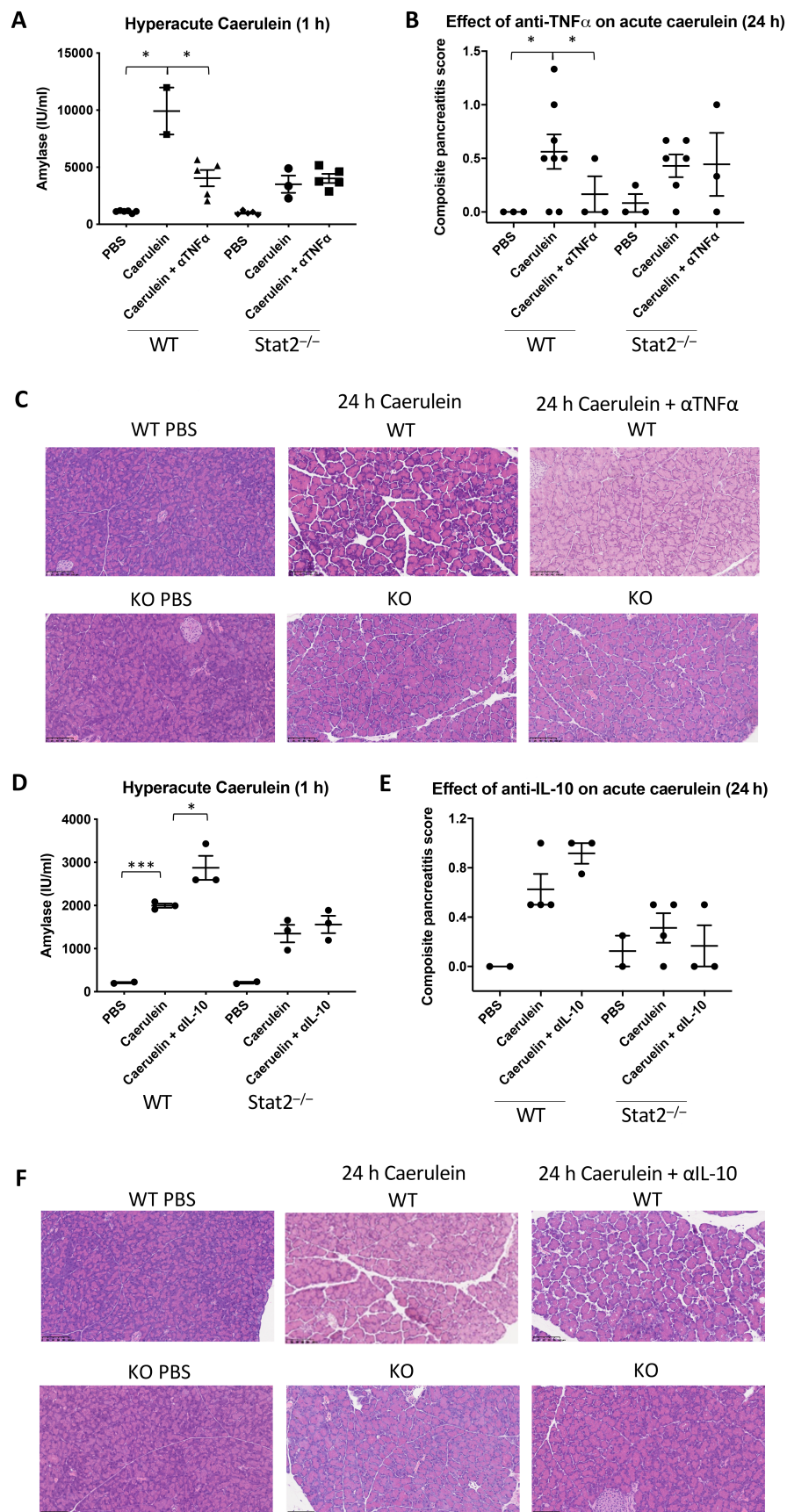


Figure 3. Effects of blocking TNF- α and IL-10 on caerulein-induced pancreatitis. WT and Stat2^{-/-} (KO) mice were given a single i.p. injection of (A–C) 100 μ g TNF- α blocking antibody or (D–F) 200 μ g IL-10 blocking antibody prior to PBS or caerulein treatment at the time points indicated (hyperacute = 1 h after four hourly injections and acute = 24 h after seven hourly injections). (A,D) Serum amylase, (B,E) tissue injury score for each pretreatment and (C,F) representative H&E-stained histology. * $p < 0.05$.

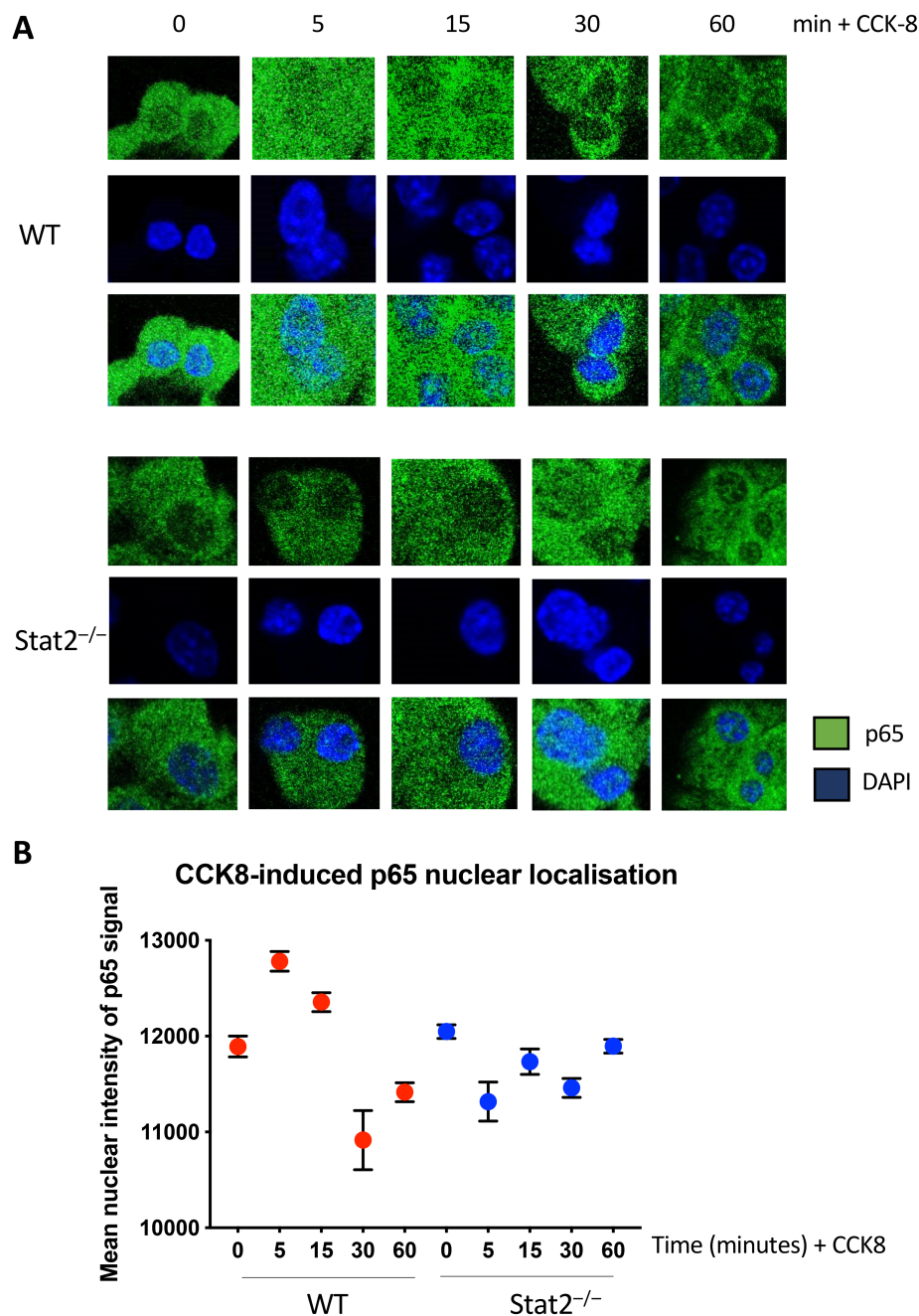


Figure 4. Loss of Stat2 disrupts NF- κ B nuclear localisation. (A) Confocal microscopy showing NF- κ Bp65 staining (green) and nuclear DAPI (blue) in primary pancreatic cells at the time points shown following stimulation with CCK-8. (B) IMARIS software quantification of nuclear localisation of NF- κ Bp65 signal in confocal images. WT, wild type, KO, Stat2^{-/-}.

reduced in Stat2^{-/-} nuclear pancreatic extracts compared with WT (see supplementary material, Figure S5A) [16].

Although we observed no difference in two upstream activators of MEK2 (MKK3 pSer189 or MKK6 pSer207) between WT and Stat2^{-/-} pancreata (see supplementary material, Figure S5B), we did find increased levels of Map3K7 (TGF- β -activated protein kinase 1: Tak1), a MAPKKK that lies upstream of MEK2 and NF- κ Bp65. The phosphorylated Ser412 modification of Tak1 was significantly more abundant in Stat2^{-/-} caerulein-treated samples compared with other conditions by mass spectrometry (Figure 5C) and in independent experiments by immunoblotting (Figure 5D). This

suggests that Ser412 phosphorylation of Tak1 is a potential point of pathway interruption in the absence of Stat2.

Cell-specific effects of Stat2 loss

To determine the cell type in which Stat2 plays a role, we generated bone marrow chimeric mice and found that loss of Stat2 in either donor or recipient cellular compartments resulted in lower serum amylase (Figure 6A) and less histological injury (Figure 6B). To further refine this, we generated mice with a conditional deletion of Stat2 (Stat2^{fllox/fllox}) induced by *Pdx1-cre* to specifically delete Stat2 in cells expressing *Pdx1* (Stat2 ^{Δ acinar}) [17]. Following hyperacute caerulein-

induced pancreatitis, the expression of inflammatory TNF- α was significantly lower in *Stat2* ^{Δ acinar} mice compared with WT (Figure 6B), in keeping with the impaired signalling above. However, this had no significant effect on serum amylase concentration (Figure 6C) nor pancreatic histology (Figure 6D). Given that cytokine blockade did modulate pancreatic inflammation, these results strongly suggest that Stat2- and cytokine-dependent tissue injury in AP is mediated by a combination of bone marrow-derived cells and/or non-acinar stromal cells.

Discussion

Our data show that Stat2 has a pathogenic, proinflammatory role in the response to sterile injury in the pancreas. In two models, assessed at different time points, *Stat2*^{-/-} mice were protected from caerulein- and L-arginine-induced AP. Using a phosphoproteomic approach, we identified signalling mediators from the MAPK cascade downstream of, and including, Tak1, that were differentially activated between WT and *Stat2*^{-/-} mice. However, acinar cell-specific deletion of Stat2 alone was

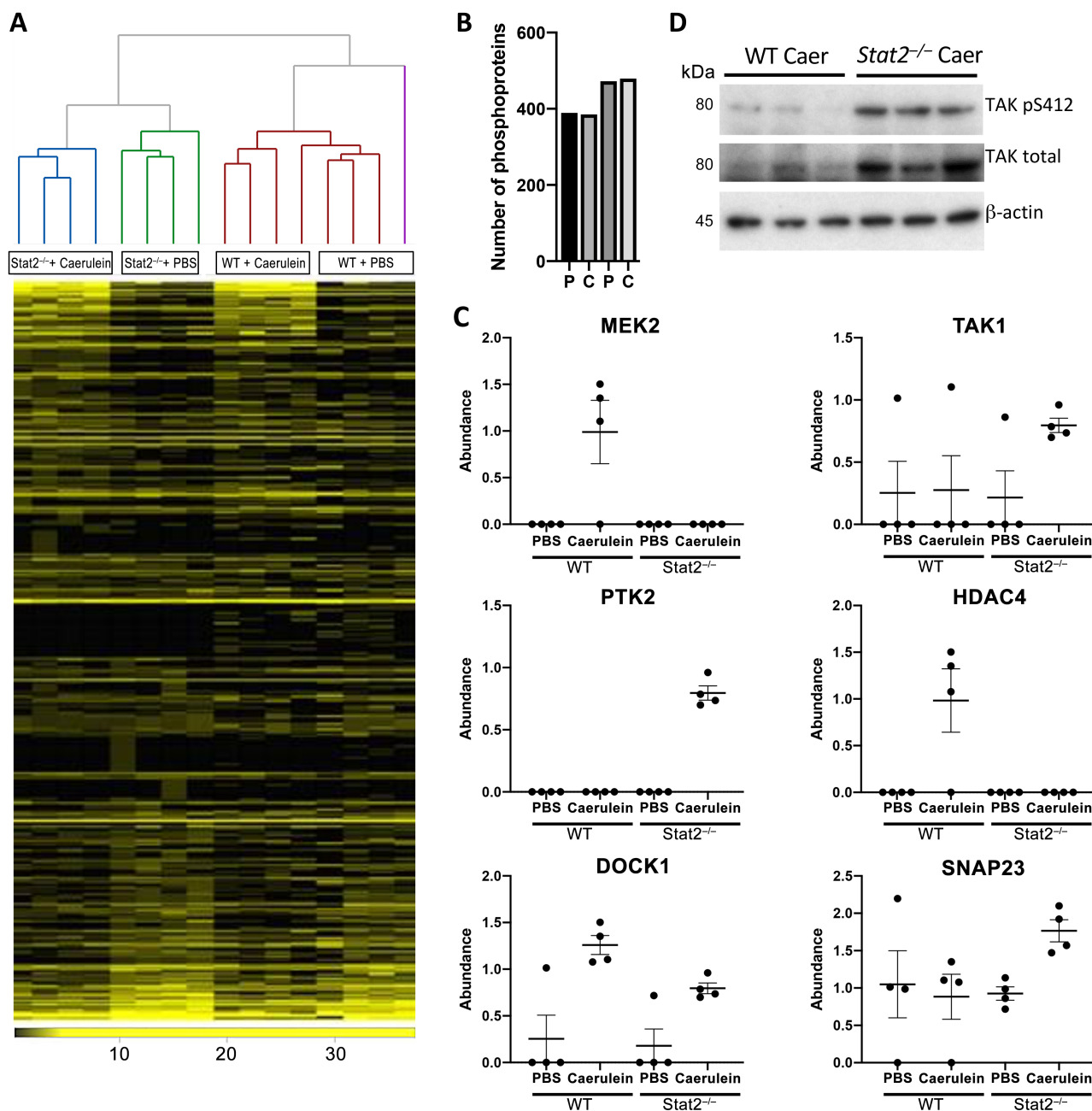


Figure 5. Global phosphoproteomic changes associated with Stat2 loss. 22 663 tandem mass spectra were identified, corresponding to 2881 unique peptide ions, of which 1650 were phosphopeptides containing 1771 phosphopeptide sites. (A) Unsupervised hierarchical clustering of 611 phosphoproteins identified using label-free mass spectrometry. Each lane represents an individual mouse and each row is one phosphoprotein. Yellow indicates high abundance and black lowest abundance. (B) Total number of phosphoproteins identified in each experimental condition. WT, wild type, KO, *Stat2*^{-/-}. (C) Abundance of key signalling phosphoproteins as determined by mass spectrometry. (D) Immunoblot for phosphorylated Tak1 (80 kDa), total Tak1 (80 kDa) and β -actin (45 kDa) in whole cell extracts and taken from WT and *Stat2*^{-/-} whole pancreata following treatment with PBS or caerulein in the hyperacute model (1 h after four hourly injections).

insufficient for histological and biochemical protection from AP, suggesting that Stat2 plays a role in non-acinar stromal and/or bone marrow-derived cells.

Caerulein is believed to cause pancreatitis by supra-physiological activation of CCK1 and CCK2 receptors, which signal through MAPK intermediates [18]. We have shown that *in vivo* cytokine gene expression in

response to caerulein is Stat2-dependent, as is NF- κ B localisation to the nucleus in primary acinar cells. L-arginine does not act through CCK receptors, yet Stat2 loss is also protective in this model of pancreatitis. We present multiple and complementary data sets which together demonstrate that, in the absence of Stat2, histological, immunological (cytokine data) and biochemical features of acute pancreatitis are improved. Serum amylase levels indicate the extent of pancreatic injury [19] but they do not predict the organ failure or other severe systemic complications observed in patients with the clinical syndrome of severe AP. The mechanism by which L-arginine causes pancreatitis is not fully understood but cellular metabolic disturbances liberate damage-associated molecular patterns that can trigger MAPK-containing signalling pathways [20,21]. Thus, Stat2 seems to play a wide role in MAPK-mediated signal transduction in response to injury that is agnostic of the initial triggering stimulus.

The main question that arises from our work is: where in these signalling pathways does Stat2 act? The data show an effect on MAPK-mediated signalling, ERK activation and NF- κ B signalling. One interpretation of

Table 1. Signalling pathways identified by GO Panther pathway analysis to be statistically significantly enriched in 278 phosphoproteins that differed between WT and *Stat2*^{-/-} pancreata either at baseline or after treatment with caerulein.

GO pathway	Number of phosphoproteins	P value
CCKR signalling	12	1.1×10^{-6}
Integrin signalling	10	1.5×10^{-4}
Gonadotropin-releasing hormone receptor pathway	10	7.4×10^{-4}
Alzheimer disease-amyloid secretase pathway	5	1.6×10^{-3}
Ras pathway	5	2.0×10^{-3}
Insulin/IGF pathway/MAPK cascade	4	1.0×10^{-3}

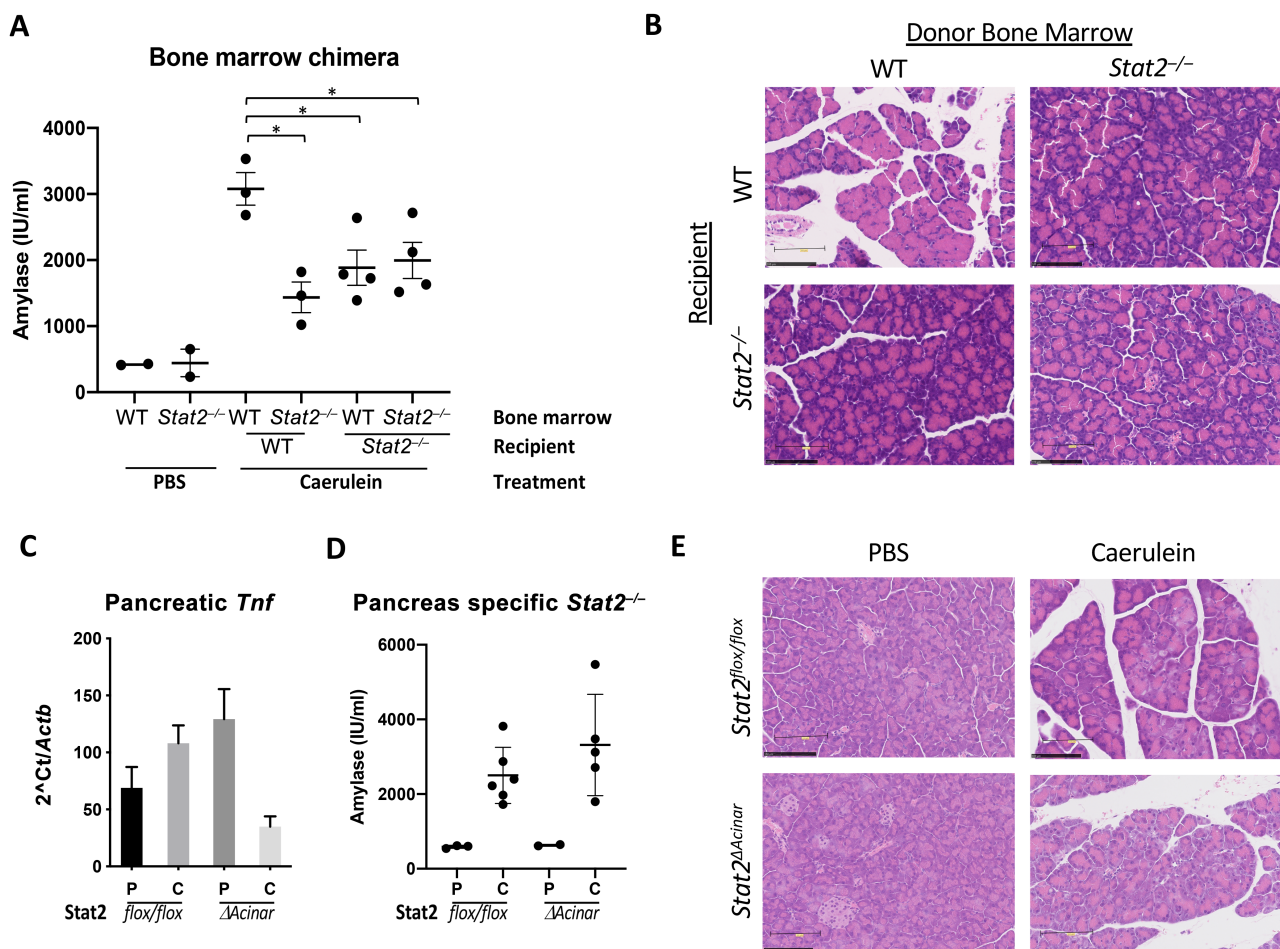


Figure 6. Effects of lineage Stat2 deletion in specific compartments on caerulein-induced pancreatitis. (A) Serum concentration of amylase and (B) representative H&E-stained histology in bone marrow chimeric mice following hyperacute caerulein-induced pancreatitis (1 h after four hourly injections). (C) RNA expression of *Tnf* in whole pancreatic extracts, (D) serum concentration of amylase and (E) representative H&E-stained histology in *Stat2*^{flox/flox} Δ Pdx1-cre mice following hyperacute caerulein-induced pancreatitis.

our results is that Stat2 sits at a common point that is proximal in these pathways. A build-up of pS413Tak1 in inflamed pancreatic tissue in *Stat2*^{-/-} mice might suggest that Stat2 is downstream of Tak1 but we have not excluded a compensatory increase in kinases or phosphatases in the absence of Stat2 that affects upstream signalling. Similarly, we have not demonstrated a direct interaction between Stat2 and Tak1 or other mediators of these pathways to see if there is physical association.

We could not recapitulate the protection seen in *Stat2*^{-/-} mice by blocking type I IFN signalling. Indeed, at the same early time points assessed in the current study, no difference in pancreatic damage was observed between WT and *Ifnar1*^{-/-} mice [22]. This suggests that IFN-mediated signals are not required for early pancreatitis and earlier work has shown that cellular responses to epidermal growth factor [23] and lipopolysaccharide (LPS) [4] are mediated by Stat2, even in the absence of the IFN phosphorylation site at Tyr689. Previous studies into the role for type I IFN signalling in AP have used prolonged injection schedules and focussed on much later time points after the final injection (3–7 days). In these models, deletion of *Ifnar1* partially improved tissue injury without an effect on cytokine mRNA levels [8,22]. Conversely, we find that pancreas-specific deletion of Stat2 reduces TNF- α levels but has no effect on tissue injury. Although pretreatment with a TNF- α blocking antibody attenuated injury in WT mice, it had no effect on the *Stat2*^{-/-}. The absence of an effect of pretreatment with recombinant TNF- α may reflect saturation or intact regulatory and control mechanisms. The lack of an effect in *Stat2*^{-/-} relates to the known defects in TNF- α responses in the absence of Stat2 [4]. Taken together, these data emphasise the IFN-independent role for Stat2 in AP and challenge the dogma that cytokine production is an obligate determinant of tissue injury. Nevertheless, we show that specific cytokine modulation does alter the severity of tissue inflammation and the main effect of Stat2 is to mediate signalling that leads to NF- κ B activation.

A consensus has yet to be reached on whether NF- κ B activation is protective or deleterious in AP [24,25]. This is largely because NF- κ B activation is the result of the timely integration of multiple pathways and NF- κ B itself can activate pro- and anti-inflammatory downstream effectors [26]. Pancreas-specific mutants of NF- κ Bp65 that are unable to dimerise or translocate to the nucleus resulted in more severe caerulein-induced injury [27] and deletion of I κ B α (which results in reduced activation of NF- κ Bp65) reduced caerulein-induced AP. Conversely, conditional overexpression of NF- κ B or constitutively active IKK2 increased activity of NF- κ B in acinar cells, and led to more severe pancreatitis compared with control mice [24]. Stat2 deficiency in our models is constitutive rather than inducible, but we do show that the absence of Stat2 is associated with disrupted nuclear entry and exit of NF- κ Bp65. This finding is complementary to our previous work showing a role for Stat2 in response to the pathogen-associated molecular pattern LPS; a model of infective inflammation [4]. We have not overexpressed Stat2, so do not know if this results in NF- κ B overactivation nor the effects that this

would have on severity. Our results suggest that at early time points, NF- κ B-related signalling and gene expression do contribute to disease severity, hence early cytokine inhibition alters the disease course. The protective effect of TNF- α inhibition is of clinical as well as pathogenic interest and a clinical trial of infliximab versus placebo in patients with severe AP is currently underway. Our data certainly support the hypothesis that early inhibition of TNF- α can be beneficial in pancreatitis, but further work is needed to determine the characteristics of patients who will benefit and the timing of treatment to maximise efficacy and minimise potential side-effects such as sepsis. We have shown that IFN-independent, Stat2-dependent signalling leads to the timely activation of NF- κ B in models of sterile pancreatic inflammation. Strategies that target Stat2 or its associated signalling mediators may have a therapeutic role in acute sterile inflammatory disease.

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Author contributions statement

HH, GB, HK, GR, MW and RG conducted experiments and/or analysed data. WA and GRF developed the study concept. WA and HH designed experiments. RH and GRF interpreted data and critically reviewed the manuscript. HH and WA wrote the paper and all authors reviewed and approved the paper. WA supervised the project.

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SUPPLEMENTARY MATERIAL ONLINE

Figure S1. Stat2 loss reduces the severity of caerulein- and L-arginine-induced pancreatitis

Figure S2. Serum cytokine concentrations in WT and Stat2^{-/-} mice following treatment with PBS (P) or caerulein (C) at the time points indicated

Figure S3. Effect of pretreatment with TNF-α on serum concentration of amylase hyperacute caerulein-induced pancreatitis

Figure S4. Principal component analysis of phosphoproteome identified by label-free mass spectrometry in whole pancreata from WT and Stat2^{-/-} (KO) mice following treatment with PBS or hyperacute caerulein-induced pancreatitis

Figure S5. Immunoblots showing abundance of proteins and phosphoproteins shown in (A) nuclear and cytoplasmic or (B) whole cell extracts from whole pancreata following hyperacute caerulein-induced pancreatitis

Table S1. Antibodies and primers used

Table S2. Mean quantitative normalised spectra for 348 unique phosphoproteins that were differentially expressed in each experimental condition shown