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Genetics and pathophysiology of pancreatitis

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Abstract

Since the discovery of the first trypsinogen mutation in families with hereditary pancreatitis, the field of pancreatic genetics has made rapid progress. The identification of mutations in genes involved in the digestive protease/antiprotease pathway has lent additional support to the notion that pancreatitis is a disease of autodigestion. Clinical and experimental observations provided compelling evidence that premature, intrapancreatic activation of digestive proteases is critical in pancreatitis onset. Disease course and severity, however, are mostly governed by inflammatory cells that drive local and systemic immune responses. Here we review the genetics, cell biology and immunology of pancreatitis with a focus on protease activation pathways and other early events.

Keywords

trypsinogen; pancreatitis; genetics; inflammation; cell death

INTRODUCTION:

Pancreatitis is the leading cause for GI-disease related hospital admissions and it is associated with considerable morbidity, mortality and socioeconomic burden[1]. Recent years shed light on the pathophysiology of pancreatitis opening up new avenues for causal treatment. In this review article, we dissect the complexity of premature protease activation and its effect on local and systemic inflammation in pancreatitis.

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This manuscript is dedicated to the memory of Walter Halangk, PhD, to honour his work as a pioneer unraveling the pathophysiology of pancreatitis.

GENETICS OF PANCREATITIS

Acute pancreatitis (AP), recurrent acute pancreatitis (RAP) and chronic pancreatitis (CP) form a disease continuum[2]. The progression of a sentinel attack of AP to RAP and eventually to CP is often driven by chronic alcohol consumption or genetic risk factors. Genetic risk for RAP and CP overlaps, while genetic studies in AP are difficult to interpret in the absence of adequate follow-up that can exclude RAP and CP cases.

The majority of the pancreatitis risk genes codes for digestive proteases, a trypsin inhibitor or other proteins highly expressed in the pancreas. Functional studies classified the various mutations and other genetic alterations into pathological pathways driving pancreatitis onset and progression. Here we discuss the trypsin-dependent, misfolding-dependent and ductal pathways of pancreatitis risk.

THE TRYPSIN-DEPENDENT PATHWAY OF GENETIC RISK IN CP

Pancreatic acinar cells secrete digestive proteases in inactive precursor forms that are flushed from the ductal system in a sodium bicarbonate-rich fluid. Trypsinogen, the precursor to trypsin, becomes activated by the serine protease enteropeptidase in the duodenum[3]. Trypsin activates chymotrypsinogens, proelastases and procarboxypeptidase B1 (CPB1) while activation of procarboxypeptidases A1 (CPA1) and A2 (CPA2) requires the concerted action of trypsin and chymotrypsin C (CTRC)[4]. Trypsinogen can be also activated by trypsin, and this process is called autoactivation[3]. Premature, intra-pancreatic activation of trypsinogen may occur via autoactivation or may be catalyzed by the lysosomal cysteine protease cathepsin B. Protective mechanisms that prevent trypsinogen activation in the pancreas include trypsin inhibition by the serine protease inhibitor Kazal type 1 (SPINK1) and trypsinogen degradation by CTRC and cathepsin L[5–7]. Although the principal action of CTRC is to promote trypsinogen degradation, it also enhances trypsinogen activation by processing the trypsinogen activation peptide to a shorter form, which is more sensitive to trypsin-mediated activation[7–9] (Figure 1). As discussed below, certain trypsinogen mutations can hijack this mechanism and thereby stimulate trypsinogen activation to a pathological extent. Human genetic studies strongly support trypsinogen autoactivation and CTRC-dependent trypsinogen degradation as key mechanisms determining intrapancreatic trypsin activity whereas similarly compelling genetic evidence for the role of cathepsins B and L has been lacking.[10].

PRSS1 mutations.

Mutations in human cationic trypsinogen cause autosomal dominant hereditary pancreatitis with incomplete penetrance or act as risk factors in sporadic CP[7,11]. Around 90% of *PRSS1*-mutation positive HP families carry the p.N29I, p.R122C, or p.R122H mutation in the heterozygous state. Mechanistically, the p.R122C and p.R122H mutations prevent CTRC-mediated trypsinogen degradation[9]. The p.N29I mutation has multiple distinct effects on trypsinogen biochemistry, the combination of which markedly increases trypsinogen autoactivation. These effects include an increase in N-terminal processing, decreased CTRC-dependent degradation and a slightly increased propensity for autoactivation[9]. The p.A16V variant, sensitizes the activation peptide of trypsinogen to CTRC-mediated processing,

which, in turn, enhances autoactivation[8,9]. Pathological trypsin levels generated by mutation p.A16V are lower than those seen with the p.R122H variant, which explains the reduced penetrance of the p.A16V variant. More recently, mutation p.P17T was found to exhibit characteristics that were similar to those of p.A16V[12]. Rare mutations affecting the activation peptide of cationic trypsinogen (p.D19A, p.D21A, p.D22G, p.K23R and p.K23_I24insIDK) robustly stimulate autoactivation independently of CTRC[13–15]. Cell culture experiments indicate that these activation peptide mutants are secreted poorly due to intracellular activation and degradation, which can lead to cellular stress and consequent acinar cell death[16]. Taken together, *PRSS1* mutations stimulate activation of cationic trypsinogen by reducing CTRC-dependent trypsinogen degradation, increasing CTRC-mediated processing of the activation peptide or directly stimulating autoactivation. GWAS studies identified a commonly occurring haplotype in the *PRSS1-PRSS2* locus that slightly decreases CP risk (OR 1.5) with a more pronounced effect in alcoholic CP[17–19]. A variant (c.−204C>A) that lies in the promoter region of *PRSS1* and reduces trypsinogen expression appears to be responsible for this small protective effect[20].

SPINK1 mutations.

The association between the most common p.N34S *SPINK1* variant and CP was first described by a candidate gene study in 2000[21]. A meta-analysis reported a carrier frequency of 9.7% in CP patients and 1% in controls with an average odds ratio (OR) of 11, making the p.N34S the clinically most significant risk factor for CP[22]. When considering European populations only, p.N34S increases CP risk by about 10-fold[23]. Although several studies attempted to identify the functional effect of p.N34S and its associated haplotype, the molecular mechanism underlying CP risk remains unclear. Neither p.N34S nor any of the four linked intronic variants affect trypsin inhibitory function or cellular expression of SPINK1[24–27]. Interestingly, in pancreatic cancer cell lines carrying the heterozygous p.N34S variant reduced expression of the mutant allele was observed in comparison to the wild-type allele[28]. The authors suggested that the c.−4141G>T variant or a hitherto unknown variant located in the 5’ region of the gene may be responsible for the reduced expression of the p.N34S allele. The second most frequently reported *SPINK1* haplotype in CP contains the c.−215G>A promoter variant and the c.194+2T>C variant in intron 3[21,29]. This haplotype was observed more frequently in East Asia than in Europe[7]. Functional studies revealed that the c.194+2T>C variant causes skipping of exon 3, which results in diminished *SPINK1* expression[27,30,31]. However, the c.−215G>A variant increases promoter activity, which might mitigate the effect of the c.194+2T>C mutation and allow for some residual SPINK1 expression even in homozygous carriers[32,33]. Finally, a large number of rare or private alterations in *SPINK1* have been found in CP, which cause loss of SPINK1 function by various mechanisms[7].

Protective anionic trypsinogen (PRSS2) variant.

Although PRSS1 and PRSS2 share 90% identity at the amino acid level and PRSS2 rapidly autoactivates, no pathogenic *PRSS2* variants were identified in HP or sporadic CP[34,35]. The absence of *PRSS2* mutations in CP may be due to the more effective CTRC-mediated degradation of anionic trypsinogen, which would prevent intra-pancreatic activation of the enzyme even if it were mutated[36]. However, a protective variant p.G191R with a ~3–6-fold

effect and circa 5% population frequency was discovered[35,37]. The mutation introduces a new trypsin cleavage site into anionic trypsinogen, which increases autocatalytic proteolysis and inactivation[35].

CTRC mutations.

Direct DNA sequencing of the *CTRC* gene in patients with nonalcoholic CP revealed heterozygous mutations in 4% of patients that increased CP risk by 5-fold on average[38,39]. The mutations cause loss of CTRC function by various mechanisms, which include defective secretion due to misfolding, resistance to trypsin-mediated activation, catalytic deficiency or increased degradation by trypsin[40,41]. Considering the clinically significant variants, p.A73T exhibits a severe secretion defect, p.K247_R254del is inactive and prone to degradation, p.R254W is degraded by trypsin and p.V235I has partially reduced activity[40]. Subsequent studies reported a frequent p.G60= variant found in about 30% of CP patients[42–45]. The heterozygous p.G60= increases the risk of CP by 2.5-fold, while the homozygous state by 10-fold[43,45]. The variant is associated with reduced *CTRC* mRNA expression (GTEx Portal), possibly due to altered pre-mRNA splicing.

CTRB1-CTRB2 locus inversion.

A recent European GWAS study identified a large inversion at the *CTRB1/CTRB2* locus that modestly (OR 1.35) modifies the risk for alcoholic and nonalcoholic CP[19]. The inversion changes the expression ratio of the CTRB1 and CTRB2 chymotrypsin isoforms in such a manner that protective trypsinogen degradation is increased and CP risk is reduced. In China the reported population frequency of the inverted (major) allele is 99.6%, thus the allele is virtually fixed and does not contribute to CP risk[46]. A mouse model with genetic deletion of the major mouse chymotrypsin CTRB1 exhibited increased intra-acinar trypsin activation and more severe pancreatitis induced by the secretagogue caerulein[47]. These observations provided the first *in vivo* proof for the protective role of chymotrypsin-mediated trypsinogen degradation against pancreatitis.

THE MISFOLDING-DEPENDENT PATHWAY OF GENETIC RISK IN CP

More recently, an alternative pathomechanism seemingly unrelated to premature intra-pancreatic trypsinogen activation has been identified, in which mutation-induced misfolding and consequent endoplasmic reticulum (ER) stress lead to acinar cell damage and pancreatitis[48].

Misfolding-associated PRSS1 mutations.

In 2009 it was demonstrated that a subset of *PRSS1* variants cause reduced secretion, intracellular retention and elevated ER stress markers, as judged by *in vitro* cell culture experiments[49]. These *PRSS1* mutations occur rarely and are mostly associated with sporadic disease (e.g., p.C139F, p.C139S, p.G208A), but were also found in HP families with incomplete penetrance (p.L104P, p.R116C)[48]. Variant p.G208A is prevalent in East Asia (4% of CP cases) and was detected in Europe only in a single case so far[50,51].

Misfolding-associated CPA1 mutations.

A candidate gene study in 2013 revealed that mutations in the *CPA1* gene are associated with CP (OR ~25), especially with early-onset disease (OR~80)[52]. The vast majority of pathogenic *CPA1* variants occur with low frequency and are mostly found in sporadic CP. The p.S282P variant was described in two HP families[53]. Pathogenic *CPA1* variants cause proenzyme misfolding resulting in a secretion defect, intracellular retention and ER stress[52,53]. In contrast to CPA1, variants of CPB1 and CPA2 are not associated with CP[54]. Interestingly, ER stress-inducing *CPA1* and *CPB1* variants were overrepresented in pancreatic cancer patients without a clinical history of RAP or CP[55]. Most of these variants caused premature truncation and did not overlap with those found in CP. A mouse model for the misfolding-dependent pathway was described recently. This study demonstrates that *CPA1 N256K* knock-in mice harboring the most frequent p.N256K human *CPA1* mutation develop spontaneous and progressive CP and exhibit signs of ER stress in their pancreas[56].

Misfolding-associated CEL mutations and CEL-HYB allele.

Single-nucleotide deletions in the last exon of the *CEL* gene encoding carboxyl ester lipase cause maturity-onset diabetes of the young type 8 (MODY8)[57]. The deletions alter the reading frame of the C-terminal variable number tandem repeat (VNTR) sequence resulting in CEL proteins with unnatural extensions that are prone to aggregation[58,59]. The exocrine dysfunction in MODY8 is in all likelihood caused by misfolding-induced ER stress and consequent acinar cell loss. A hybrid *CEL* allele (*CEL-HYB1*) formed between *CEL* and its neighboring pseudogene *CELP* was found about 5-fold overrepresented in idiopathic CP versus the average population frequency of 0.5–1%[60]. In cell culture experiments, the hybrid protein was secreted poorly due to intracellular retention, suggesting that the *CEL-HYB1* variant may increase CP risk via the misfolding-dependent pathway. A second hybrid *CEL* allele (*CEL-HYB2*) that does not associate with CP was described in Asian populations[61]. Interestingly, the CEL protein carries blood group antigens and a GWAS study in 2015 indicated that fucosyltransferase 2 (FUT2) non-secretor status and blood group B are risk factors for CP[62]. Although other studies in ethnically mixed cohorts failed to replicate this association[63,64] with the exception of azathioprine-induced pancreatitis in IBD patients[65] it is still interesting to speculate that the observed effects may have been due to changes in CEL folding or trafficking.

THE DUCTAL PATHWAY OF GENETIC RISK IN CP

CFTR variants.

The cystic fibrosis transmembrane conductance regulator (CFTR) is a cyclic AMP-regulated chloride/bicarbonate channel localized to the apical plasma membrane of epithelial cells[66] (Figure 2). *CFTR* mutations disrupt channel activity or affect membrane levels and are associated with various phenotypes, ranging from asymptomatic state to multi-organ symptoms leading to the diagnosis of cystic fibrosis (CF) in homozygous carriers of severe mutations. Observations that heterozygous and compound heterozygous *CFTR* mutations are associated with CP were reported by two papers in 1998[67,68]. In the first analysis of the entire *CFTR* coding region, the frequency of abnormal *CFTR* alleles in CP patients was 18.6% in comparison to 9.2% in controls[69]. More recent, large cohort analyses corroborate

the pathogenic role of *CFTR* variants in CP although the effect and frequency of *CFTR* variants was less pronounced than reported previously[39,70,71]. Heterozygous carrier status of the severe p.F508del mutation confers a small risk for CP with an OR of 2.5, whereas the mild p.R117H mutation increases risk by about 4-fold. Compound heterozygous state for one severe and one mild *CFTR* allele represents strong risk for CP and may be considered causative[70]. The role of common polymorphic *CFTR* alleles (e.g. T5, TG12) and the non-CF-causing, so-called bicarbonate-defective *CFTR* variants in CP remains controversial as the preponderance of data does not support their association with CP[66]. Unlike *CFTR*, variants in the solute-linked carrier 26 member 6 anion transporter (*SLC26A6*) do not alter the genetic risk in CP[72].

CLDN2 variants.

GWAS studies of CP identified several SNPs in the *CLDN2-MORC4* locus to be associated with CP risk[17,19]. The OR was about 2 and the effect was more pronounced in alcoholic CP. Within this locus, *CLDN2* seems to be the clinically relevant risk gene, as it is expressed in pancreatic ducts at low levels as a tight junction protein. It was proposed that *CLDN2-MORC4* variants might cause CLDN2 mislocalization. Additional work is required to clarify the mechanism of action of this risk locus and to confirm whether assignment to the ductal pathway is appropriate (Figure 2).

CASR variants.

The calcium-sensing receptor (CASR) regulates calcium homeostasis through parathyroid hormone secretion and renal tubular calcium reabsorption. Functional CASR is also expressed in the pancreas, including ductal cells where CASR may respond to high calcium concentrations in the juice by increasing ductal fluid secretion, thereby preventing stone formation and pancreatitis[73] (Figure 2). A US population based study failed to demonstrate the previously anticipated association between *CASR* variants and the *SPINK1* p.N34S haplotype, but reported the p.R990G variant to increase CP risk, especially in subjects with moderate or heavy alcohol consumption[74]. More recently, a French study found overrepresentation of rare *CASR* coding variants in idiopathic CP and significant association of the p.A986S variant, but only in the homozygous state, with CP[75]. However, the previously reported association with the p.R990G variant was not observed in this cohort. Taken together, current evidence does not support a clear role for *CASR* variants in CP pathogenesis.

In summary, human genetic data indicate that premature activation or mifolding of pancreatic proteases play a central role in the onset of pancreatitis and progression to chronic pancreatitis (see suppl. table 1).

THE ROLE OF PROTEASES IN THE PATHOPHYSIOLOGY AND CELL BIOLOGY OF PANCREATITIS

While genetic evidence for the involvement of the protease/antiprotease balance in the pathogenesis of pancreatitis dates back only two decades and mainly focusses on chronic pancreatitis, pathophysiological and biochemical investigations have implicated this system

for over a century. Due to lack of adequate animal models and the inability to keep isolated pancreatic acinar cells in culture for long periods of time, experimental studies has focussed primarily on acute pancreatitis. The relative importance of the pathways discussed below might change with respect to etiology. It is our general understanding that these mechanisms are also relevant to chronic pancreatitis, although experimental evidence is mostly lacking.

Autodigestion by pancreatic proteases.

The pathophysiological concept of autodigestion was first developed by the Austrian pathologist Hans Chiari in Prague more than 120 years ago. He claimed that pancreatitis was caused and driven by the glands own digestive properties[76]. Ever since the pathomechanism of premature activation of pancreatic enzymes and its contribution to disease severity and progression has captured the attention of many pancreatologists. Bialek and colleagues first showed, that protease activation during pancreatitis begins in the exocrine pancreas[77] and Saluja et al. reported that it begins in a membrane-confined vesicular compartment and parallels acinar cell damage[78]. Although the fact that activation of digestive proteases is an early event during acute pancreatitis is widely accepted, the question where and through what mechanism this process is initiated and whether it plays a role in chronicity remains under debate.

Protease activation during pancreatitis – clues from mechanistic studies

Role of calcium for intracellular protease activation.—States of hypercalcemia such as primary hyperparathyroidism are a risk factor for the development of acute pancreatitis in humans and rats[79–81]. Intracellular calcium concentrations and compartmental distribution in acinar cells are tightly regulated as calcium serves as a second messenger for the physiological release of digestive enzymes in response to vagal nerve stimulation or humoral activation[82,83]. Upon intraperitoneal treatment of rats with supramaximal doses of caerulein, an analogue of cholecystokinin[84], a time-dependent disruption of the physiological oscillating intracellular calcium signal was observed in rat acini using the Ca^{2+}-sensitive dye fura-2[85]. Employing for the first time fluorescent trypsin substrates that allow the subcellular imaging localization and quantification of protease activation[86], Krüger et al. could demonstrate that a specific extended plateau release of calcium at the apical pole of acinar cells is necessary for premature trypsin activation to occur, which is different from the calcium oscillations required for enzyme secretion[87]. These findings where confirmed by others who also demonstrated that acetylcholine- or CCK-induced intracellular protease activation was associated with formation of cytoplasmic vacuoles in acinar cells that resemble those overserved in experimental pancreatitis *in vivo*[88] and that inhibition of calcium release also inhibits the formation of these vesicles[89]. Acinar cells can replace calcium for magnesium in its stores and when this is done not only the premature activation of proteases *in vitro* and *in vivo* but also the severity of pancreatitis is significantly reduced[90]. Two ongoing clinical trials (one in acute and one in chronic pancreatitis) have now been based on this observation. Orabi et al. took the concept of calcium- dependent protease activation further by showing that acute pancreatitis induced by supramaximal doses of the muscarinic agonist carbachol can be abrogated by inhibition of the intracellular calcium channel ryanodine receptor, which is located at the basolateral membrane of acinar cells. Therefore, the importance of spatial distribution of calcium to the apical pole might be

a specific feature of the caerulein model. Interestingly, carbachol-induced protease activation is more severe if cells are pre-treated with ethanol[91]. Calcium signaling may also play a role in other forms of experimental pancreatitis, like bile acid-induced pancreatitis[92] as well as pressure induced pancreatitis/experimental post-ERCP pancreatitis[93]. Experimental pancreatitis can be ameliorated by modulating calcium release from intracellular stores or influx through the plasma membrane via pharmacological inhibition of inositol-tri-phosphate-receptor (IP3R, predominantly types 2 and 3) signaling[94,95] or calcium release-activated calcium modulator 1 (ORAI1)[96,97]. The calcium-dependent protease activation also heavily depends on calcineurin, a calcium-activated phosphatase, and its downstream signaling via the transcription factor NFAT. Inhibition of calcineurin by inhibitors or in mice lacking calcineurin-subunits causes reduced intracellular protease activity in either secretagogue or bile acid induced pancreatitis, without affecting vesicular transport[98,99].

Mechanisms of protease activation.—Three major concepts have been investigated over the past decades: autoactivation, spatial redistribution and fusion of zymogen granules with other organelles and failure of protective mechanisms.

Autoactivation of trypsin.

There is strong evidence from human genetic studies, indicating that autoactivation of trypsinogen[100] causes chronic pancreatitis in affected humans (chapter 1.). In contrast, trypsinogen autoactivation is unimportant in experimental caerulein induced pancreatitis. It could be shown that inhibition of active trypsin by the reversible chemical inhibitor S124 could prevent trypsin activity in experimental caerulein-induced acute pancreatitis, but had no effect on the generation of the trypsin-activation-peptide (TAP) or active trypsin after wash-out of S124, thus indicating that hydrolysis of trypsinogen to trypsin appeared in a trypsin-independent fashion[101].

Activation by lysosomal proteases.

Inhibition of the lysosomal hydrolase cathepsin B by the cysteine protease inhibitor E-64d, leads to a significant decrease in trypsin activity and TAP-formation, indicating that the trypsin activation in response to caerulein depends on cathepsin B[101–103]. Similarly, in cathepsin B knock-out mice, the trypsin-activation is significantly inhibited after caerulein administration[104]. However, active cathepsin B (but inactive[105]) and trypsinogen under physiological conditions are not located in the same cell organelles (zymogen granules for exocytosis vs. lysosomes for degradation of content of endosomes and autophagosomes). In 1998 the group of Michael Steer showed in subcellular fractions of caerulein-treated acini a co-localization of cathepsin B and digestive enzymes, including trypsin and TAP, in heavy fractions[78] containing zymogen granules and lysosomes early in the disease course and later a shift of trypsin and cathepsin B activity to the cytosol[106]. This confirmed earlier findings from *in vivo* studies that indicated a fusion of zymogen containing vacuoles with lysosomes in secretagogue-, duct-obstruction- or diet-induced acute pancreatitis[107–112]. Intracellular activation of proteases other than trypsin, like chymotrypsin and carboxypeptidase B, also depend on non-physiological co-localization with other cell components, but are independent of cathepsin B[113]. A misorting in the exocrine machinery as well as secretion blockage and re-uptake of previously secreted proteases via endocytosis have been described

under experimental conditions. The fact that an acidic pH, as found in lysosomes, enhances secretagogue-dependent zymogen activation supports the fusion hypothesis[114]. A very recent study reports that CCK or ethanol treatment depletes acinar cells of syntaxin 2, a key regulator of apical exocytosis, thus leading to increased basolateral exocytosis and formation of autolysosomes mediated by syntaxin 3 and 4, in which trypsinogen activation takes place[115]. Inhibition of syntaxin-4-mediated basolateral exocytosis in experimental pancreatitis decreases disease severity[116]. Another concept introduces endocytic vacuoles (EVs) as the site of intracellular trypsinogen activation. These occur under physiological and pathophysiological conditions following compound exocytosis of zymogen granules[117]. EV formation is calcium dependent[118]. Their content is acidic and calcium rich and after supramaximal CCK- or taurocholate-stimulation, trypsin activity within these post-exocytic structures can be visualized using fluorescent dyes[119]. During pancreatitis, EVs are larger than normal; tend to fuse with the plasma membrane or even rupture, discharging active trypsin into the cytosol or extracellular space. This instability is thought to be caused by disruption of otherwise protective actin filaments surrounding the EV[120]. Rupture of EVs is however, independent of trypsin or cathepsin B activity. Secretory blockage may contribute to these events. In experimental pancreatitis vesicle-associated membrane protein-8 (VAMP-8) mediated secretion is impaired, due to a loss of early endosomal proteins, resulting in retention of trypsinogen and transformation to active trypsin in a cathepsin-dependent manner. Knock-out of VAMP-8 protects from pancreatitis and restoration of early endosomal trafficking decreases severity of pancreatitis[121,122]. Vesicular trafficking is regulated in a calcium dependent manner[123,124].

Loss of trypsin-inhibitors.

Little is known about the role of failing protective mechanisms for protease activation in the early phase of pancreatitis. The most potent cellular trypsin-inhibitor is the before mentioned SPINK1. Although SPINK1 mutations are among the most common genetic risk-factors for the development of recurrent acute and chronic pancreatitis, so far none of the described mutations seemed to impair the SPINK-function, and therefore do not explain the increased risk for pancreatitis (see chapter 1). In mice, carrying a heterozygous SPINK3-deletion, a significant reduction of functional SPINK does not lead to development of spontaneous pancreatitis or more severe disease after supramaximal caerulein administration when compared to wild type controls[125]. The fact that mice with a homozygous SPINK3-deletion suffer from pancreatic atrophy and that this phenotype can be rescued by transgenic expression of rat PSTI-1[125], points towards a role of trypsin-inhibitors during pancreatic development, but does not explain its role in pancreatitis.

In conclusion, the current cumulative evidence suggests a cathepsin B-dependent mechanism of protease activation in experimental pancreatitis.

Cell death cause or consequence of protease activation?

Premature intracellular protease activation in acinar cells leads to cell injury. The type of cell death, be it necrosis, apoptosis, autophagy, necroptosis or pyroptosis determines disease severity[126]. Necrosis is understood as an unregulated response to damage. In animal models of acute pancreatitis approximately 1 to 5% of acinar cells undergo apoptosis and severity of

pancreatitis is inversely correlated to the rate of apoptosis[127]. Macroautophagy is a multistep, lysosomal driven, adaptive process by which cells degrade cytoplasmic organelles and long-lived protein[128]. Pancreatitis presents with impaired autophagic flux evident by vacuole accumulation[129]. Currently it is debated, whether impaired autophagy stimulates cell death through accumulation of damaged mitochondria mediating an inflammatory response via a ROS-dependent mechanism as in LAMP2[130] or Atg7-knock-out animals[131] or whether autophagy prevents an inflammatory response as in Atg5 knock-out mice[132,133]. The regulated process of necrosis is termed necroptosis and triggered by TNF, TRAIL, FasL, type 1 interferon and TLRs all of which are released in the early phase of acute pancreatitis in response to protease activation[134]. RIP-1 (receptor-interacting protein) and RIP-3 form a phosphorylated complex the necrosome and phosphorylate MLKL (mixed lineage kinase domain-like) resulting in membrane rupture. 40% of cells undergo necroptosis in pancreatitis and RIP3 deletion or treatment with necrostatin ameliorates pancreatitis[135,136]. Necroptosis releases DAMPs and those will activate the NLRP3 pathway resulting in pyroptosis[137]. Pyroptosis is an innate immune sensing mechanism with poorly understood upstream signaling. Nevertheless, some interesting inhibitors are known such as lactate, beta-hydroxybutyrate and aspartate. The term pyroptosis describes activation of the inflammasome via NLRP3. The inflammasome is a macroscopic cytosolic protein complex which proteolytically cleaves IL1β pro-IL18 and releases HMGB1. NLRP3 activation requires lysosomal rupture and cathepsin release, calcium influx and mitochondria derived ROS production and thus is closely linked to pancreatitis[138,139]. However, NLRP3 expression is restricted to innate immune cells[140].

Cell death pathways in pancreatitis intersect. Caspase 3 activation cannot only induce apoptosis but pyroptosis. Necroptosis can shift to pyroptosis via caspase 8 activation and necroptosis activates pyroptosis[141]. As of today, we have not understood why some of our patients deteriorate and develop a necrotizing course of pancreatitis. A shift of regulated cell death from apoptosis to pyroptosis as well as stimulation of necroptosis might explain this observation. Inhibition of pyroptosis e.g. by using Ringer's lactate for volume resuscitation[142] or inhibition of necroptosis by necrostatin[143] might well be a way forward in the treatment of pancreatitis.

An interesting question is whether cell death is a result of premature protease activation or a consequence of inflammation. The answer is ambiguous: Saluja and co-workers show a direct effect on lysosomal stability mediated by active trypsin and lysosomal rupture leads to the release of cathepsins into the cytosol causing dose dependently apoptosis or necrosis[106]. Our own group showed that inhibition of protease activation, especially trypsin by specific inhibitors results in a decreased rate of apoptosis, but did not affect necrosis[144]. Thus, cell death is a result of intracellular protease activation, but this has only been shown for isolated acinar cells mimicking the early phase of pancreatitis. Taking into account pyroptosis and necroptosis inflammation is the origin and consequence of cell death in pancreatitis (figure 3).

Protease activity and disease severity

This raises the question whether intracellular protease activation of trypsin as it is linked to cell death can also mediate systemic disease severity. The notion that trypsin activation is linked to disease severity is supported by the correlation of TAP urine levels to severity in patients with acute pancreatitis[145]. However, several studies question the role of trypsin for severity of pancreatitis. Expression of mutant human trypsin bearing the hereditary pancreatitis mutation R122H in mice leads to slightly more severe caerulein pancreatitis[146], but, when compared to mice expressing normal human trypsinogen, there is no increase in disease severity. Moreover, the effect seems to be not solely dependent on trypsin, since mice with human trypsin mutations (R122H or N29I) show lower trypsin activity after caerulein hyperstimulation. This might in part be explained by a higher rate of acinar cell apoptosis even in untreated animals transgenic for human trypsinogen[147]. A similar effect was seen in the PACE-tryp(on) mice, which conditionally express an endogenously activated trypsinogen within pancreatic acinar cells. Those mice will develop acute pancreatitis in a trypsin activity dependent way, which can lead to organ dysfunction and mortality, but they also show a pronounced caspase-3 activation with consecutive apoptotic loss of acinar cells and replacement by fatty tissue[148]. Similarly, the group of Bar-Sagi described a transgenic mouse, where the human mutation R122H was inserted in the murine PRSS1 gene. Those mice develop spontaneous pancreatitis and show a more pronounced inflammatory infiltrate as well as cellular damage in response to caerulein[149]. The fact that the predominant way of cell death determines the overall severity of experimental pancreatitis in mice has been demonstrated in animals deleted for cathepsin L. Cathepsin L degrades trypsinogen into an inactive elongated TAP, but cathepsin L deficiency leads to a milder form a of caerulein induced pancreatitis, which is linked to a shift from necrosis to apoptosis[6].

The most convincing data, questioning the role of trypsin for disease severity was generated by using a mouse strain lacking trypsinogen 7 (T7 ko), the murine counterpart of human cationic trypsinogen. Dawra et al showed that a significant reduction in trypsin and chymotrypsin activity after caerulein hyperstimulation in these mice had no effect on disease severity in terms of cell death, local or systemic inflammation in an acute and chronic pancreatitis model, which they claim is mediated by a NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) dependent mechanism[150,151]. This confirms previous findings generated in the PACE-tryp(on) model, where NFκB -activation was independent of increased intracellular trypsin activity *in vitro*[152].

THE ROLE OF SYSTEMIC INFLAMMATION IN PANCREATITIS

NFκB activation - initial step of inflammation.

The activation of NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) is an early event during pancreatitis and occurs within the first minutes after onset of the disease[153,154]. One main function of NFκB is the transcriptional regulation of the immune response[155]. The principle pathway of NFκB signal transduction is depicted in figure 4. The fact that NFκB is already present in the cytoplasm explains its rapid activation after induction of pancreatitis[153,154].

Intraacinar protease activation and NFκB activation are early cellular events during pancreatitis[153,154], which have been suggested to occur independent from each other[151], but follow a similar kinetic. Trypsinogen activation depends on intracellular Ca^{2+} signaling[87] and NFκB activation can also be induced by protein kinase C and Ca^{2+}, which could be the reason for parallel kinetics[156]. The deletion of T7 trypsinogen or cathepsin B, which both result in greatly reduced protease activation[104,144,151], do not influence NFκB activation during caerulein-induced pancreatitis[151]. This can be regarded as evidence that protease activation does not directly lead to NFκB activation in acinar cells while NFκB activation can still interact with protease activity: the transcriptional regulation of Spi2A (serine protease inhibitor 2a) is mediated by NFκB and is able to inhibit trypsin activity in a mouse model of acute pancreatitis (figure 4)[157]. These results suggest a more complex role of NFκB during disease progression which is not restricted to pro-inflammation[158]. Surprisingly, the pancreas-specific deletion of Rel-A(p65) resulted in increased systemic inflammation and pancreatic necrosis[159]. In contrast, the pancreas-specific deletion of IκBα results in nuclear translocation of Rel-A and ameliorated pancreatitis[157]. The same phenotype could be observed in MyD88 deficient mice, which develop a more severe disease phenotype compared to controls[160]. MyD88 is a central adaptor for the TLR/IL1-receptor signaling pathway which induces NFκB activation. These results ultimately suggest a protective role of NFκB activation in pancreatic acinar cells. Other studies attributed a more critical role to NFκB regarding disease severity. Mice which constitutively over-express active IKKβ under a pancreas-specific promoter, showed chronic infiltration of immune cells, associated with an increased disease severity after caerulein stimulation[161,162]. However, the infiltration of immune cells alone, or the constitutive activity of NFκB in acinar cells, did not result in acute pancreatitis, organ damage and necrotic or apoptotic cell death[162]; an additional pathophysiological stimulus was still needed. The increased severity of pancreatitis in IKKβ over-expressing mice can be explained by the presence of leukocytes within the pancreas, which become rapidly activated after disease induction and do not need to infiltrate the pancreas. The pancreas-specific deletion of IKKα, another IκBα phosphorylating kinase, caused spontaneous pancreatitis in mice[163], but this process appeared to be independent of NFκB. IKKα also regulates autophagic flux which is essential for pancreas homeostasis[164]. These data demonstrate the complexity of the NFκB network which often hampers the interpretation of results. Taken together the constitutive activation of NFκB leads to a chronic infiltration of immune cells, but pancreatitis only develops after induction by an external stimulus[161,162]. The presence of immune cells within the pancreas is required but insufficient for pancreatitis to develop and these cells need to be activated in order to contribute to disease severity. On the other hand, data from pancreas-specific RelA deleted mice show that NFκB activation in acinar cells is not essential for the recruitment of immune cells to the side of damage[159]. Quite to the contrary, the absence of p65 in acinar cells result in greater disease severity. While all the mentioned studies investigated the role of NFκB in acinar cells NFκB activation may play a much greater role in infiltrating immune cells, which directly regulate the immune response.

Another master switch in the transcriptional machinery of acinar cells is AP1 (activator protein 1). AP1 is implicated in multiple transcriptional networks within the acinar cells regulating pancreatic development, differentiation, cell death and inflammation. Mice

heterozygous for the orphan nuclear receptor NR5A2 develop an acinar-cell-autonomous AP1 dependent pre-inflammatory state, which, on a transcriptome level, mimics that of early acute pancreatitis[165].

It seems however, that the extent of NFκB and AP1 activity greatly differs with respect to the cause of pancreatitis. In caerulein models, an activation of both transcription factors is described and submaximal CCK stimulation induces acinar cell dedifferentiation and proliferation via the MAPK/c-Jun/AP-1 pathway probably as part of a pancreatic regeneration program[166,167]. In contrast, the metabolites occurring in ethanol-induced experimental pancreatitis can positively and negatively regulate NFκB and AP1 depending on the predominance of oxidative or non-oxidative alcohol metabolism in the pancreas[168].

The role of infiltrating immune cells.

The infiltration of immune cells start within minutes after the onset of disease and plays a crucial role for the severity and prognosis of pancreatitis[169–172]. Cells of the innate immune system like neutrophil granulocytes and monocytes/macrophages represent the majority of infiltrating cells. NFκB plays a crucial role for the activation of leukocytes and is a central mediator of the innate and adaptive immune system[173]. Pancreatitis is primarily a sterile inflammation, so pathogen-associated molecular patterns (PAMPs) play no role in the activation of immune cells during the early phase of disease. In pancreatitis, the activation of immune cells is thus mediated by cytokines or damage-associated molecular patterns (DAMPs) that arise from acinar cell necrosis. Acinar cells release various cytokines and chemokines in response to CCK stimulation and NFκB activation such as TNFα[174], IL6 or MCP-1[175]. DAMPs and cytokines result in the nuclear translocation of p65/p50 within infiltrating immune cells, which enhances the cytokine storm via the secretion of pro-inflammatory mediators[175] (figure 5). DAMPs can act in the same way as PAMPs via Toll-like receptors (TLRs)[176], or specific receptors like P2RX7 which uses extracellular ATP as a ligand. Acinar cells, which undergo necrotic cell death, release a multitude of different DAMPs, like free DNA[177], histones or free ATP[138] which can act as immune activators. Finally, activated immune cells increase pancreatic damage and contribute to systemic inflammation[169,175,178]. Several studies have focused on different populations of immune cells and their role during acute pancreatitis.

Neutrophil granulocytes are often used as reference marker for pancreatic inflammation via measurements of myeloperoxidase (MPO) activity in tissue and reflects the amount of infiltrating neutrophils. One major function of neutrophils is removing pathogens by the release of proteases, antimicrobial peptides and reactive oxygen species (ROS). During pancreatitis neutrophils are the major source of ROS production, they can induce oxidative damage on acinar cells and enhance trypsinogen activation[178]. The release of proteases like PMN-elastase contributes to tissue destruction and acinar cell dissociation[179]. These data indicate that neutrophils have a direct effect on disease severity. This was confirmed by the depletion of neutrophils using anti-neutrophils serum, which resulted in reduced pancreatic damage and protease activation[169,178]. Recent studies investigated the role of neutrophil extracellular trap formation (NETs) in the context of pancreatitis. NETs are extracellular networks consisting of neutrophil DNA and used to bind pathogens[180]. This suicide

mechanism of neutrophils is a last line of defense against bacterial infections. NET formation is induced via TLR-4 activation, and the activation of NADPH-oxidase which lead to the oxidation of peptidylarginine-deiminase-4 (PAD4)[181,182]. TLR4 is not only responsible for the detection of pathogens but also DAMPs can activate the TLR-signaling pathway[176]. Merza *et.al.* could show that NET formation enhances the immune response during severe acute pancreatitis and is accountable for trypsinogen activation[183]. Treatment with DNAses prevents NET formation and reduces disease severity[183]. Beside bacterial infections, also crystals can induce NET formation[184]. Another group has shown that NET formation is a critical step in bile stone development and plays an important role for ductal obstruction contributing to onset and severity of pancreatitis[185,186]. Neutrophil infiltration during acute pancreatitis is an unspecific reaction of the immune system, which enhances local damage by formation of NETs and the release of ROS, or activates digestive enzymes.

Monocytes/macrophages belong to the cells of the innate immune system. In contrast to neutrophil granulocytes macrophages are characterized by a high plasticity. Classical activated macrophages (M1) act in a pro-inflammatory manner and secrete high amounts of IL6, TNFα, IL12 and IL1β, an increased expression of inducible nitric oxide synthases (iNOS) results in the release of NO[187]. Alternatively activated macrophages (M2) are associated with wound healing, tissue regeneration and fibrogenesis and act in an anti-inflammatory manner via the release of IL10 or TGFβ. They are characterized by reduced iNOS- and increased Arginase-1 (Arg1) expression[187]. Macrophages are phagocytosing cells which remove tissue debris, necrotic and apoptotic cells. During acute pancreatitis, macrophage infiltration correlates to a greater extent with pancreatic damage and necrosis than the number of infiltrating neutrophils[175]. The reason for that is that macrophages are required for the removal of necrosis and thus mitigate pancreatic damage. Phagocytosing macrophages could be observed in different models of acute and chronic pancreatitis[130,164,169,175]. In contrast to apoptosis, necrosis is a pro-inflammatory cell death because it entails the release of multiple DAMPs which induce an M1 polarization of macrophages[188]. Therefore acinar cell apoptosis is suggested to be protective against hyperinflammation and decreases disease severity[189]. M1 macrophages release high amounts of TNFα which have a direct effect on pancreatic acinar cells[174]. Two independent groups could show that TNFα secreted from infiltrating monocytes is responsible for pancreatic damage and digestive protease activation[169,190]. Depletion of macrophages by clodronate containing liposomes decreases disease severity and protected mice from caerulein-induced pancreatitis[169,191]. TNFα acts on cells via cell death receptors and is necessary to induce necroptotic cell death via the RIP1/RIP3 pathway[135], which has been suggested to be the major cell death pathway during acute pancreatitis[136]. Therefore infiltrating macrophages are responsible for induction of necroptosis as well as for the clearance of necrotic areas within the damaged pancreas[175].

Besides TNFα, macrophages also produce high amounts of IL1β a pro-inflammatory cytokine associated with the acute disease phase. In contrast to other cytokines IL1β needs to be processed. During maturation pro-IL1β and pro-IL18 undergo activation by the caspase-1/inflammasome complex and are released by the gasdermin D pore complex from the cytosol to the extracellular space[192]. In consequence to this process the cell undergoes

pyroptotic cell death[192,193]. During pancreatitis the activation of the inflammasome complex and the release of IL1β contribute critically to disease severity[138,139,175,177]. Inflammasome complex formation and pyroptotic cell death is mainly known from macrophages and not present in acinar cells[175]. Inflammasome activation is a complex mechanism requiring two signals for activation: the first signal induces the transcriptional up-regulation of inflammasome components by NFκB and the second signal induces the oligomerization of the inflammasome complex and the activation of pro-caspase-1. Major inducer of the inflammasome pathway is the TLR/MyD88 cascade. The second signal, which is necessary for inflammasome complex formation, can be a high potassium influx, TNFα, or the release of cathepsins from phagosomes into the cytosol[194]. Phagocytosis of zymogens by macrophages results in a co-localization of trypsinogen and cathepsin B in non-acinar cell phagolysosomes and results activation of trypsinogen[175] and macrophage activation. The rupture of these trypsin and cathepsin B containing vesicles leads to a cytosolic redistribution of cathepsins which acts as a second signal of inflammasome activation and indirectly links trypsinogen activation to the NFκB pathway via IL1β release. IL1β acts as activator for other immune cells, the IL1 receptor being directly linked to the MyD88 NFκB pathway. The importance of IL1β during pancreatitis could nicely be shown by a transgenic mouse model of pancreas-specific, IL1β over-expressing mice which develop chronic pancreatitis including complete loss of pancreatic function within weeks after birth[195]. Therefore M1 macrophages contribute prominently to the systemic immune response syndrome (SIRS) which is associated with multi-organ dysfunction syndrome (MODS) and increased mortality[170]. In contrast to M1 macrophages, the M2 phenotype is associated with organ regeneration and fibrosis development[196–198].

In conclusion, early protease activation as well as NFκB activation are essential characteristics of pancreatitis, both events occur in parallel during disease manifestation and strongly influence each other. Recent prove that not only the activation of proteases and NFκB play a critical role, but also the type of cell, in which their activation takes place is of importance. Pancreatitis is not a disease of acinar cells alone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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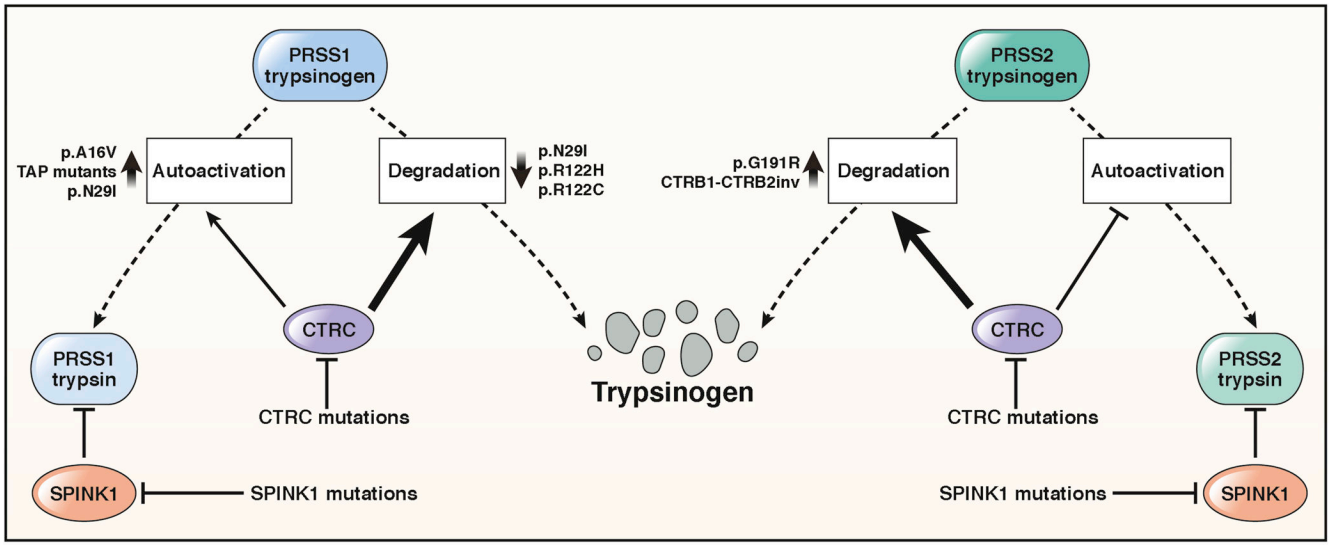

Figure 1.

Genetic risk factors associated with the trypsin-dependent pathological pathway. See text for details.

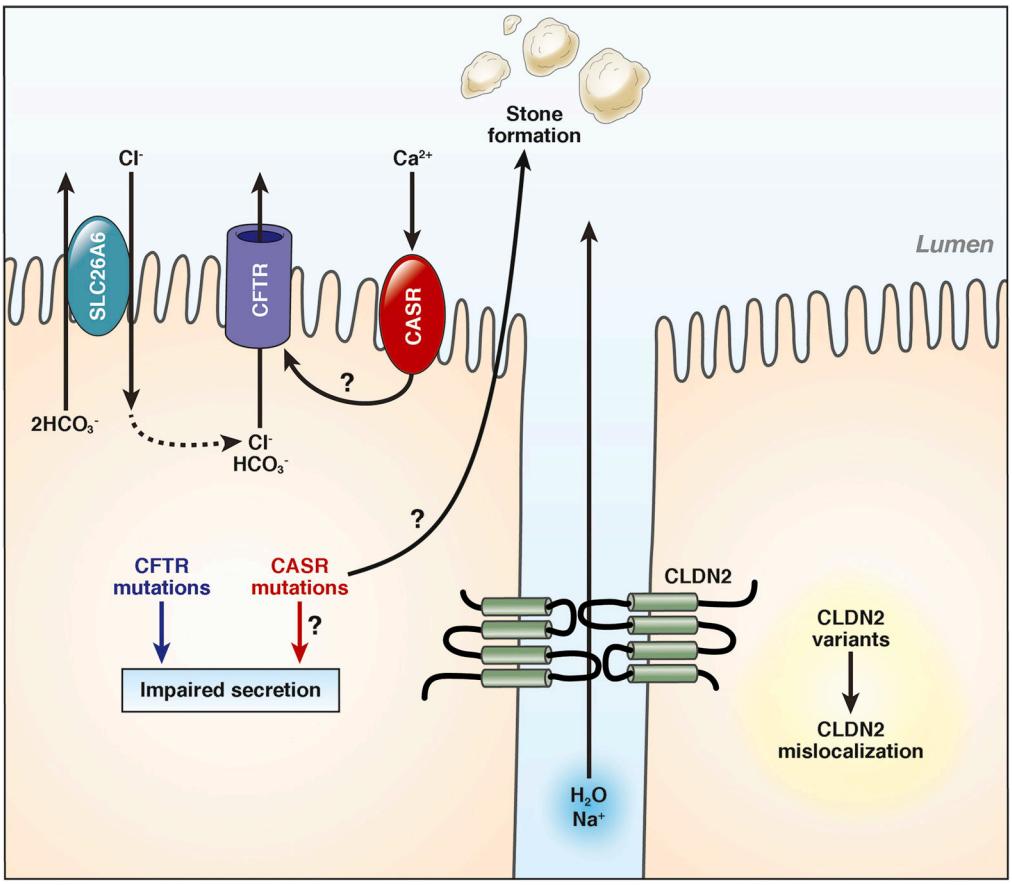

Figure 2.
Genetic risk factors associated with the ductal pathological pathway. See text for details.

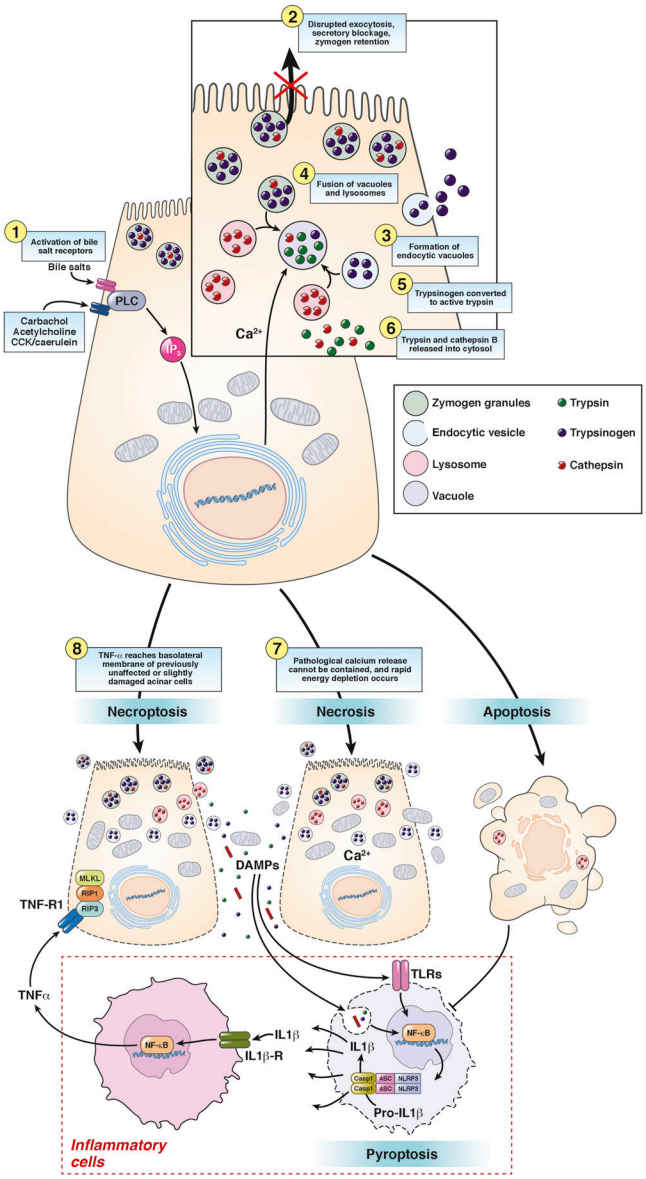

Figure 3.

Trypsinogen activation and cell death in pancreatic acinar cells. Intracellular trypsinogen activation is an early event at the onset of acute experimental pancreatitis. Supramaximal secretagogue-receptor stimulation or activation of bile salt receptors leads to an un-physiological peak-plateau calcium signal. This results in disrupted exocytosis of zymogen granules, secretory blockage, zymogen retention and formation of endocytic vacuoles, which contain trypsin and trypsinogen, taken up from the extracellular space. Those vacuoles co-localize and fuse with lysosomes containing cathepsin B, which in turn transforms trypsinogen into active trypsin. Due to increasing instability endocytic vacuoles often rupture, releasing trypsin and cathepsin B into the cytosol. Active trypsin is thought to induce mainly apoptosis, a silent form of cell death, which suppresses inflammation. In contrast, if the pathological calcium release cannot be contained, rapid energy depletion occurs and cells undergo necrosis during which the plasma membrane becomes leaky and

cellular components e.g. DNA or mitochondria reach the extracellular space. Those will be recognized by leukocytes, which will be activated via the inflammasome signaling pathway. IL1β- and TNFα-release as well as pyroptosis occur. If TNFα reaches the basolateral membrane of previously unaffected or slightly damaged acinar cells it can induce another form of programmed cell death, called necroptosis.

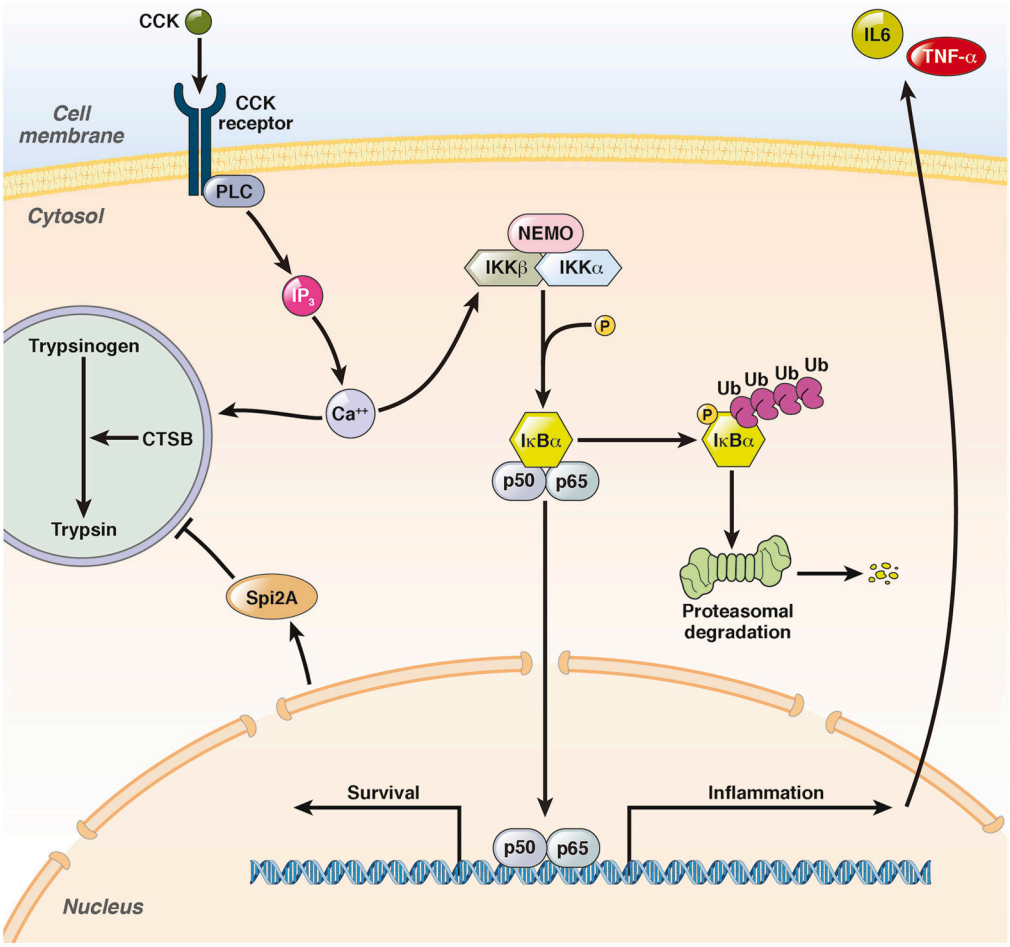

Figure 4:

NFκB pathway in pancreatic acinar cells. The early activation of NFκB follows the same time pattern as trypsinogen activation. Both are induced by cytoplasmatic Ca^{2+} influx, but NFκB did not depend on trypsinogen activation. The phosphorylation of IκBα, followed by proteasomal degradation and the nuclear translocation of NFκB (p65/p50) occurs in parallel to protease activation. NFκB as transcription factor acts in two directions; first the transcriptions of pro-inflammatory genes like IL6 or TNFα to initiate the immune response and second the transcription of pro-survival genes. Therefore NFκB can directly influence protease activity to protect cells by the up-regulation of Spi2A, a serine protease inhibitor.

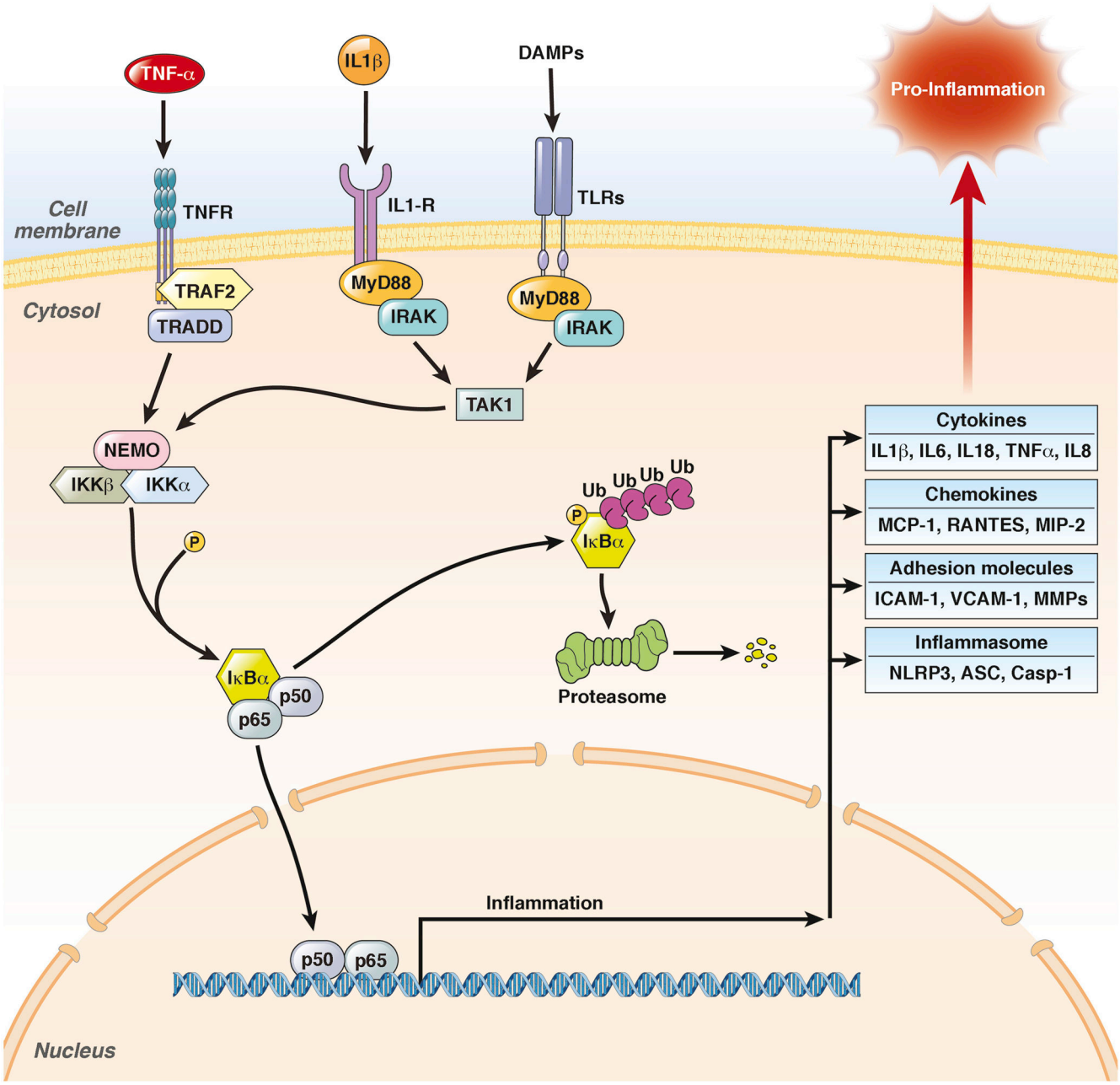

Figure 5:

NFκB activation in inflammatory cells. There are multiple pathways how NFκB could be activated within leukocytes; here are the major pathways which play a role during acute pancreatitis. Cytokines like IL1β or TNFα as well as DAMP signals acting via Toll-like receptors can induce the translocation of p65/p50 into the nucleus. In leukocytes the majority of inflammatory mediators are under the control of NFκB: cytokines, chemokines, adhesion molecules and components of the inflammasome pathway. Leukocyte mediated NFκB activation enhances the immune response in a very prominent manner and therefore has a different role in pancreatitis compared to NFκB activation within acinar cell.