Genetics, Cell Biology, and Pathophysiology of Pancreatitis

Since the discovery of the first trypsinogen mutation in families with hereditary pancreatitis, 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 have provided compelling evidence that premature intrapancreatic activation of digestive proteases is critical in pancreatitis onset. However, disease course and severity are mostly governed by inflammatory cells that drive local and systemic immune responses. In this article, 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.

Pancreatitis is the leading cause for gastrointestinal disease-related hospital admissions and it is associated with considerable morbidity, mortality, and socioeconomic burden. Recent years have 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.

Genetics of Pancreatitis

Acute pancreatitis (AP), recurrent AP (RAP), and chronic pancreatitis (CP) form a disease continuum. 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, whereas genetic studies in AP are difficult to interpret in the absence of adequate follow-up that can exclude RAP and CP cases.

Most pancreatitis risk genes code for digestive proteases, a trypsin inhibitor, or other proteins highly expressed in the pancreas. Functional studies have classified the various mutations and other genetic alterations into pathologic pathways driving pancreatitis onset and progression. We discuss the trypsin-dependent, mis-folding-dependent, and ductal pathways of pancreatitis risk.

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 enteroproteidase in the duodenum. Trypsin activates chymotrypsinogens, proelastases, and procarboxypeptidase B1 (CPB1), whereas activation of procarboxypeptidases A1 (CPA1) and A2 (CPA2) requires the concerted action of trypsin and chymotrypsin C (CTRC). Trypsinogen also can be activated by trypsin, and this process is called autoactivation. Premature, intrapancreatic activation of trypsinogen can occur by autoactivation or can 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.\(^8\)–\(^9\) (Figure 1). As discussed below, certain trypsinogen mutations can hijack this mechanism and thereby stimulate trypsinogen activation to a pathologic 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.\(^10\)

**Cationic Trypsinogen Mutations**

Mutations in human cationic trypsinogen (PRSS1) cause autosomal dominant hereditary pancreatitis (HP) with incomplete penetrance or act as risk factors in sporadic CP.\(^11\) Approximately 90% of PRSS1-mutation-positive HP families carry the p.N34S, 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.\(^9\) Pathologic trypsin levels generated by mutation p.A16V are lower than those seen with the p.R122H variant, which explains the decreased 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 have indicated that these activation peptide mutants are secreted poorly because of 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 decreasing CTRC-dependent trypsinogen degradation, increasing CTRC-mediated processing of the activation peptide, or directly stimulating autoactivation. Genomewide association studies (GWASs) have identified a commonly occurring haplotype in the PRSS1 and anionic trypsinogen (PRSS2) locus that slightly decreases CP risk (odds ratio [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 decreases 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 patients with CP and 1% in controls with an average OR of 11, making the p.N34S the clinically most significant risk factor for CP.\(^22\) When considering European populations only, p.N34S increased CP risk by approximately 10-fold.\(^23\) Although several studies have 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 4 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, decreased expression of the mutant allele was observed compared with the wild-type allele.\(^28\) The investigators suggested that the c.–4141G>T variant or a hitherto unknown variant located in the 5’ region of the gene might be responsible for the decreased 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 have shown 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.\(^32\)\(^,\)\(^33\) 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 PRSS2 Variant**

Although PRSS1 and PRSS2 share 90% identity at the amino acid level and PRSS2 rapidly auto-activates, no pathogenic PRSS2 variants have been identified in HP or sporadic CP.\(^34\)\(^,\)\(^35\) The absence of PRSS2 mutations in CP could be due to the more effective CTRC-mediated degradation of anionic trypsinogen, which would prevent intrapancreatic activation of the enzyme even if it were mutated.\(^36\) However, a protective variant p.G191R with a ~3- to 6-fold effect and approximately 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 showed 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 from mis-folding, 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 partly decreased activity.\(^42\)\(^,\)\(^43\) Subsequent studies reported a frequent p.G60= variant found in approximately 30% of patients with CP.\(^42\)\(^–\)\(^45\) The heterozygous p.G60= increases
the risk of CP by 2.5-fold, whereas the homozygous state increases the risk by 10-fold.\textsuperscript{43,45} The variant is associated with decreased $CTRC$ mRNA expression (GTEx Portal), possibly because of altered pre-mRNA splicing.

**CTRB1–CTRB2 Locus Inversion**

A recent European GWAS identified a large inversion at the $CTRB1$–$CTRB2$ locus that modestly (OR 1.35) modifies the risk for alcoholic and nonalcoholic CP.\textsuperscript{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 decreased. 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.\textsuperscript{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 cerulein.\textsuperscript{47} These observations provided the first in vivo proof for the protective role of chymotrypsin-mediated trypsinogen degradation against pancreatitis.

**Mis-folding–Dependent Pathway of Genetic Risk in CP**

More recently, an alternative pathomechanism seemingly unrelated to premature intrapancreatic trypsinogen activation has been identified, in which mutation-induced mis-folding and consequent endoplasmic reticulum (ER) stress lead to acinar cell damage and pancreatitis.\textsuperscript{48}

**Mis-folding–Associated PRSS1 Mutations**

In 2009, a subset of $PRSS1$ variants was found to cause decreased secretion, intracellular retention, and increased ER stress markers, as judged by in vitro cell culture experiments.\textsuperscript{49} These $PRSS1$ mutations occur rarely and are mostly associated with sporadic disease (eg, p.C139F, p.C139S, p.G208A), but also have been found in HP families with incomplete penetrance (p.L104P, p.R116C).\textsuperscript{48} Variant p.G208A is prevalent in East Asia (4% of CP cases) and was detected in Europe only in a single case thus far.\textsuperscript{50,51}

**Mis-folding–Associated CPA1 Mutations**

A candidate gene study in 2013 reported that mutations in the $CPA1$ gene are associated with CP (OR ~25), especially with early-onset disease (OR ~80).\textsuperscript{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 2 HP families.\textsuperscript{53} Pathogenic $CPA1$ variants cause proenzyme mis-folding, resulting in a secretion defect, intracellular retention, and ER stress.\textsuperscript{52,53} In contrast to $CPA1$, variants of $CPB1$ and $CPA2$ are not associated with CP.\textsuperscript{54} Interestingly, ER stress-inducing $CPA1$ and $CPB1$ variants were over-represented in patients with pancreatic cancer without a clinical history of RAP or CP.\textsuperscript{55} Most of these variants caused premature truncation and did not overlap with those found in CP. A mouse model for the mis-folding–dependent pathway was described recently. This study showed 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.\textsuperscript{56}

**Mis-folding–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).\textsuperscript{57} The deletions alter the reading frame of the C-terminal variable number tandem repeat sequence, resulting in CEL proteins with unnatural extensions that are prone to aggregation.\textsuperscript{58,59} The exocrine dysfunction in MODY8 is in all likelihood caused by mis-folding–induced ER stress and consequent acinar cell loss. A hybrid $CEL$ allele ($CEL$-HYB1) formed between $CEL$
Ductal Pathway of Genetic Risk in CP

**CFTR Variants**

The cystic fibrosis transmembrane conductance regulator (CFTR) is a cyclic adenosine monophosphate–regulated chloride–bicarbonate channel localized to the apical plasma membrane of epithelial cells (Figure 2). CFTR mutations disrupt channel activity or affect membrane levels and are associated with various phenotypes, ranging from asymptomatic state to multiorgan symptoms leading to the diagnosis of cystic fibrosis in homozygous carriers of severe mutations. Observations that heterozygous and compound heterozygous CFTR mutations are associated with CP were reported by 2 studies in 1998. In the first analysis of the entire CFTR coding region, the frequency of abnormal CFTR alleles in patients with CP was 18.6% compared with 9.2% in controls. More recent large cohort analyses have corroborated the pathogenic role of CFTR variants in CP, although the effect and frequency of CFTR variants was less pronounced than reported previously. Heterozygous carrier status of the severe p.F508del mutation confers a small risk for CP (OR 2.5), whereas the mild p.R117H mutation increases risk by approximately 4-fold. Compound heterozygous state for 1 severe and 1 mild CFTR allele represents strong risk for CP and can be considered causative. The role of common polymorphic CFTR alleles (eg, T5, TG12) and the non-cystic fibrosis–causing, so-called bicarbonate-defective CFTR variants in CP remains controversial because the preponderance of data does not support their association with CP. Unlike CFTR, variants in the solute-linked carrier 26 member 6 anion transporter (SLC26A6) do not alter the genetic risk in CP.

**CLDN2 Variants**

GWASs of CP identified several single-nucleotide peptides in the claudin 2 (CLDN2) and MORC4 locus to be...
associated with CP risk. The OR was approximately 2 and the effect was more pronounced in alcoholic CP. Within this locus, CLDN2 seems to be the clinically relevant risk gene, because it is expressed in pancreatic ducts at low levels as a tight junction protein. It was proposed that CLDN2–MORC4 variants might cause CLDN2 mis-localization. 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 also is expressed in the pancreas, including ductal cells where CASR can respond to high calcium concentrations in the juice by increasing ductal fluid secretion, thereby preventing stone formation and pancreatitis (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 increased CP risk, especially in subjects with moderate or heavy alcohol consumption. More recently, a French study found over-representation of rare CASR coding variants in idiopathic CP and a significant association of the p.A986S variant, but only in the homozygous state, with CP. 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 mis-folding of pancreatic proteases play a central role in the onset of pancreatitis and progression to CP (Supplementary Table 1).

Role of Proteases in Pathophysiology and Cell Biology of Pancreatitis

Although genetic evidence for the involvement of the protease–antiprotease balance in the pathogenesis of pancreatitis dates back only 2 decades and focuses mainly on CP, pathophysiologic and biochemical investigations have implicated this system for more a century. Because of the lack of adequate animal models and the inability to keep isolated pancreatic acinar cells in culture for long periods, experimental studies have focused primarily on AP. The relative importance of the pathways discussed below might change with respect to etiology. It is our general understanding that these mechanisms also are relevant to CP, although experimental evidence is mostly lacking.

Autodigestion by Pancreatic Proteases

The pathophysiologic 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’ digestive properties. Since then, the pathomechanism of premature activation of pancreatic enzymes and its contribution to disease severity and progression have captured the attention of many pancreatologists. Bialek et al first reported that protease activation during pancreatitis begins in the exocrine pancreas and Hofbauer et al reported that it begins in a membrane-confined vesicular compartment and parallels acinar cell damage. Although the fact that activation of digestive proteases is an early event during AP is widely accepted, the question of where and through what mechanism this process is initiated and whether it plays a role in chronicity remain 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 AP in humans and rats. Intracellular calcium concentrations and compartmental distribution in acinar cells are tightly regulated because calcium serves as a second messenger for the physiologic release of digestive enzymes in response to vagal nerve stimulation or humoral activation. After intraperitoneal treatment of rats with supramaximal doses of cerulein, an analogue of cholecystokinin, a time-dependent disruption of the physiologic oscillating intracellular calcium signal was observed in rat acini using the Ca**2+-sensitive dye fura-2. When using for the first time fluorescent trypsin substrates that allow the subcellular imaging, localization, and quantification of protease activation, Krüger et al found 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. These findings were confirmed by others who also found that acetylcholine- or cholecystokinin (CCK)-induced intracellular protease activation was associated with the formation of cytoplasmic vacuoles in acinar cells that resemble those overserved in experimental pancreatitis in vivo and that inhibition of calcium release also inhibits the formation of these vesicles. 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 decreased. Two ongoing clinical trials (1 in AP and 1 in CP) are based on this observation. Orabi et al took the concept of calcium-dependent protease activation further by showing that AP 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 cerulein model. Interestingly, carbachol-induced protease activation is more severe if cells are pretreated with ethanol. Calcium signaling also could play a role in other forms of experimental pancreatitis, such as bile acid-induced pancreatitis, pressure-induced pancreatitis, and pancreatitis after endoscopic retrograde cholangiopancreatography.
calcium release from intracellular stores or influx through the plasma membrane by pharmacologic inhibition of inositol triphosphate receptor (predominantly types 2 and 3) signaling44,95 or calcium release-activated calcium modulator 1.66,97 The calcium-dependent protease activation also heavily depends on calcineurin, a calcium-activated phosphatase, and its downstream signaling through the transcription factor nuclear factor of activated T cells. Inhibition of calcineurin by inhibitors or in mice lacking calcineurin subunits causes decreased intracellular protease activity in secretagogue- or bile acid–induced pancreatitis, without affecting vesicular transport.38,99

**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 trypsinogen100 causes CP in affected humans.9 In contrast, trypsinogen autoactivation is unimportant in experimental cerulein-induced pancreatitis. Inhibition of active trypsin by the reversible chemical inhibitor S124 could prevent trypsin activity in experimental cerulein-induced AP, but had no effect on the generation of the trypsin activation peptide (TAP) or active trypsin after washout 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 cerulein depends on cathepsin B.101–103 Similarly, in cathepsin B knockout mice, trypsin activation is significantly inhibited after cerulein administration.104 In the past, it was thought that cathepsin B105 and trypsinogen under physiologic 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, investigators showed in subcellular fractions of cerulein-treated acini a colocalization of cathepsin B and digestive enzymes, including trypsin and TAP, in heavy fractions76 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 AP.107–112 Intracellular activation of proteases other than trypsin, such as chymotrypsin and carboxypeptidase B, also depend on non-physiologic colocalization with other cell components, but are independent of cathepsin B.113 Mis-sorting in the exocrine machinery, secretion blockage, and reuptake of previously secreted proteases by 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 reported 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 syntaxins 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 physiologic and pathophysiologic conditions after 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 and tend to fuse with the plasma membrane or even rupture, discharging active trypsin into the cytosol or extracellular space. This instability is believed to be caused by disruption of otherwise protective actin filaments surrounding the EV.120 However, rupture of EVs is independent of trypsin or cathepsin B activity. Secretory blockage can contribute to these events. In experimental pancreatitis, vesicle-associated membrane protein-8–mediated secretion is impaired because of a loss of early endosomal proteins, resulting in retention of trypsinogen and transformation to active trypsin in a cathepsin-dependent manner. Knockout of vesicle-associated membrane protein-8 protects against 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 SPINK1. Although SPINK1 mutations are among the most common genetic risk factors for the development of recurrent AP and CP, thus far, none of the described mutations seemed to impair SPINK function and therefore do not explain the increased risk for pancreatitis.24–27 In mice carrying a heterozygous SPINK3 deletion, a significant decrease of functional SPINK does not lead to the development of spontaneous pancreatitis or more severe disease after supramaximal cerulein administration compared with wild-type controls.125 The fact that mice with a homozygous SPINK3 deletion develop pancreatic atrophy and that this phenotype can be rescued by transgenic expression of rat pancreatic secretory trypsin inhibitor 1125 point toward a role of trypsin inhibitors during pancreatic development but do not explain that 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. 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 AP, approximately 1%–5% of acinar cells undergo apoptosis and severity of pancreatitis is inversely correlated to the rate of apoptosis. Macroautophagy is a multistep, lysosomal-driven, adaptive process by which cells degrade cytoplasmic organelles and long-lived protein. Pancreatitis presents with impaired autophagic flux evident by vacuole accumulation. Currently, it is debated whether impaired autophagy stimulates cell death through accumulation of damaged mitochondria mediating an inflammatory response through a reactive oxygen species (ROS)-dependent mechanism as in lysosome-associated membrane protein 2 or Atg7-knockout animals, or whether autophagy prevents an inflammatory response as in Atg5-knockout mice. The regulated process of necrosis is termed necroptosis and is triggered by tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand, Fam ligand, interferon type 1, and Toll-like receptors (TLRs), which are released in the early phase of AP in response to pyroptosis. Receptor-interacting protein 1 (RIP1) and RIP3 form a phosphorylated complex, the necroosome, and phosphorylates mixed-lineage kinase domain-like, resulting in membrane rupture. Forty percent of cells undergo necroptosis in pancreatitis and RIP3 deletion or treatment with necrostatin ameliorates pancreatitis. Necroptosis releases damage-associated molecular patterns (DAMPs) and those will activate the nucleotide-binding oligomerization-domain-like receptor protein 3 (NLRP3) pathway, resulting in pyroptosis. Pyroptosis is an innate immune sensing mechanism with poorly understood upstream signaling. Nevertheless, some interesting inhibitors are known, such as lactate, β-hydroxybutyrate, and aspartate. The term pyroptosis describes activation of the inflammasome through NLRP3. The inflammasome is a macroscopic cytosolic protein complex that proteolytically cleaves interleukin (IL) 1β and pro-IL1β and releases high mobility group box 1 protein. NLRP3 activation requires lysosomal rupture and cathepsin release, calcium influx, and mitochondria-derived ROS production and thus is closely linked to necroptosis. However, NLRP3 expression is restricted to innate immune cells.

Cell death pathways in pancreatitis intersect. Caspase 3 activation can induce not only apoptosis but also pyroptosis. Necroptosis can shift to pyroptosis by caspase 8 activation and necroptosis activates pyroptosis. Currently, 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 and stimulation of necroptosis might explain this observation. Inhibition of pyroptosis, for example, by using Ringer’s lactate for volume resuscitation or inhibition of necroptosis by necrostatin, 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: Talukdar et al reported a direct effect on lysosomal stability mediated by active trypsin and lysosomal rupture leading to the release of cathepsins into the cytosol, causing dose-dependently apoptosis or necrosis. Our group showed that inhibition of protease activation, especially trypsin by specific inhibitors, results in a decreased rate of apoptosis but does not affect necrosis. Thus, cell death is a result of intracellular protease activation, but this has been shown only 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 of whether intracellular protease activation of trypsin as it is linked to cell death also can 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 AP. However, several studies have questioned the role of trypsin for severity of pancreatitis. Expression of human trypsin bearing the HP mutation R122H in mice leads to slightly more severe cerulein pancreatitis, but when compared with mice expressing normal human trypsin, there is no increase in disease severity. Moreover, the effect seems to be not solely dependent on trypsin, because mice with human trypsin mutations (R122H or N29I) show lower trypsin activity after cerulein hyperstimulation. This might be explained in part by a higher rate of acinar cell apoptosis even in untreated animals transgenic for human trypsinogen. A similar effect is seen in PACE-tryp(on) mice, which conditionally express an endogenously activated trypsinogen within pancreatic acinar cells. Those mice will develop AP in a trypsin activity-dependent way, which can lead to organ dysfunction and mortality, but they also show pronounced caspase 3 activation with consecutive apoptotic loss of acinar cells and replacement by fatty tissue. Similarly, Archer et al described a transgenic mouse, in which the human mutation R122H was inserted in the murine PRSS1 gene. Those mice developed spontaneous pancreatitis and showed a more pronounced inflammatory infiltrate and cellular damage in response to cerulein. The fact that the predominant way of cell death determines the overall severity of experimental pancreatitis in mice has been demonstrated in animals with deletion for cathepsin L. Cathepsin L degrades trypsinogen into an inactive elongated trypsinogen, such as lactate, β-hydroxybutyrate, and aspartate. The term pyroptosis describes activation of the inflammasome through NLRP3. The inflammasome is a macroscopic cytosolic protein complex that proteolytically cleaves interleukin (IL) 1β and pro-IL1β and releases high mobility group box 1 protein. NLRP3 activation requires lysosomal rupture and cathepsin release, calcium influx, and mitochondria-derived ROS production and thus is closely linked to necroptosis. However, NLRP3 expression is restricted to innate immune cells.

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Figure 3. Trypsinogen activation and cell death in pancreatic acinar cells. Intracellular trypsinogen activation is an early event at the onset of experimen
tial AP. Supramaximal secretagogue-receptor stimulation or activation of bile salt re
ceptors leads to an unphysiologic peak–plateau calcium signal. This results in disrupted exocytosis of zymogen granules, secretory blockage, zymogen retention, and formation of EVs, which contain trypsin and trypsinogen, taken up from the extra
cellular space. Those EVs colocalize and fuse with lysosomes containing cathepsin B, which in turn transforms trypsinogen into active trypsin. Owing to increasing instability, EVs often rupture, releasing trypsin and cathepsin B into the cytosol. Active trypsin is believed to induce mainly apoptosis, a silent form of cell death, which suppresses inflammation. In contrast, if the pathologic calcium release cannot be contained, rapid energy depletion occurs and cells undergo necrosis, during which the plasma membrane becomes leaky and cellular components (eg, DNA or mito
chondria) reach the extracellular space. Those will be recognized by leukocytes, which will be activated by the inflammasome signaling pathway. IL1β and TNFα release and pyroptosis occur. If TNFα reaches the basolateral membrane of previously unaffected or slightly damaged acinar cells, then it can induce another form of programmed cell death (ie, necroptosis). PLC, phospholipase C.
Role of Systemic Inflammation in Pancreatitis

**NFκB Activation—Initial Step of Inflammation**

The activation of NFκB is an early event during pancreatitis and occurs within the first minutes after onset of the disease. One main function of NFκB is transcriptional regulation of the immune response. The principal 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.

Intra-acinar protease activation and NFκB activation are early cellular events during pancreatitis, which have been suggested to occur independent of each other, but follow a similar kinetic. Trypsinogen activation depends on intracellular Ca2+ influx, but NFκB does not depend on trypsinogen activation. The phosphorylation of IκBα, followed by proteosomal degradation and nuclear translocation of NFκB (p65–p50) occurs in parallel to protease activation. NFκB as a transcription factor acts in 2 directions; first, transcription of proinflammatory genes, such as IL6 or TNFα, initiate the immune response; and second, the transcription of prosurvival genes. Therefore, NFκB can directly influence protease activity to protect cells by the up-regulation of serine protease inhibitor 2a.

**Figure 4.** NFκB pathway in pancreatic acinar cells. The early activation of NFκB follows the same time pattern as trypsinogen activation. They are induced by cytoplasmic Ca2+ influx, but NFκB does not depend on trypsinogen activation. The phosphorylation of IκBα, followed by proteosomal degradation and nuclear translocation of NFκB (p65–p50) occurs in parallel to protease activation. NFκB as a transcription factor acts in 2 directions; first, transcription of proinflammatory genes, such as IL6 or TNFα, initiate the immune response; and second, the transcription of prosurvival genes. Therefore, NFκB can directly influence protease activity to protect cells by the up-regulation of serine protease inhibitor 2a.
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 NFκB inhibitor α 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.64 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 develops only 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 to contribute to disease severity. Conversely, 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. Although all these studies investigated the role of NFκB in acinar cells, NFκB activation could play a much larger role in infiltrating immune cells, which directly regulate the immune response.

Another master switch in the transcriptional machinery of acinar cells is activator protein 1 (AP1). 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 AP.165 However, the extent of NFκB and AP1 activity seems to greatly differ with respect to the cause of pancreatitis. In cerulein models, activation of the 2 transcription factors is described and submaximal CCK stimulation induces acinar cell dedifferentiation and proliferation through the mitogen-activated kinase-c-Jun–AP1 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

Role of Infiltrating Immune Cells

Infiltration of immune cells starts within minutes after the onset of disease and plays a crucial role in the severity and prognosis of pancreatitis.169–172 Cells of the innate immune system, such as neutrophil granulocytes and monocytes and macrophages, represent the majority of infiltrating cells. NFκB plays a crucial role in 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 play no role in the activation of immune cells during the early phase of disease. Thus, in pancreatitis, activation of immune cells is mediated by cytokines or 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, IL6, or monocyte chemoattractant protein 1.175 DAMPs and cytokines result in the nuclear translocation of p65 and p50 within infiltrating immune cells, which enhances the cytokine storm through the secretion of proinflammatory mediators175 (Figure 5). DAMPs can act in the same way as pathogen-associated molecular patterns through TLRs176 or specific receptors such as P2RX7, which uses extracellular adenosine triphosphate as a ligand. Acinar cells, which undergo necrotic cell death, release a multitude of different DAMPs, such as free DNA,177 histones, or free adenosine triphosphate138 which can act as immune activators. 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 AP.

Neutrophil granulocytes are often used as reference markers for pancreatic inflammation by measurements of myeloperoxidase activity in tissue and reflect the amount of infiltrating neutrophils. One major function of neutrophils is removing pathogens by the release of proteases, antimicrobial peptides, and 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 such as 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 antineutrophil serum, which resulted in decreased pancreatic damage and protease activation.169,175,178 Several studies have investigated the role of neutrophil extracellular trap (NET) formation in the context of pancreatitis. NETs are extracellular networks consisting of neutrophil DNA and are used to bind pathogens.180 This suicide mechanism of neutrophils is a last line of defense against bacterial infections. NET formation is induced by TLR4 activation and the activation of nicotinamide adenine dinucleotide phosphate oxidase, which lead to the oxidation of peptidyl-arginine-deiminase 4.181,182 TLR4 is responsible for the detection of pathogens, although DAMPs can activate the TLR-signaling pathway.176 Merza et al183 reported that NET formation enhances the immune response during severe AP and is accountable for trypsinogen activation. Treatment with DNase prevents NET formation and decreases disease severity.183 In addition to bacterial infections, crystals can induce NET formation.183 Another group showed that NET formation is a critical step in bile stone development and plays an important role in ductal obstruction contributing to onset and severity of pancreatitis.185,186 Neutrophil infiltration during AP is an unspecific reaction of the immune system, which enhances local damage by the formation of NETs and the release of ROS or activates digestive enzymes.

Monocytes and macrophages belong to cells of the innate immune system. In contrast to neutrophil granulocytes, macrophages are characterized by high plasticity. Classic activated macrophages (M1) act in a proinflammatory manner and secrete large amounts of IL6, TNFα, IL12, and IL1β, and increased expression of inducible nitric oxide
Alternatively, activated macrophages (M2) are associated with wound healing, tissue regeneration, and fibrogenesis and act in an anti-inflammatory manner through the release of IL10 or transforming growth factor β. They are characterized by decreased inducible nitric oxide synthase and increased arginase 1 expression.\textsuperscript{187} Macrophages are phagocytosing cells that remove tissue debris and necrotic and apoptotic cells. During AP, macrophage infiltration correlates to a greater extent with pancreatic damage and necrosis than with the number of...
infiltrating neutrophils. The reason for that is that macrophages are required for the removal of necrosis and thus mitigate pancreatic damage. Phagocytosing macrophages have been observed in different models of AP and CP. In contrast to apoptosis, necrosis is a proinflammatory cell death because it entails the release of multiple DAMPs that induce an M1 polarization of macrophages. Therefore, acinar cell apoptosis is suggested to be protective against hyperinflammation and decrease disease severity. M1 macrophages release large amounts of TNFα, which have a direct effect on pancreatic acinar cells. Two independent groups reported that TNFα secreted from infiltrating monocytes is responsible for pancreatic damage and digestive protease activation. Depletion of macrophages by clodronate-containing liposomes decreased disease severity and protected mice from cerulein-induced pancreatitis. TNFα acts on cells by cell death receptors and is necessary to induce cell death by necroptosis through the RIP1–RIP3 pathway, which has been suggested to be the major cell death pathway during AP. Therefore, infiltrating macrophages are responsible for the induction of necroptosis and for the clearance of necrotic areas within the damaged pancreas.

In addition to TNFα, macrophages produce large amounts of IL1β, a proinflammatory 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 complex and the release of the cytosol to the extracellular space. In conclusion, early protease activation and NFκB activation are essential characteristics of pancreatitis; these 2 events occur in parallel during disease manifestation and strongly influence each other. Recent studies have proved that not only the activation of proteases and NFκB but also the type of cell play a critical role, in which their activation takes place is of importance. Pancreatitis is not a disease of acinar cells alone.

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Supplementary Material
Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2018.11.081.

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Conflicts of interest
The authors disclose no conflicts.

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### Supplementary Table 1. Genetic Risk Factors in Chronic Pancreatitis

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<th>Phenotype</th>
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<tr>
<td><strong>PRSS1/cationic trypsinogen</strong></td>
<td>p.R122C, p.R122H</td>
<td>Increased trypsinogen activation owing to prevention of CTRC-mediated degradation</td>
<td>Autosomal dominant HP, familial pancreatitis, or sporadic CP</td>
</tr>
<tr>
<td></td>
<td>c.–204C&gt;A</td>
<td>Decreased trypsinogen expression</td>
<td>Protection against alcoholic CP</td>
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<td><strong>PRSS2/anionic trypsinogen</strong></td>
<td>p.G191R</td>
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<td><strong>SPINK1/trypsin inhibitor</strong></td>
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<td>Skipping of exon 3 resulting in lower SPINK1 levels</td>
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<td><strong>CTRB1–2/chymotrypsin B1 and B2</strong></td>
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<tr>
<td><strong>CEL/carboxy ester lipase</strong></td>
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<td>Protein retention and ER stress</td>
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<tr>
<td><strong>CFTR/cystic fibrosis transmembrane conductance regulator</strong></td>
<td>CEL-HYB1, p.F508del, p.R117H</td>
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<td>Altered bicarbonate and fluid secretion from disrupted ductal calcium sensing?</td>
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