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Autophagy, Inflammation, and Immune Dysfunction in the Pathogenesis of Pancreatitis

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Abstract

Pancreatitis is a common disorder with significant morbidity and mortality, yet little is known about its pathogenesis, and there is no specific or effective treatment. Its development involves dysregulated autophagy and unresolved inflammation, demonstrated by studies in genetic and experimental mouse models. Disease severity depends on whether the inflammatory response resolves or amplifies, leading to multi-organ failure. Dysregulated autophagy might promote the inflammatory response in the pancreas. We discuss the roles of autophagy and inflammation in pancreatitis, mechanisms of deregulation, and connections among disordered pathways. We identify gaps in our knowledge and delineate perspective directions for research. Elucidation of pathogenic mechanisms could lead to new targets for treating or reducing the severity of pancreatitis.

Keywords

Lysosome; Mitochondrial Dysfunction; Macrophage; Cytokine

Pancreatitis has been associated with genetic factors, gallstones, and alcohol abuse. The current paradigm is that pancreatitis is initiated by acinar cell injury, leading to parenchymal necrosis and inflammation, which are the main pathologic features of the disease. It is believed that chronic pancreatitis (CP) results from repetitive subclinical or clinically evident bouts of acute pancreatitis (AP), or can develop without prior AP.[1–3]

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Conflicts of interest

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AP is a leading cause for gastrointestinal-related hospital admissions, with gallstones as a frequent etiologic factor. Although AP is most commonly mild to moderate in severity, as many as 30% of patients with severe disease associated with persistent multi-organ dysfunction die.[1–3] The pathophysiology of severe AP with multi-organ dysfunction is poorly understood. For many years, researchers believed that AP, and particularly severe pancreatitis, resulted from activation of digestive enzymes within the pancreas.[2,4] Indeed, patients with hereditary pancreatitis carry mutations in genes encoding for digestive enzymes.[3] However, these patients develop recurrent attacks and have a high risk of developing CP rather than severe AP.

There is now evidence that the systemic complications associated with AP result from uncontrolled or deregulated activation of the immune system.[5–7] Interestingly, the idea that severe pancreatitis is a consequence of excessive inflammation was proposed 30 years ago.[8] Despite significant progress in understanding disease pathophysiology and some potentially promising therapeutic approaches (eg, see references[5,6,9–15]), no drugs have been approved by the Food and Drug Administration for treatment of AP or CP, and morbidity remains high. There is, therefore, an urgent need to elucidate the mechanisms that initiate and promote pancreatitis.

Autophagy is the major catabolic process by which cells eliminate damaged, defective, or unwanted cytoplasmic organelles, long-lived proteins, and lipids, and recycle their constituents for energy and biogenesis needs. Hundreds of studies on the functions and mechanisms of autophagy during the last decade revealed its key homeostatic roles in metabolic adaptation, “quality control” of intracellular organelles, and differentiation and development. Deregulation of autophagy has been associated with the pathogenesis of many diseases, including neurodegenerative and inflammatory disorders and cancer. The physiologic and pathologic roles of autophagy in mammalian cells have been extensively reviewed.[16–19]

Inflammation eliminates cells that have been damaged during injury or infection and initiates tissue repair. Efficient development and resolution of the inflammatory response and restoration of tissue homeostasis depend on coordinated interactions between neutrophils, macrophages, and other types of immune cells, controlled by secreted mediators, such as cytokines and chemokines. Cytokines and chemokines recruit leukocytes and other immune cells to the inflamed pancreas,[5,20] as demonstrated by various blockade approaches.[5,6,9–15]

Deregulated autophagy and unresolved inflammation contribute to development of pancreatitis. Disordered acinar cell autophagy has been implicated in disease initiation, whereas the severity of AP is largely determined by whether the inflammatory response resolves or amplifies, leading to multi-organ dysfunction. Persistent low-grade inflammation is a characteristic of CP and an important factor in the development of pancreatic ductal adenocarcinoma.

We review the roles of autophagy and inflammation in pancreatitis, the mechanisms of their deregulation, and the pathways that link these processes. We discuss findings from in vitro

studies, mouse models of AP and CP, and patients. We identify gaps in our knowledge and delineate perspective directions for research.

Autophagy in Pancreatitis

Autophagy is a collective term for several pathways through which cytoplasmic materials are delivered to the lysosome and degraded by lysosomal hydrolases.[16–18] The resultant degradation products, such as amino and fatty acids, are recycled back to the cytosol through transporters and permeases.[16,17] The types of autophagy differ by how the cargo is delivered to lysosomes. Macroautophagy requires de novo formation of double-membraned structures termed *autophagosomes*, which sequester cargo and ultimately fuse with lysosomes to form autolysosomes, where degradation occurs (Figure 1). In chaperone-mediated autophagy, cytosolic proteins carrying a specific sequence motif bind to a receptor on the lysosome and are delivered into the lysosomal lumen. Microautophagy is mediated by direct lysosomal engulfment of small cytoplasmic components. In crinophagy, secretory vesicles fuse with the lysosomes and are degraded by lysosomal hydrolases[21]; this process was noted in pancreatitis decades ago,[22] but the fusion mechanism remains unknown.

Macroautophagy (hereafter referred to as autophagy) is the best studied form of autophagy and the only one examined in normal exocrine pancreas and in pancreatitis. It starts with the formation of autophagosomes, which in mammalian cells derive from various donor membranes, including the endoplasmic reticulum (ER), Golgi complex, or the plasma membrane. The process begins with the formation of so-called isolation membrane, or phagophore, followed by its elongation and closure to form the mature autophagosome, a globular double-membraned organelle (Figure 1). These steps are mediated by hierarchically recruited complexes of evolutionary conserved ATG (autophagy-related) proteins. Autophagy initiation is controlled by ULK1/ATG1-mediated complex, followed by the formation of another multiprotein complex involving phosphate-dylinositol 3-kinase catalytic subunit type 3 (PIK3C3 or VPS34) and beclin1, which nucleates the phagophore. Phagophore expansion and elongation are controlled by the ubiquitin-like conjugation systems involving the ATG5 ATG12 ATG16 complex and microtubule-associated protein 1 light chain 3a (LC3). LC3, the mammalian paralog of yeast ATG8, is necessary for phagophore closure; during this process, its cytosolic form (LC3-I) is lipidated to become LC3-II, which specifically translocates to the autophagosome membrane. Autophagosomes fuse with late endosomes and, ultimately, lysosomes, forming single-membraned autolysosomes, where cargo breakdown occurs.

Recent studies have advanced our knowledge of the lysosome as a dynamic organelle that regulates autophagy to meet the cell's degradation and recycling needs.[23] A master regulator of lysosomal biogenesis, the transcription factor EB coordinates the expression of lysosomal proteins and proteins involved in autophagy.[24]

Formation of autophagosomes and autolysosomes are key steps in autophagy. Autophagosomes sequester cargo (eg, organelles) destined for degradation. Their luminal pH is neutral and they do not contain hydrolases. In contrast, the lumen of the autolysosome is acidic and contains multiple hydrolases. Because LC3-II is almost exclusively associated

with the autophagosome's membranes, it is commonly used to monitor autophagy.[18] LC3-II remains present on early autolysosome's membrane until being degraded; autolysosomes, but not autophagosomes, also bear on their surface lysosomal markers, such as lysosome-associated membrane proteins (LAMPs). Recent studies[25] have demonstrated the complexity of autophagosome transport along microtubules to the site of late endosomes and lysosomes, which are predominantly located in the perinuclear region, and of the fusion process.

Macroautophagy can be selective and nonselective.[26–28] Autophagic responses induced by nutrient deprivation are nonselective; portions of the cytoplasm are randomly sequestered by autophagosomes, degraded, and recycled. On the other hand, organelle damage induces selective forms of macroautophagy, which direct specific cargo to lysosomes. The most-investigated types of selective autophagy are those that remove damaged/defective mitochondria (termed *mitophagy*), misfolded protein aggregates (aggrephagy), lipids, pathogenic bacteria inside a cell, and peroxisomes.

Selective autophagy is initiated through specific receptors on the cellular component destined for degradation, which interact with LC3 (or functionally equivalent protein GABARAP) to deliver this component to autophagosomes. One receptor is sequestosome 1 (SQSTM1, also known as p62), a multi-domain protein that serves as signaling scaffold and chaperone for polyubiquitinated proteins; it regulates a variety of cellular processes, including autophagy and oxidative stress.[16,17,29] For example, the process of aggrephagy starts with p62/SQSTM1 binding to polyubiquitinated protein aggregates through its ubiquitin-associated domain; and to LC3, through its LC3-interacting region.[29] It is believed that in normal tissue, different forms of selective autophagy co-exist with nonselective autophagy at a low basal level to maintain cellular homeostasis, and that selective autophagy protects against diseases.[27,28]

In normal physiologic conditions, autophagic flux (ie, turnover of autophagic vacuoles, from the formation of autophagosomes to cargo degradation in autolysosomes) is efficient, and there is no vacuole accumulation. Accumulation of autophagosomes could be via increased formation or defective fusion with lysosomes, whereas accumulation of autolysosomes indicates defective lysosomal degradation. p62 is specifically degraded by autophagy and is kept at a low level in normal conditions. Thus, excessive cell vacuolization and accumulation of p62 (especially together with LC3-II) are markers of impaired autophagy.

Functions of Basal Autophagy in Pancreatic Acinar Cells

Mouse exocrine pancreas has a high basal level of autophagy, which is activated in response to starvation to a greater extent than in liver, kidney, heart, or endocrine pancreas.[30,31] Exocrine pancreas has among the highest rates of protein synthesis and trafficking; these processes require coordinated actions of the ER, mitochondria, Golgi, endo-lysosomal system, and zymogen granules compartment. To function efficiently, pancreatic acinar cells might have a greater need to remove defective (or unneeded) cytoplasmic organelles.

Recent studies reveal that genetic alterations specifically targeting autophagic or lysosomal pathways cause pancreas damage. Disruption of genes encoding proteins that mediate autophagosome formation (ATG5 or ATG7) or lysosomal function (LAMP2) causes

autophagy blockade or impairment in the pancreas, resulting in severe acinar cell degeneration, exocrine pancreas atrophy, fibrosis, and inflammation.[32–35] This indicates that basal autophagy is critical for maintaining pancreatic acinar cell homeostasis. Impaired autophagy also dysregulates secretion, as shown in LAMP2-deficient acinar cells.[35]

In Experimental Pancreatitis, Autophagy Is Activated but Its Completion Is Inhibited

We only review acinar cell autophagy and its role in pancreatitis, as there is little information on the effects of pancreatitis on autophagy in other cell types, such as inflammatory or stellate cells. A prominent feature of pancreatitis is the accumulation of large vacuoles in acinar cells.[31,36–38] This is observed in human disease[35,36,38,39]; in rodent models of AP induced by administration of caerulein, alcohol, or coxsackievirus[31,37,40,41]; and in genetically engineered mice that develop pancreatitis spontaneously.[32,34,35,42,43] Histologic and electron microscopy analyses, and the presence of LC3-II on these vacuoles, indicate their autophagic nature. The autophagic vacuoles that accumulate in acinar cells in experimental pancreatitis are predominantly large autolysosomes, indicating inefficient lysosomal degradation.[31,37] Furthermore, acinar cell vacuolization in pancreatitis models is associated with decreased rates of degradation of long-lived proteins and accumulation of p62,[31,32,34,35,37,43] indicating impaired autophagic flux.

Efficient lysosomal degradation depends on activities of acid hydrolases and on lysosomal membrane proteins, such as LAMP1 and LAMP2, which protect the cytoplasm (and the limiting lysosomal membrane) from acid hydrolases and regulate various functions of lysosomes.[44] Experimental pancreatitis is associated with several defects in lysosome function. One is defective processing (maturation) of cathepsins, major lysosomal proteases, manifested by decreased level of fully processed (mature) forms of cathepsins and accumulation of intermediate forms in pancreas of mice and rats with L-arginine or caerulein-induced pancreatitis.[31,37,45] Concomitantly, cathepsins' enzymatic activities decrease in lysosome-enriched pancreatic subcellular fractions from these animals,[31,37] as reported decades ago.[46] Pancreatic tissues from patients and rodent models of AP have also reduced levels of LAMP1 and LAMP2 compared to normal pancreas.[35,39] In normally functioning lysosomes, hydrolases are thought to form large luminal complexes, ensuring their spatial separation from lysosome membrane proteins.[47,48] In AP, accumulation of the intermediate forms of cathepsins might compromise this separation; recent findings indicate that the intra-lysosomal part of LAMP molecules becomes susceptible to cleavage by cathepsin B, resulting in LAMP degradation.[35]

To evaluate the effect of an agent on autophagy induction (autophagosome formation) vs autophagic flux, cells are incubated in the absence and presence of lysosomal inhibitors. In the presence of the inhibitors, when lysosomal degradation is blocked, agent-induced changes in LC3-II level result from changes in autophagosome formation.[18] A recent study found that acinar cells from mice with L-arginine or caerulein-induced pancreatitis had higher levels of LC3-II in the presence of lysosomal inhibitors than cells from control mice, indicating that autophagosome formation is stimulated during development of AP.[45] Findings from these experimental models, therefore, indicate that in pancreatitis, autophagy is activated but its completion (lysosomal degradation) is inhibited. This conclusion is

supported by findings from genetic models of pancreatitis. The imbalance between increased autophagosome formation and decreased lysosomal degradation results in impaired autophagic flux.

Mitochondrial Dysfunction Impairs Autophagy in Mouse Models of Pancreatitis

Mitochondrial dysfunction is an early event in development of AP in rodent in vivo and ex vivo models, and is also associated with human disease.[41,49,50] It is manifested by loss of mitochondrial membrane potential[41,50] and decreased activity of F-ATP synthase.[45] Both defects are caused by deregulation of the permeability transition pore, a nonselective channel that traverses the outer and inner mitochondrial membranes; recent studies have identified F-ATP synthase as its central component.[51] Opening of the permeability transition pore is controlled by the mitochondria resident protein peptidylprolyl isomerase D (also called cyclophilin D [CypD]); its genetic or pharmacologic inactivation restores mitochondrial polarity and F-ATP synthase activity in pancreatitis.[41,45,49] Remarkably, deletion of CypD normalizes lysosomal function and autophagic flux in mice with L-arginine or caerulein-induced pancreatitis, revealing that mitochondria regulate lysosomes, and thereby autophagy, in the exocrine pancreas.[45] A similar relationship between the mitochondria and lysosome was also reported in T cells.[52] Little is known about the mechanisms of this interaction, which can involve lysosomal damage by reactive oxygen species (ROS) generated by dysfunctional mitochondria or impaired delivery of hydrolases to lysosomes due to ATP decrease.[53]

Selective and Nonselective Autophagy

Electron microscopy has shown that autophagic vacuoles accumulating in acinar cells of humans and rodents with pancreatitis contain various organelles, such as mitochondria, ER, and zymogen granules,[36–38,54] indicating both nonselective and selective autophagy. Induction of AP in mice induces mitophagy, the selective autophagy of mitochondria.[45] Pancreatitis induces mitochondrial depolarization and fragmentation, which activate mitophagy,[55] and up-regulates Parkin, a E3 ubiquitin-protein ligase that initiates mitophagy by decorating depolarized mitochondria.[26] Restoring mitochondrial function by deletion of CypD largely prevented induction of autophagy in mice with L-arginine or caerulein-induced pancreatitis, and increased autophagic flux.[45]

Researchers have shown that pancreas-specific over-expression of vacuole membrane protein 1, a protein mediating autophagosome formation, promotes formation of autophagosomes, which sequester zymogen granules.[56,57] Pancreatic level of vacuole membrane protein 1 increases in rodents with caerulein-induced AP. These findings prompted the concept of zymophagy—an autophagy pathway that selectively removes zymogen granules in pancreatitis.[57]

One likely activator of autophagy in pancreatitis is calcium.[41,58] Aberrant increases in acinar cell cytosolic Ca^{2+}, observed in several rodent models of AP, could mediate selective and nonselective autophagy.[59–61] In particular, Ca^{2+} overload causes mitochondrial depolarization that activates mitophagy.[41,45,62] On the other hand, the increase in cytosolic Ca^{2+} could stimulate nonselective autophagy through mitochondria-independent activation

of transcription factor EB.[63] Selective (ie, mitophagy) and nonselective autophagy both appear to become activated during development of pancreatitis.

Does Disrupted Autophagy Cause Pancreatitis?

Genetic alterations that specifically target autophagic and lysosomal pathways induce pancreatitis-like injuries. Disruption of genes encoding ATG5, ATG7, or LAMP2[32,34,35,64] block or impair autophagy in the pancreas and induce spontaneous pancreatitis, with inflammation, fibrosis, acinar-to-ductal metaplasia, and pancreas atrophy (Figure 2). Pancreatitis develops regardless of whether autophagy is disrupted at the level of autophagosome formation, as in ATG5-and ATG7-knockout mice,[32,34,64] or at the completion of autophagy, as in LAMP2-knockout mice.[35] Researchers also found that mice with deletion of inhibitor of the nuclear factor [NF]-$_\kappa$B kinase subunit α (IKK-α) develop spontaneous pancreatitis.[43] This serine/threonine protein kinase is a member of the IKK complex that activates NF-B transcription factors (see *Inflamatory Transcription Factor Activation*). Interestingly, the effects of IKK-α knockout in the pancreas[43] are unrelated to NF-$_\kappa$B, but are caused by impaired completion of autophagy, resulting in damage to acinar cells that progresses from vacuole accumulation to pancreatitis.

All of these knockout mice[32,34,35,43] display increased levels of active trypsin, accumulation of p62-positive protein aggregates, dilated ER, and oxidative and ER stress responses in exocrine pancreas (Figure 2). Blocking autophagy also causes accumulation of damaged mitochondria in acinar cells. Conversely, mitochondrial damage impairs autophagy.[41,45,49] These findings demonstrate the cross-regulation between autophagy and mitochondrial homeostasis in exocrine pancreas.

To determine whether impaired to autophagy makes the pancreas more susceptible to acute insult, pancreatitis was induced in LAMP2-null mice by administration of caerulein.[35] The effects of LAMP2 knockout and administration of caerulein on acinar cell vacuolization, necrosis, and apoptosis partially overlap, corroborating the notion that defects in lysosomal and autophagic pathways contribute to pathogenesis of pancreatitis.

In patients, pancreatitis is associated with decreases in pancreatic levels of LAMPs[35,39] and accumulation of p62[43] and LC3-II in acinar cells.[31,45] These changes might be targeted for treatment of pancreatitis. Although no mutations in *ATG5* have been identified in patients with pancreatitis,[32] it is possible that alterations in ATG5 or other ATG proteins could affect their levels or ability to regulate autophagy.

Most studies of pancreas-specific disruption of *Atg5* or *Atg7* used Cre recombinase regulated by developmental transcriptional factors PTF1A or PDX1.[32,34,64] However, the study in which *Atg5* was disrupted using Cre recombinase regulated by the elastase promoter (*Atg5*$^{F/F}$;*Ela-Cre* mice)[65] observed no pancreatic injury and less severe AP after administration of caerluein. Possible reasons for this discrepancy include differences in timing of recombination effects and cell types affected (more acinar cell specific disruption with Ela-Cre compared to Pdx1-Cre, which is active in exocrine and endocrine pancreas), as well as in recombination efficiency, which is suboptimal for the Ela-Cre vector used.[66] Researchers, however, reported that disruption of *Atg5* with Ela-Cre in >70% of adult acinar

cells did not induce spontaneous pancreatitis,[32] suggesting that autophagy is important in the pancreas development, but adult acinar cells could have compensatory mechanisms to overcome effects of loss of ATG5.

Restoring Autophagy

Impaired autophagy has been associated with several diseases, so there is considerable interest in pharmacologic approaches to modulate autophagy. Most focus has been on neurodegenerative and infectious diseases and aging.[67,68] Damaged organelles, protein aggregates, lipid droplets, or pathogens accumulate during development of these diseases; therefore, the goal of therapeutic strategies is to increase or promote autophagy-mediated delivery of deleterious structures for lysosomal destruction. Screens have identified modulators of autophagic and lysosomal path-ways that enhance autophagic activity.[67] In particular, the natural disaccharide trehalose stimulates autophagic flux and clearance of autophagic vacuoles.[67] A recent study[45] found that trehalose increased autophagic flux, prevented acinar cell vacuolization, and markedly reduced the severity of AP induced in mice by L-arginine or caerulein.

CypD is another promising target, as its deletion or pharmacologic inhibition restored mitochondrial function, normalized autophagic flux, and reduced the severity of pancreatitis in mice. This approach might be developed for treatment of pancreatitis.[41,45,49,69]

Autophagy is regulated by mechanistic target of rapamycin and adenosine monophosphate activated kinase signaling.[70,71] Both kinases are active in pancreatitis.[72,73] Studies are needed to determine whether they regulate autophagy in acinar cells, and whether modulating the mechanistic target of rapamycin and/or adenosine phosphate-activated kinase can increase autophagy in pancreatic tissues. Small molecules that affect the mechanistic target of rapamycin and adenosine monophosphate activated kinase are available and used in treatment of cancer, diabetes, and neurologic disorders; they might also modulate autophagy in patients with pancreatitis.[74,75] Little is known about other signaling pathways that regulate autophagy and are deregulated in pancreatitis, such as Ca^{2+}, phosphoinositide 3 kinase, and heat-shock proteins.[69,76]

Inflammatory Response of Pancreatitis

Immune Cells

Intrinsic (genetic) and extrinsic factors (such as alcohol, impacted biliary stones, drugs, and metabolic disorders) incite initial injury to acinar cells, triggering a cascade of events resulting in the release of pancreatic enzymes into the tissue, leading to local destruction. Injured acinar cells activate a number of inflammatory pathways[5,6,15,77–84] (Figure 3). Necrotic cells activate inflammatory and immune-stimulatory responses by releasing damage-associated molecular patterns (DAMPs) and other molecules. DAMPs can be also released through other pathways, such as necroptosis, pyroptosis, and autophagy. DAMPs interact with membrane-bound and cytosolic pattern recognition receptors expressed by immune and nonimmune cells to activate signaling and sterile inflammation.[85]

There is increasing evidence that DAMPs contribute to pathogenesis of AP, linking local tissue damage to systemic complications that can lead to death (Figure 3). DAMPs derived from damaged cells, such as the high mobility group box 1 (HMGB1) DNA-binding protein, bind to Toll-like receptors (TLRs). TLR4 is involved in development of AP[86,87]; its inhibition protects mice from pancreatitis.[11,14] Circulating levels of HMGB1 correlate with the severity of AP in patients.[88] Pancreas-specific deletion of HMGB1 makes mice more susceptible to experimental AP; thus, intracellular HMGB1 limits the inflammatory response and resultant tissue damage.[89] In contrast, the extracellular HMGB1 (derived from necrotic cells or secreted by the infiltrating infammatory cells) increases the severity of AP.[89]

These findings show an important role for DAMPs in activating innate immune cells and recruiting them to sites of inflammation to increase the severity of AP. During early stages of pancreatitis, neutrophils and monocytes are recruited to the pancreas, followed by dendritic cells, mast cells, T cells, and platelets. Migration of immune cells is a multi-step process that involves many adhesion molecules and their receptors.[90] Neutrophil adhesion to endothelial and epithelial cells is partly mediated by intercellular adhesion molecule 1 (ICAM1).[91] ICAM1 is constitutively expressed at a low level on endothelium and some epithelium. It is produced in higher amounts at the sites of inflammation, such as damaged acinar cells, leading to increased neutrophil adhesion.[84] Serum, pancreatic, and lung levels of ICAM1 increase during development of AP in rodents, and ICAM1-knockout mice are protected from pancreatitis induced by caerulein or a choline-deficient, ethionine-supplemented diet, compared to mice without disruption of *Icam1*.[78,84]

Infiltrating neutrophils produce an oxidative burst during the inflammatory response; nicotinamide adenine dinucleotide phosphate (NADPH) oxidase mediates this process.[92] Levels and activity of NADPH oxidase increase in the pancreas after administration of caerulein and neutrophil infiltration.[93] Furthermore, infiltrating neutrophils contribute to increased intra-acinar trypsin activity, a hall-mark response of AP thought to be acinar cell autonomous. This mechanism involves NADPH oxidase, as neutrophil depletion and genetic inactivation of NADPH oxidase inhibit trypsinogen activation (as well as other responses of caerulein-induced AP).[93] In addition to neutrophils, infiltrating macrophages also contribute to intra-acinar trypsinogen activation (Figure 3), which is mediated by tumor necrosis factor (TNF).[83]

Activated neutrophils expel nuclear DNA and histones to form extracellular web-like structures called neutrophil extracellular traps (NETs). NETs contribute to organ dysfunction in patients with infectious diseases, and (as shown more recently) in sterile inflammation.[94] The role of NETs in AP has been illuminated in recent studies.[95,96] NETs are formed in pancreatic tissues of mice during experimental AP and promote organ inflammation and injury. Importantly, levels of NETs are increased in plasma from patients with severe AP. Interestingly, the addition of NETs to acinar cells causes trypsinogen activation and activates the signal transducer and activator of transcription 3 (STAT3).[95]

Monocytes also mediate the inflammatory response during development of AP. Similar to neutrophils, monocytes are recruited by signals deriving in part from damaged acinar cells. It is believed[10,97,98] that activation of primary monocytes is mediated by chemokines C-C

motif ligand 2 (CCL2, also called monocyte chemokine protein 1), CCL3, and CCL5 (for chemokine nomenclature see Zlotnik and Yoshie[99]). Signals originating from injured acinar cells are multiplied by activated monocytes. As a result, TNF, interleukin (IL) 1, IL6, and ICAM1 are produced in greater amounts, promoting tissue damage and disease progression. This signal amplification primarily affects lung, liver, and kidney, leading to systemic inflammation.[100]

Distinct populations of macrophage mediate systemic inflammation in pancreatitis. In rodents with severe AP, peritoneal macrophages are rapidly activated due to large amounts of pancreatic enzymes and cytokines; these macrophages are associated with complications of severe pancreatitis.[101,102] Importantly, AP also activates alveolar macrophages, which produce cytokines and nitric oxide to attract large number of leukocytes to the lungs.[103,104] Kupffer cells are also associated with AP; these liver macrophages are activated by inflammatory mediators released into the blood by damaged pancreas and promote systemic inflammation.[105,106] Activation of Kupffer cells by pancreatic enzymes was demonstrated in vitro.[107] However, liver damage is evident only in late, but not early, stages of AP; and it is not clear whether inflammatory mediators originating in the pancreas during experimental pancreatitis induce acute liver responses.[108] However, Kupffer cells might be activated in pancreatitis via other mechanisms.[109]

In mice and rats with CP, macrophages are found near areas of fibrosis.[110,111] Lipopolysaccharide-activated macrophages produce cytokines that active pancreatic stellate cells and promote collagen and fibronectin synthesis in cultured pancreatic stellate cells.[112,113] Lipopolysaccharide binds to TLR4; $TLR4^+$ monocytes and macrophages contribute to development of AP.[14,86] Unlike in AP, alternatively activated (M2) macrophages predominate in mice and patients with CP; blocking IL4Ra signaling in macrophages decreases pancreatic stellate cell activation, fibrosis, and progression of caerulein-induced CP.[111]

Other innate immune cells, such as dendritic cells (DCs) and mast cells, are also likely to be involved in development of pancreatitis. Depletion of DCs, unlike depletion of neutrophils, increases the severity of AP in mice.[114] Major histocompatibility complex II^+CD11c^+ DCs increase 100-fold in pancreas of mice with AP, and account for nearly 15% of intra-pancreatic leukocytes.[114] DCs regulate T-cell responses to both promote and suppress inflammation. During the development of AP, DCs produce high levels of inflammatory cytokines and chemokines (TNF, IL6, and CCL2), but also protect pancreas from cellular stress. Little is known about the switch that regulates DC function during development of AP.[114] One mechanism by which DCs could modulate inflammation in AP is by inducing $CD4^+CD25^+Foxp3^+$ regulatory T cells. Inhibition of regulatory T cells results in uncontrolled inflammation, as these cells inhibit the inflammatory activities of macrophages and direct their differentiation toward an anti-inflammatory (M2) phenotype.[115] DCs mediate clearance of byproducts of tissue injury, and they also clear antigens (in addition to apoptotic and necrotic cell debris). Thus, the finding that depletion of DCs promotes inflammation in mice with AP suggests that DCs may limit sterile inflammation in pancreatitis.

Activation of mast cells could also contribute to development of AP and CP. In acute inflammation, mast cells initiate and promote multi-organ failure, by disrupting endothelial barrier function in the pancreas and other organs. These cells are also implicated in chronic inflammatory processes with fibrous tissue deposition and in tissue destruction and remodeling.[116]

Activation of Inflammatory Transcription Factors

Transcription factors, such as NF-$_k$B, STAT3, and nuclear factor of activated T cells, regulate activities of immune cells and their production of cytokines during the inflammatory response. NF-$_k$B is activated rapidly upon induction of inflammation, up-regulating expression of TNF, IL6, IL1β, inducible nitric oxide synthase, and ICAM1; all of these cytokines have been associated with AP.[82,117,118] Another major function of NF-$_k$B is regulation of cell proliferation and apoptosis. NF-$_k$B comprises homo- and heterodimers of the members of Rel family, and is activated by cytokines, lipopolysaccharide, oxidative stress, and activators of protein kinase C.[119,120] Early and prominent activation of NF-$_k$B in pancreatic tissues of rodents with caerulein-induced AP was reported almost 20 years ago; [117,118] these 2 studies, however, arrived at different conclusions regarding its role in development of AP. Since then, NF-$_k$B activation in pancreatitis has been studied extensively.[82,121]

In most studies, pharmacologic inhibition of NF-$_k$B reduced the inflammatory response, necrosis, and other parameters of AP severity. However, the pharmacologic agents used were largely nonspecific, such as antioxidants and proteasome inhibitors. Studies with genetically engineered mice were expected to clarify the role of NF-$_k$B in development of pancreatitis. However, disagreement continued with evidence that activation of IKK–NF-$_k$B pathway could either increase or reduce the severity of pancreatitis, depending on the approach used to manipulate this pathway.[121–124] These controversies might be explained by the observation that in acinar cells, activation of NF-$_k$B induces both inflammatory and anti-inflammatory signaling pathways, as is also observed in other cells and organs.[121,125]

The IKK complex has 2 catalytic subunits (IKK-α and IKK-β) and the noncatalytic subunit NEMO. The IKKs phosphorylate inhibitory proteins (I$_k$Bs), which normally sequester NF-$_k$B dimers in an inactive state in the cytoplasm; phosphorylation marks I$_k$Bs for degradation in the proteasome and allows NF-$_k$B translocation into the nucleus.[125,126] NF-$_k$B activation in experimental AP involves IKK-β; the expression of a constitutively active IKK-β in acinar cells promotes features of CP.[124,127] Persistent acinar cell activation of NF-$_k$B is also associated with inflammation and other manifestations of CP in humans.[128] A recent study showed that the I$_k$B protein BCL3 is up-regulated in inflamed pancreatic tissues from mice and patients with pancreatitis; its deletion prolongs NF-$_k$B activation and aggravates the inflammatory response and severity of AP in mice.[129]

Pancreas-specific deletion of IKK-α induces features of CP[43]; as stated above, this is unrelated to NF-$_k$B but is due to defective completion of autophagy in the pancreas. This study also reported down-regulation of IKK-α in human CP.[43]

There are therefore complex and multiple mechanisms determining the role of IKK–NF-$_k$B pathway in pancreatic (particularly, acinar cell) homeostasis and in the inflammatory response of pancreatitis. Researchers also showed the importance of this pathway for other responses of pancreatitis; for example, activation of NF-$_k$B in myeloid cells induces pancreatic fibrosis during experimental CP in mice.[130] Further studies are needed to develop therapeutic strategies to modify these pathways in pancreatitis.

STAT3 mediates inflammation signaling pathways and is dysregulated during development of pancreatitis. Its pancreas-specific deletion or inactivation affects the severity of AP in different models.[15,131] Although NF-$_k$B is activated during the onset of inflammation, phosphorylation and nuclear translocation of STAT3 occur at later stages, linking local damage to multi-organ failure.[15,128] Pharmacologic inhibitors of this pathway reduced the severity of pancreatitis, whether given before or after the induction of pancreatitis, so this strategy might have clinical application.[132]

Mediators of Inflammation

NF-$_k$B activation triggers transcription of proteins that mediate inflammation. Injured acinar cells express cytokines and chemokines that promote development of AP; in particular, expression of TNF is induced during early stages of AP in rodents.[80] Along with up-regulated adhesion molecules, such as ICAM1, these cytokines and chemokines recruit neutrophils as first-responder inflammatory cells. Disruption of *Icam1* or depletion of neutrophils reduces the severity of AP in mice or rats.[78,133] (Interestingly, depletion of neutrophils from ICAM1-knockout mice does not have an additive effect in reducing AP severity,[78] indicating over-lapping functions of ICAM1 up-regulation and infiltrating neutrophils.) Similarly, blocking other chemoattractants of neutrophils, such as CXCL1, its receptor CXCR2, or (more recently) CX3CL1, reduces the severity of AP and its complications in mice and rats.[9,12,13]

Progression of AP is associated with further increases in expression of TNF, IL1, IL6, ICAM1, and multiple chemokines. These recruit other inflammatory cells and amplify signals to promote local and extrapancreatic multi-organ injury.[5,100] Similar to other models of acute injury, activation of the innate immune system, including neutrophils and monocytes, contributes to pathogenesis of AP (Figure 3).

Activation of NF-$_k$B in myeloid cells and expression of IL6 by macrophages mediate development of AP in mice.[15] IL6 can signal by binding to its membrane receptor (IL6R) or by forming a complex with soluble IL6R. Both lead to activation of IL6 signal transducer (also called GP130), which activates the kinase JAK2 to phosphorylate STAT3 at Y705. In infiltrating myeloid cells, NF-$_k$B regulates production of IL6 at sites of inflammation; the ensuing persistent activation of STAT3 results in high levels of CXCL1, which (in conjunction with ICAM1 in lung endothelial cells) mediate granulocyte infiltration into the lung, promoting lethal acute lung injury.[15,132] Cytokines and chemokines in the systemic circulation induce secretion of acute-phase proteins in the liver and activate complement factors and the bradykinin-kinin system. This increases capillary permeability, leading to hypovolemia and edema.[134]

Interestingly, IL22, a member of the IL10 cytokine family and inducer of STAT3, protects mice from AP.[135,136] Expression of IL22 is regulated by the aryl hydrocarbon receptor, a ligand-dependent transcription factor. Aryl hydrocarbon receptor up-regulation of IL22 promotes interactions between leukocytes and pancreatic epithelial cells and reduces the severity of AP in mice.[136]

Early recruitment of inflammatory monocytes to the pancreas requires the chemokine CCL2; thus agents that block CCL2 or its receptor reduce the severity of AP in rodents.[10,97,98] In experimental AP, inflammatory monocytes differentiate and polarize into classically activated (M1) macrophages; whereas pancreatic tissues from patients with CP contain higher numbers of alternatively activated (M2) macrophages,[111,137] indicating differential immune response mechanisms involved. Unlike M1 macrophages, the alternatively activated macrophages associated with CP promote pancreatic stellate cell activation and fibrosis.

Links Between Autophagy and Inflammation

Immunologic Roles of Autophagy

One function of autophagy is to regulate the inflammatory response. The effects of autophagy on the immune response are complex; autophagy can promote or reduce inflammation by regulating pathogen clearance, antigen presentation, and innate and adaptive immune responses.[138,139] Normal/efficient autophagy has a dual effect on formation and activation of inflammasomes. It limits inflammasome activation by clearing endogenous sources of inflammasome inducers, such as damaged or depolarized mitochondria, which leak ROS or mitochondrial DNA.[139,140] Autophagy degrades several inflammasome components, including NLRP3, to reduce inflammasome formation. However, once the inflammasome is activated, autophagy supports secretion of IL1β, as well as IL18 and HMGB1, to perpetuate inflammation.[139–141] On the other hand, autophagy provides negative feedback to inflammasomes. DAMPs (such as HMGB1, ATP, and mitochondrial DNA released into the cytosol) and ROS activate autophagy to limit inflammasome activation.

Autophagy also inhibits expression of inflammatory cytokines, in particular by eliminating p62/SQSTM1, the adaptor protein that usually promotes activation of NF-$_k$B.[29,32] Autophagy also affects the ER stress response; activation of eukaryotic translation initiation factor 2a kinase 3 (EIF2AK3 or PERK) signaling to eukaryotic translation initiation factor 2 subunit a (EIF2-α) arrests translation, reducing levels of I$_k$B and activating NF-$_k$B.[142,143] Finally, autophagy prevents activation of type I interferon (IFN) by inhibiting TANK binding kinase 1(TBK1), a ubiquitously expressed serine/threonine kinase that regulates production of type I IFN.[144] These findings reveal complex regulatory feedback, leading to the notion that autophagy “rations” the innate immunity response through balancing positive and negative effects, depending on context.[138] This process is likely to regulate sterile inflammation in patients with pancreatitis.

Blocking Autophagy in Acinar Cells Induces an Inflammatory Response

Autophagy blockade through disruption of genes encoding ATG5, ATG7, LAMP2, or IKK-α stimulates the inflammatory response, manifested by up-regulation of cytokines and chemokines and inflammatory cell infiltration of the pancreas (Figure 4).[32,34,35,43] Most of these cells are macrophage, typically associated with CP. Autophagy blockade or impairment promotes infiltration of the pancreas by inflammatory (M1) and fibrogenic (M2) macrophages; inflammatory M1 are predominant in LAMP2-null mice.[35] Interestingly, the neutrophil infiltration that characterizes AP is decreased in pancreatic tissues of LAMP2-null mice with caerulein-induced pancreatitis; in contrast, the macrophage infiltration increases, indicating a shift toward a chronic inflammatory response.[35]

Investigation of the mechanisms through which defective autophagy promotes the inflammatory response in pancreatitis has only started (Figure 4).[5,33,145] In ATG5-deficient mice,[32] autophagy blockade causes activation of NF-$_k$B and STAT3, as well as p38 and c-Jun N-terminal kinases, which all stimulate production of cytokines by pancreatic acinar cells.[77,117,146] The mechanisms of these processes are likely to involve increases in ROS due to defective clearance of damaged or depolarized mitochondria, and accumulation of p62-containing protein aggregates; both pathways activate NRF2 and p53.[32] ATG5 deficiency in the pancreas also up-regulates TBK1, resulting in increased levels of type I IFN and the IFN-regulated chemokine CCL5.[147]

Although persistent ER stress occurs in mouse models of AP[148] and CP,[149] and autophagy blockade induces ER stress in experimental and genetic models of pancreatitis,[32,34,43,45] the mechanisms linking ER stress, impaired autophagy, and the inflammatory response have not been determined. Studies are also needed to determine the role of autophagy in inflammasome activation in pancreatitis[79,81,150,151] (Figure 4).

Future Directions

We have made progress in elucidating the roles and mechanisms of impaired autophagy in pancreatitis. There is an essential homeostatic role of basal autophagy in pancreatic acinar cells to maintain protein synthesis and secretion. However, we have much to learn about how autophagy regulates these and other functions of acinar cells. The development of spontaneous pancreatitis in mice with disruption of *Atg5*, *Atg7*, or *Lamp2* provides evidence that defects in autophagic and lysosomal pathways contribute to development of AP and CP. Detailed analyses of these pathways in exocrine pancreas are underway. It is important to determine which signaling pathways are deregulated to impair autophagy, how errors in distinct steps of autophagy lead to pancreatitis (such as during autophagosome formation, fusion with lysosomes, or lysosome function), and how we might restore efficient autophagy in pancreatic cells. Open questions include the contribution of different types of autophagy (particularly chaperone-mediated autophagy) to pathogenesis of pancreatitis and the mechanisms of impaired autophagic flux. Studies are also needed to determine how disruptions in autophagy lead to pancreatitis responses, such as trypsinogen activation in AP or fibrosis in CP. An important area for investigation is to determine relationships between impaired autophagy and dysfunction of other organelles in pancreatic cells. Studies discussed in this review provide evidence for interactions between autophagy and

mitochondria in the exocrine pancreas; little is known about how these might involve the ER, Golgi, or the secretory compartment.

Our understanding of the inflammatory response during development of AP has also advanced, and some blockade approaches have therapeutic potential. However, we know less about the inflammatory response during development of CP. Compared to activation of inflammatory transcription factors, little is known about the role of inflammasome activation and the involvement of DAMPs (apart from HMGB1). Detailed information is still lacking on how the inflammatory response develops in models of pancreatitis on time-dependent changes in profiles of inflammatory mediators or infiltrating immune cells (especially M1 vs M2 macrophages and distinct subsets of T cells). Such information is necessary to elucidate the central unanswered question about the mechanism of the uncontrolled, non-resolving inflammation in pancreatitis.

It will be important to study the relationships between impaired autophagy and inflammation in development of pancreatitis. Findings from knockout and transgenic mice indicate that defects in autophagy promote the inflammatory response by unclear mechanisms. Studies of other organs and diseases have shown this interaction to be complex. Essentially nothing is known about the effects of inflammatory mediators, such as cytokines, on the process of autophagy during pathogenesis of pancreatitis. Further-more, studies on autophagy have been almost exclusively performed in acinar cells; little is known about the roles of autophagy in other cells involved in the genesis of pancreatitis, such as immune or stellate cells. Elucidation of the mechanisms that disrupt autophagy and how these affect inflammation and development of pancreatitis, could lead to new therapeutic targets or approaches to treat or reduce severity of AP and CP.

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Abbreviations used in this paper:

AP	acute pancreatitis
ATG	autophagy-related (gene or protein)
CCL	chemokines C-C motif ligand
CP	chronic pancreatitis
CypD	cyclophilin D
DAMP	damage-associated molecular pattern
DC	dendritic cell

ER	endoplasmic reticulum
HMGB1	high mobility group box 1 protein
ICAM1	intercellular adhesion molecule 1
I$_k$B	inhibitor of $_k$B
IKK	inhibitor of $_k$B kinase
IL	interleukin
LAMP	lysosome-associated membrane protein
LC3	microtubule-associated protein 1 light chain 3 (mammalian paralog of yeast ATG8)
NADPH	nicotinamide adenine dinucleotide phosphate
NET	neutrophil extracellular trap
NF-$_k$B	nuclear factor $_k$B
ROS	reactive oxygen species
STAT3	signal transducer and activator of transcription 3
SQSTM1	seques-tosome 1
TLR	Toll-like receptor
TNF	tumor necrosis factor

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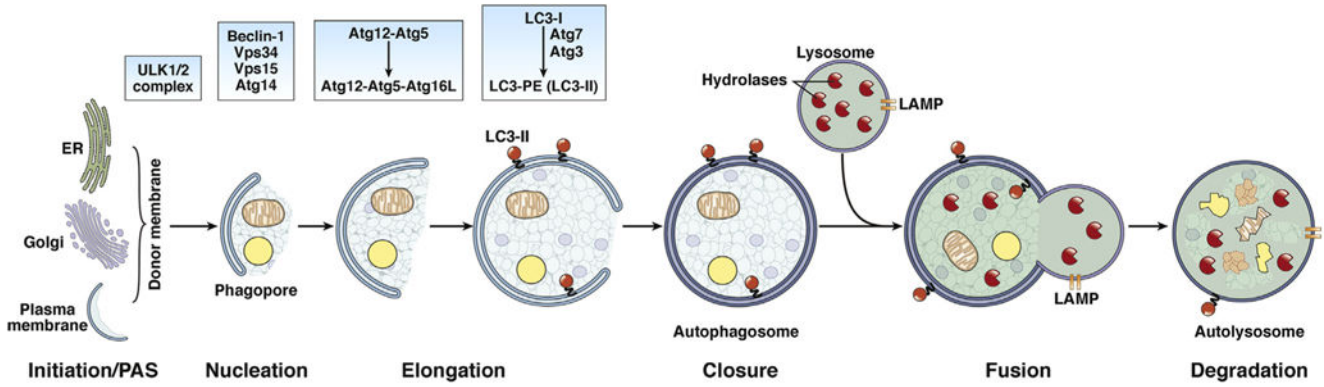

Figure 1.

Macroautophagy. Cellular material destined for degradation in the lysosome is sequestered in double-membraned autophagosomes, which are formed in a stepwise process involving phagophore nucleation, elongation, and closure. This process is controlled by hierarchically assembled multi-protein complexes comprising ATGs and other proteins. Autophagosomes ultimately fuse with lysosomes, forming single-membraned autolysosomes in which cargo is degraded. Lipidated LC3 (LC3-II) translocates to autophagosome membranes. LAMPs are present on autolysosomes but not autophagosomes. PAS, pre-autophagosomal structure.

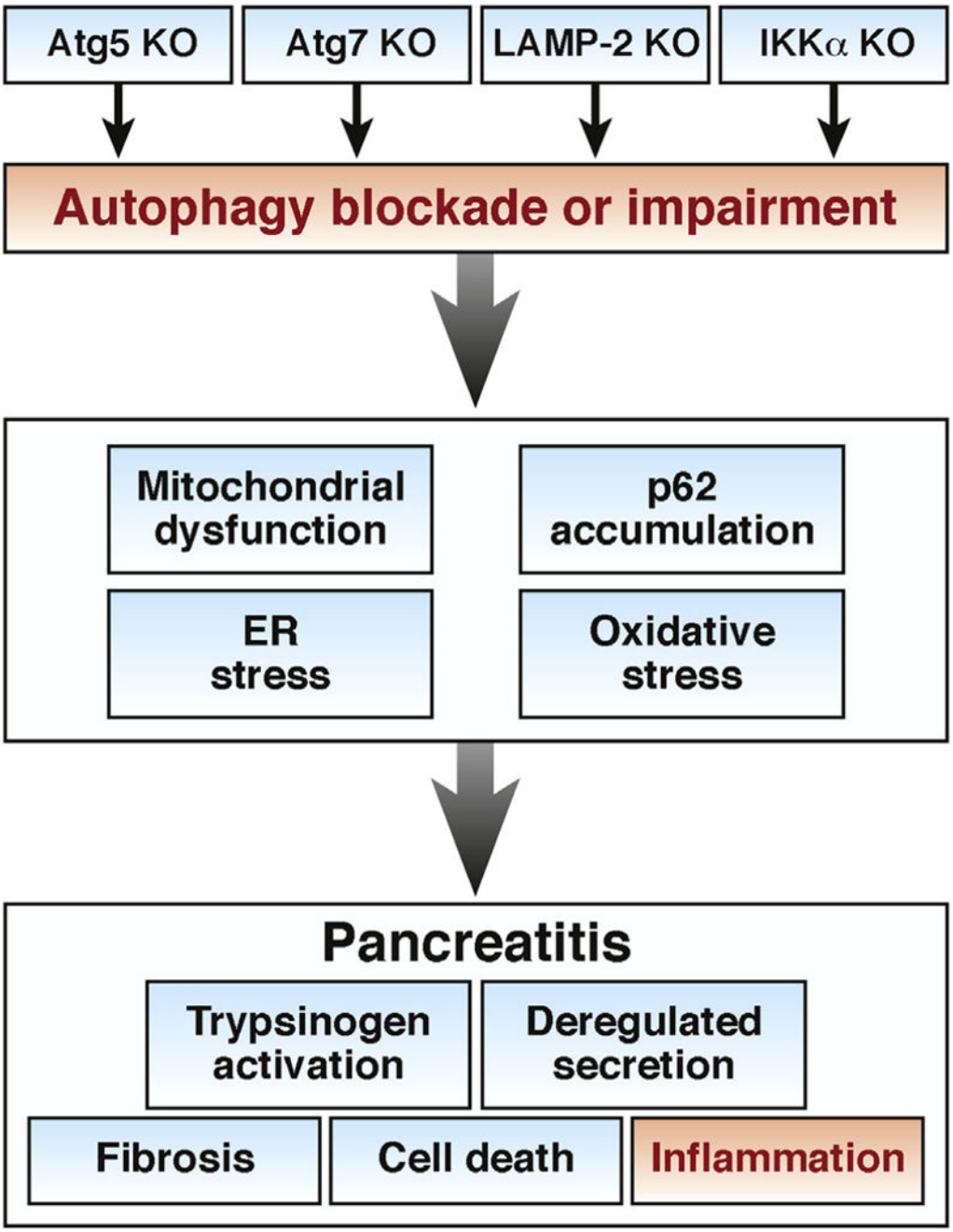

Figure 2.

Autophagy blockade or impairment by deletion of Atg5, Atg7, Lamp2, or IKK-α causes spontaneous pancreatitis, characterized by trypsinogen activation, deregulated secretion from acinar cells, fibrosis, loss of parenchymal tissue, and inflammation. Mechanisms of pathogenesis involve mitochondrial dysfunction in acinar cells, accumulation of p62, and ER and oxidative stresses.

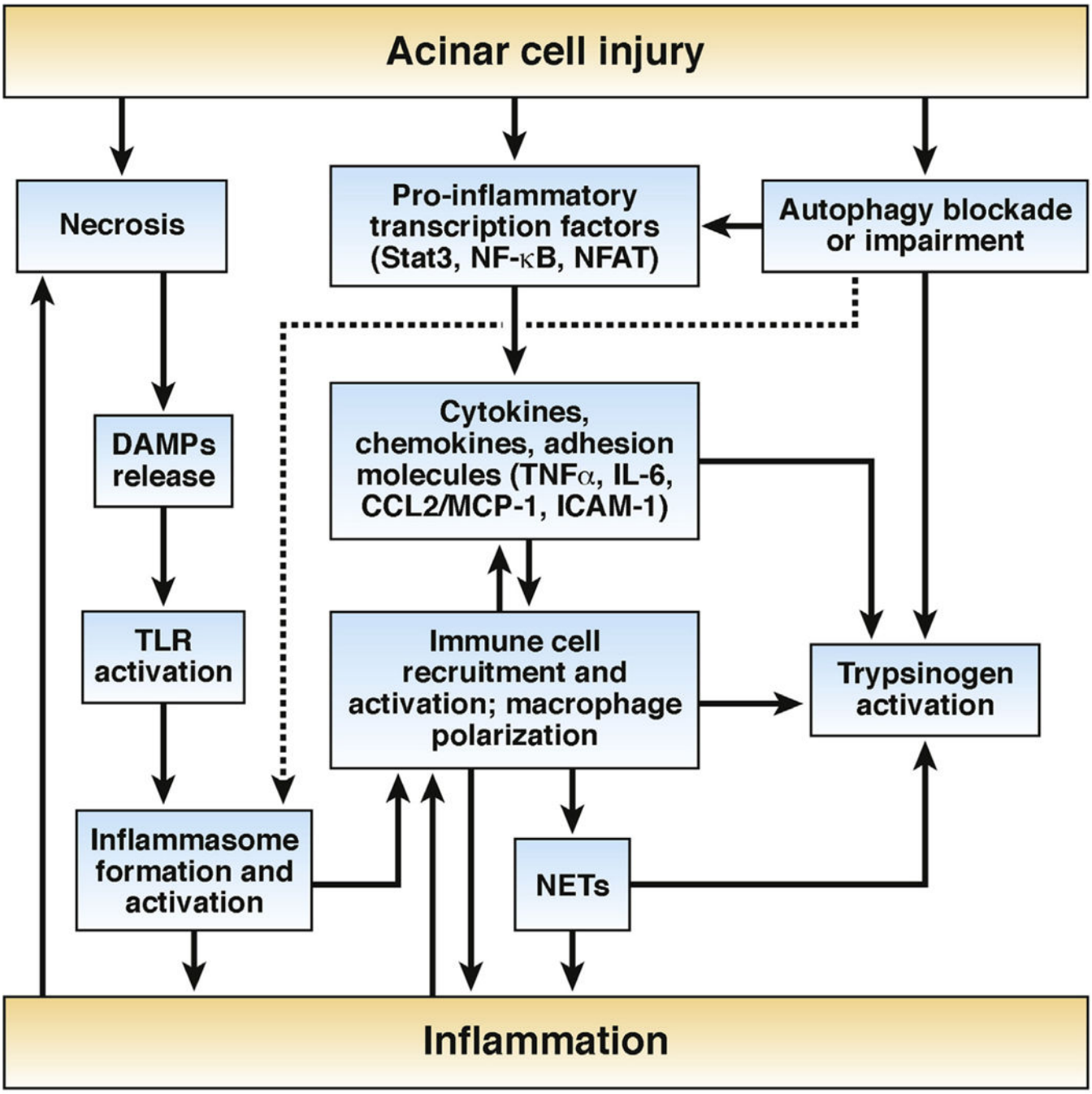

Figure 3.

Pathways that mediate inflammation during development of pancreatitis, and their involvement in intraacinar trypsinogen activation. *Dashed lines* indicate pathways that are likely but not yet proven to be involved in the pancreas. For detailed links between impaired autophagy and inflammatory responses, see Figure 4. Not shown are other inflammatory pathways involved in development of pancreatitis, such as abnormal Ca^{2+} signaling or ER stress, which are not discussed in this review.

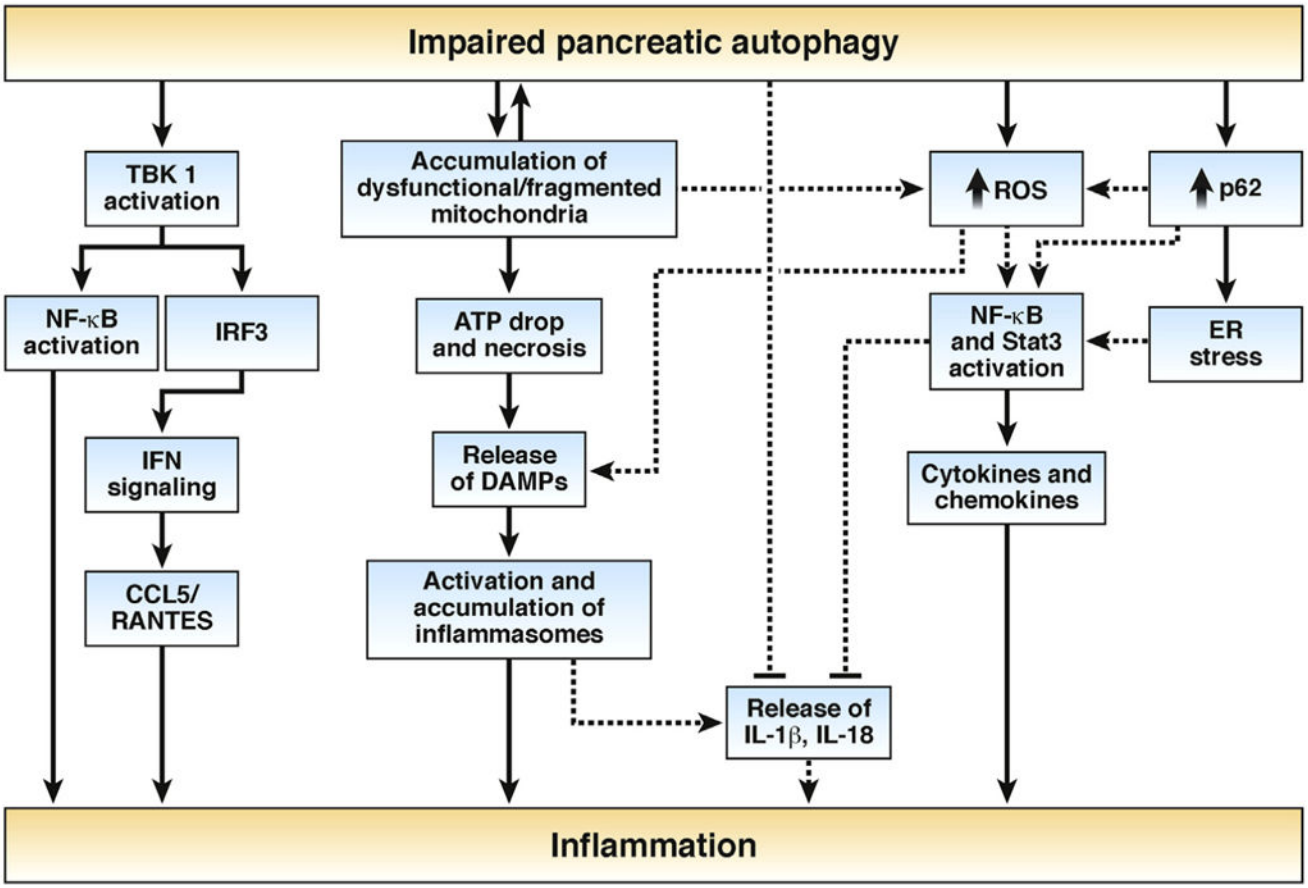

Figure 4.

Mechanisms that link impaired autophagy to inflammation during development of pancreatitis. *Dashed lines* indicate pathways that are likely but not yet proven to be involved in the pancreas.