Pancreas-specific RelA/p65 truncation increases susceptibility of acini to inflammation-associated cell death following cerulein pancreatitis

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Activation of the transcription factor NF-κB/Rel has been shown to be involved in inflammatory disease. Here we studied the role of RelA/p65, the main transactivating subunit, during acute pancreatitis using a Cre-loxP strategy. Selective truncation of the relα gene in pancreatic exocrine cells led to both severe injury of the acinar cells and systemic complications including lung and liver damage. Our data demonstrated that expression and induction of the protective pancreas-specific acute phase protein pancreatitis-associated protein 1 (PAP1) depended on RelA/p65. Lentiviral gene transfer of PAP1 cDNA reduced the extent of necrosis and infiltration in the pancreata of mice with selective truncation of RelA/p65. These results provide in vivo evidence for RelA/p65 protection of acinar cell death via upregulation of PAP1. Moreover, our data underscore the pancreas-specific role of NF-κB/Rel and suggest multidimensional roles of NF-κB/Rel in different cells and contexts during inflammation.

Introduction

Acute pancreatitis (AP) is a common clinical problem (1, 2). Approximately 25% of patients with AP develop a severe disease course that leads to systemic inflammatory response syndrome (SIRS) and sequelae such as multiorgan dysfunction syndrome (MODS) and acute respiratory distress syndrome (ARDS) with mortality rates up to 50%. Although intra-acinar cell activation of digestive enzymes such as trypsinogen is likely to be the triggering event for acinar cell injury, the exact mechanisms that regulate the severity of AP are unknown (3–5).

An important reorganization of the gene expression pattern occurs during inflammation of the pancreas as part of a well-structured response to exogenous or endogenous damage. Digestive enzymes such as amylase and lipase are downregulated and newly synthesized proteins are overexpressed, including secretory proteins such as pancreatitis-associated protein 1 (PAP1; also known as peptide 23, Reg-2, or RegIIIα) and the pancreatic stone protein (PAP/Reg) constitute a family of proteins that belong to the C-type lectin family (9). While the expression of PAP1 and the severity of cerulein-induced pancreatitis are strongly correlated, the physiological role of PAP1 remains unclear (10). Recently, a regulatory link between PAP1 and the transcription factor NF-κB/Rel was proposed (11, 12).

Nonstandard abbreviations used: AP, acute pancreatitis; IkB, inhibitor of NF-κB; IKK, IkB kinase; MEF, murine embryonic fibroblast; MPO, myeloperoxidase; PAP, pancreatitis-associated protein; SDI, scrambled duplex siRNA; SIRS, systemic inflammatory response syndrome.

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Ptf1a locus (19, 20). Cre-mediated deletion resulted in a functional inactive form of RelA/p65 in the pancreas, which does not dimerize or translocate to the nucleus. We found that truncation of RelA/p65 did not ameliorate the course of AP, but increased the susceptibility of acinar cells to inflammation-associated cell death and resulted in severe necrotizing pancreatitis with severe lung inflammation and liver damage. This unexpected phenotype was due, at least in part, to impaired induction of PAP1. Taken together, these data suggest a protective and organ-specific role of RelA/p65 in acute pancreatitis.

Results

Pancreas-specific truncation of RelA/p65 increases tissue damage during inflammation. To direct Cre expression to the exocrine pancreas, we generated a knockin mouse (Ptf1a-creex1) with the gene encoding for the Cre recombinase within the Ptf1a locus (21). PTF1 is a heteromeric basic helix-loop-helix protein complex composed of 3 subunits: p48, p64, and p75. The tissue-specific component, p48, is essential for exocrine pancreatic development (19, 20). Thus expression of Cre resulted in the development of mice with pancreas-specific deletion of the floxed allele (exons 7–10; relaΔ/Δ mice; Figure 1, A and B) and accordingly in a truncated form of RelA/p65 in the pancreas (Figure 1C). The protein expression level of pancreatic IκBα, a NF-κB/Rel target, was lower in the relaΔ/Δ pancreas (Figure 1C). The expression level of p50, a subunit of the NF-κB/Rel heterodimer, was not altered in the relaΔ/Δ pancreas. The relaΔ/Δ mice developed normally with no signs of obvious dysfunction.

To analyze the function of RelA/p65 in AP, we induced AP by repetitive i.p. injections of sulfated cerulein, a decapeptide analog of the pancreatic secretagogue cholecystokinin. Application of cerulein i.p. in relaflx/flx mice induced rapid and pronounced NF-κB/Rel activation as early as 30 minutes after the first injection (Figure 2A). Kinetics of IκBα degradation in relaflx/flx and relaΔ/Δ mice were assessed by Western blot analyzing the protein content of 40 μg of pancreatic whole-cell protein lysates using an antibody against IκBα. Phosphorylated IκBα (p-IκBα; Ser32) was monitored by Western blotting using a phospho-specific antibody.

Figure 1
Pancreas-specific truncation of RelA/p65 using a Cre-loxP system. (A) Two identically oriented loxP sites (triangles) flank exons 7 and 10 of the rela gene. loxPmod, modified loxP site. (B) Recombination of genomic DNA from the pancreata of relaΔ/Δ mice was detected by Southern blot analysis using a probe external to the 5’ end of the targeting construct. No recombination was detected in the other organs. (C) Deletion of rela (exons 7–10) in the mouse pancreas was demonstrated at the protein level by Western blot of isolated acini from mice with the floxed allele in the presence or absence of Cre as indicated. In contrast to relaflx/flx mice, relaΔ/Δ mice display a truncated form of RelA/p65 (Δp65). Pancreas protein extracts (40 μg) were analyzed using antibodies against p50, IκBα, and β-actin (as a loading control). The protein marker (PM) indicates the size of detected protein bands.

Figure 2
Inhibition of nuclear translocation of RelA/p65 in the pancreas following cerulein stimulation. (A) relaflx/flx and relaΔ/Δ mice were injected i.p. with 50 μg/kg cerulein at hourly intervals. Pancreatic nuclear protein extracts (10 μg) at the indicated time points were subjected to gel retardation assays with an NF-κB consensus probe. (B) Kinetics of IκBα degradation in relaflx/flx and relaΔ/Δ mice were assessed by Western blot analyzing the protein content of 40 μg of pancreatic whole-cell protein lysates using an antibody against IκBα. Phosphorylated IκBα (p-IκBα; Ser32) was monitored by Western blotting using a phospho-specific antibody.
Pancreas-specific truncation of RelA/p65 exacerabrates AP. (A) rela^{lox/lox} and rela^{Δ/Δ} mice were given 8 hourly i.p. injections of cerulein (50 μg/kg) and sacrificed 8, 12, or 24 hours after the first injection. Histological sections of rela^{lox/lox} and rela^{Δ/Δ} mice were analyzed at the indicated time points. Note the increased vacuolization, morphologically apoptotic cells, ghost cells, edema, infiltration, and massive necrosis in the rela^{Δ/Δ} pancreata. (B) Pancreatic injury was determined by measuring amylase and LDH enzyme activity in serum. Total tissue homogenates were obtained from pancreata of cerulin-injected mice at the indicated time points and subjected to trypsin activity analysis. Pancreatic edema was determined indirectly by increase in pancreatic weight. (C) H&E-stained pancreas sections from rela^{lox/lox} and rela^{Δ/Δ} mice 24 hours after cerulein-induced inflammation were used to measure and quantify necrotic parenchymal surface area. TUNEL assay results are expressed as the apoptotic index of pancreata from mice with AP. Apoptotic cells exhibited black nuclei. (D) L-Arginine–induced pancreatitis was evaluated 72 hours after induction. Pancreata and lungs were removed for morphological analysis by H&E. Note the appearance of focal necrosis in the pancreata of both groups. (E) Serum was removed for amylase and lipase evaluation at the indicated time points. Note the significant release of amylase and lipase into the serum in rela^{Δ/Δ} mice. Lung inflammation was evaluated as described in Methods. Values are mean ± SD for independent animals (n = 5). *P < 0.05 versus rela^{lox/lox}. Original magnification, ×50 (A, inset); ×200 (A and D); ×100 (C, inset); ×100 (C).

Next, we determined whether the lack of functional RelA/p65 in the pancreas was responsible for the impaired induction of PAP1. To identify the genes responsible for the RelA/p65-mediated protective effect in the pancreas, we performed microarray analysis and real-time PCR. The pancreas-specific acute phase protein PAP1 was downregulated in resting pancreas and was not induced following acute experimental pancreatitis in rela^{Δ/Δ} mice (Figure 6, A and B). In contrast, previous studies showed that PAP1 was induced during pancreatitis and correlated with its severity (6, 10). We generated an antibody to an N-terminal peptide sequence of murine PAP1 in rabbits and then immunoblotted pancreatic protein lysates to determine the expression level of PAP1 (Supplemental Figure 2; supplemental material available online with this article; doi:10.1172/JCI29882DS1). While PAP1 was induced 12 hours after the first injection of cerulein and peaked after 24 hours in littermate controls, the induction of PAP1 was virtually absent in rela^{Δ/Δ} mice (Figure 6C).

Figure 4
Analysis of MAPK modules during cerulein-induced pancreatitis. Pancreata were removed at the indicated time points. Whole-cell lysates (30 μg) were blotted against phosphorylated p42, p44, p38, p46, and p54 and their respective unphosphorylated proteins.
TATA box of the PAP1 gene, which could be a target of nuclear RelA/p65. To directly analyze the interaction of RelA/p65 with the PAP1 promoter/enhancer region, chromatin immunoprecipitations with a RelA/p65 antibody were performed on pancreas lysates at various time points following induction of inflammation. As shown in Figure 6D, RelA/p65 recruitment to the κB1 site in the PAP1 promoter was evident in control mice, with a peak at 24 hours after the onset of pancreatitis. In relaΔ/Δ mice, RelA/p65 was not recruited to the promoter of PAP1. Thus, this recruitment was specific. The κB2 site was not targeted by RelA/p65 protein in either type of mice (Figure 6D). Immunohistochemical analysis of the pancreatic tissues from relaΔ/Δ mice showed localized PAP1 expression at the apical regions of acini, which is typical for secretory proteins. No PAP1 expression was detected in the pancreata of relaΔ/Δ mice 12 or 24 hours after cerulein injections (Figure 6E). Taken together, these data indicate that PAP1 regulation is impaired in the relaΔ/Δ pancreas.

PAP1 is involved in acinar cell death during pancreatitis. To clarify whether the extent of tissue damage during AP is regulated by overexpression of PAP1, we used siRNA to knock down PAP1 expression during inflammation in control littersmates (32). First, we tested the ability of i.p. injected siRNA to penetrate the pancreas by using FITC-marked constructs (Supplemental Figure 3, A–D). Large areas of the liver and most of the pancreatic acinar cells were targeted with FITC-marked siRNA. Second, we tested 2 different siRNAs for their ability to inhibit cerulein-induced overexpression of PAP1 in vivo. Scrambled duplex siRNA (SDI) or specific siRNAs (PAP1 90 and PAP1 288) were administered to mice, which were then subjected to cerulein-induced pancreatitis (Figure 7A). PAP1 288 reduced PAP1 overexpression by nearly 80%, while PAP1 90 reduced PAP1 overexpression by 40% (Figure 7B and Supplemental Figure 3, E and F). Specific and nonspecific siRNAs did not substantially alter early NF-κB/Rel activation, as assessed by EMSA (Figure 7C). Mice that received PAP1 288 displayed a worse histology phenotype, with extensive areas of necrosis and increased LDH serum levels, compared with mice treated with SDI or PAP1 90 (Figure 7, D and E). MPO activity levels in the pancreas were also higher in mice pretreated with PAP1 288 (Figure 7F). These data suggest that interference with PAP1 expression promotes a course of pancreatitis with increased severity.

We next investigated whether delivery of PAP1 cDNA into relaΔ/Δ mice could attenuate the severity of AP. Therefore, we generated lentivirus vectors that contain the full-length murine PAP1 cDNA. A lentivirus containing the lacZ gene served as control. We treated relaΔ/Δ and relaΔ/Δ mice according to an established schedule (Figure 8A and Supplemental Figure 4). Pancreatic homogenates were analyzed for PAP1 expression 12 hours after the first cerulein injection. Cell death was evaluated morphologically 12 and 24 hours after the first injection of cerulein. ImmunobLOTS confirmed that lentiviral gene transfer of PAP1 in relaΔ/Δ mice resulted in protein levels comparable to those following cerulein-induced pancreatitis in littermate controls. Transfer of the lacZ gene did not alter the basal expression levels of PAP1. Transfer of the lacZ gene into relaΔ/Δ mice displayed numerous TUNEL-positive cells (Figure 8C), mice infected with lentiviral PAP1 displayed reduced areas of necrosis, indicating that PAP1 partially rescued the necrotic phenotype (Figure 8D). Morphologically, lentiviral expression of PAP1 in relaΔ/Δ mice slightly decreased the characteristic lung inflammation. This might be simply a manifestation of the more severe pancreatitis in relaΔ/Δ mice.

These results indicate that RelA/p65 is involved in the transcriptional regulation of the acute-phase protein PAP1. Furthermore, upregulation of PAP1 was crucial for the protection of acinar cells following cerulein-induced pancreatitis. PAP1 had no significant effect on apoptosis, while it significantly reduced necrotic cell
death. Because PAP1 did not fully rescue the inflammatory phenotype in relaΔ/Δ mice, it is likely that other NF-kB targets are also involved in the protective effects of NF-kB.

**Figure 6**

Impaired upregulation of murine PAP1 during AP in relaΔ/Δ mice. (A) and (B) Pancreata from relaflox/fl ox and relaΔ/Δ mice were removed at the indicated times. (A) Total pancreatic RNA (8 μg; n = 2) was labeled and hybridized to Affymetrix MOE430A GeneChips, and pancreas-specific genes were clustered hierarchically. (B) Relative levels of PAP1 mRNA were determined by real-time PCR and expressed as mean ± SD (n = 5). (C) Pancreatic tissues were removed at the indicated times. Whole-tissue extracts were prepared and subjected to Western blot analysis using a newly generated antibody to murine PAP1. (D) Chromatin immunoprecipitation experiments were performed with relaΔ/Δ and relaflox/fl ox pancreatic tissue at the indicated times after stimulation with cerulein using an antibody to p65. Precipitated DNA was analyzed by PCR using primers surrounding the positions of both κB sites in the respective promoters. PCR was also performed with 2.5% of input chromatin to ensure equal loading. (E) Sections of snap-frozen pancreata and duodenum were prepared and analyzed for PAP1 expression in relafl ox/fl ox and relaΔ/Δ mice in unstimulated pancreas and 12 and 24 hours after the first cerulein injection, respectively. Snap-frozen duodenum served as a positive control, because Paneth cells are known to express PAP1. Signals in the pancreas were localized to the apical regions of acini (arrows), typical for secretory proteins like PAP1.

Ablation of RelA/p65 in acinar cells increased extrapancreatic damage during AP. Severe necrotizing pancreatitis is characterized by a systemic inflammatory response that mostly targets the lung.
and causes ARDS in humans. In order to determine the systemic inflammatory response following pancreatitis in our mouse model, we studied serum levels of IL-6, morphological changes in the lung, and the degree of leukocyte infiltration to the lung. While the expression levels of wild-type RelA/p65 in the lung remained unaffected in both mouse lines (Figure 9A), the morphological appearance following AP displayed obvious differences. In contrast to littermate controls, rela−/− mice exhibited severe inflammation of the lungs as indicated by alveolar fluid accumulation and progressive thickening, hyperemia, and neutrophil infiltration of the interalveolar tissue (Figure 9, B–H). Serum levels of IL-6 were significantly higher in rela−/− mice than in littermate controls (Figure 9I). Extensive lung inflammation and the subsequent reduced oxygenation of the blood produced morphological changes of the liver including centrilobular cell swelling with small vacuoles and fatty deposits, probably a result of disturbed β-oxidation (data not shown). From these data, we conclude that deletion of the rela gene (exons 7–10) in the pancreas resulted in SIRS.

**Discussion**

Using a genetic approach, we demonstrated that truncation of RelA/p65 in acinar cells did not ameliorate the course of AP, but provoked a severe necrotic AP characterized by extensive cell death, mononuclear infiltration, and signs of both MODS and ARDS. Therefore, our results clearly challenge the current view of the role of NF-κB/Rel in the initiation and course of AP and demonstrate a protective and organ-specific role of endogenous RelA/p65 during AP (Figure 10).

Our data provide unambiguous and clear proof that endogenously activated NF-κB/Rel in acinar cells is not just an indicator of a cellular stress response, but plays a crucial role in limiting tissue injury and spread of the inflammatory response in the cerulein pancreatitis model. While induction of apoptotic cell death has been suggested to improve the outcome in AP, acinar cell necrosis results in a serious course of AP (33–37). In our model, the increase in necrosis was far greater than the induction of apoptosis. The mechanism by which the decision between apoptosis and necrosis is arbitrated is not well understood. NF-κB/Rel may act as a switch in the decision process in acinar cells. This effect seems to be cell specific. Wild-type MEFs are resistant to TNF-α–induced cell death. Upon TNF-α stimulation, MEFs that are deficient for RelA/p65 display signs of apoptotic cell death but predominantly display features of necrosis, specifically the loss of membrane integrity without apparent damage of nuclei. Thus, necrosis was proposed to be a result of intracellular accumulation of ROS, which mediate pro-
longed activation of the MAPK modules in their system (24, 38). MAPK modules are known to be transiently activated during cerulein-induced pancreatitis (25–31). Inhibition of these modules has been associated with an improvement in the outcome during AP (27, 39–41). By analyzing these pathways in our mouse model, we also detected a prolonged activation of MAPK modules (ERK1/2 [p42/44], p38, and SAPK [p46/54]) during inflammation in the relaΔ/Δ pancreas (Figure 4). Taken together, our data and the results from these previous studies suggest that functional RelA/p65 is required to prevent cell death in the inflamed pancreas.

Intracellular conversion of zymogens to their activated forms is a critical step in the initiation of AP. It has been suggested that premature intra-acinar activation of trypsinogen catalyzes the intra-pancreatic activation of digestive zymogens and pancreatic autodigestion in experimental animal models as well as in humans, although other studies discussed the dispensability of trypsin activity in this scenario (42–47). Because cleavage of trypsinogen and activation of NF-kB/Rel display similar kinetics, previous studies assessed the relationship between trypsinogen activation and NF-kB/Rel induction (48, 49). We found that early activation of trypsinogen was not affected in relaΔ/Δ mice compared with littermate controls, while late activation was dramatically increased in relaΔ/Δ mice. This finding demonstrates that the initiation of trypsinogen activation is independent of NF-kB/Rel, as previously proposed (50, 51). Late, pronounced conversion of trypsinogen to trypsin might result from the increased tissue damage and non-specific activation of serine proteases. Pronounced injury in the pancreata of relaΔ/Δ mice was not a consequence of increased early trypsin activity, because trypsin activity in these mice was even lower 3 hours after the onset of AP. Therefore, other mechanisms must account for the protective effects mediated by NF-kB/Rel.

To identify potential protective NF-kB/Rel target genes, we performed microarray analysis using the pancreata from relaΔ/Δ mice and from control littermates. We found that PAP1 was downregulated in unstimulated pancreas and was not induced during AP in relaΔ/Δ mice. Several functions have been proposed for PAP1 (6, 10, 52). PAP1 prevented apoptosis and necrosis following oxidative stress or TNF-α stimulation in an AR42J pancreatic acinar cell line (12, 53, 54). Anti-inflammatory activity has been attributed to PAP1 in AR42J cells and in RAW cells, in which exogenous

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**Figure 8**

Protective effect of PAP1 on cerulein-induced pancreatitis in relaΔ/Δ mice. (A) Lentivirus harboring the full-length cDNA of murine PAP1 was generated in HEK 293T cells and injected i.p. into relaΔ/Δ mice to express PAP1 (pLenti4-PAP1) in the pancreas (circles). Four age- and sex-matched mice were used. Littermate control mice were infected with lentivirus (pLenti4-LacZ) containing the lacZ gene. Mice were subjected to cerulein-induced pancreatitis (arrows) 7 days after infection and then were sacrificed 12 and 24 hours after the first cerulein injection. (B) Pancreatic homogenates were obtained 12 hours after the first cerulein injection and analyzed for PAP1 expression. (C) Representative H&E-stained sections of pancreata and lungs from mice treated as in A. Original magnification, ×100. (D) To assess the extent of tissue injury, necrosis and apoptosis were evaluated. Areas of necrotic parenchymal surface were measured and quantified. TUNEL assay was used to determine the apoptotic index of the pancreas. Values are mean ± SD for independent animals (n = 4). *P < 0.05.
purified PAP1 inhibited both TNF-α-mediated NF-κB/Rel activation and IL-6 and TNF-α production (11, 12). In this context, it is conceivable that the lack of PAP1 in relaΔ/Δ mice causes prolonged activation of NF-κB/Rel in inflammatory cells. Indeed, in contrast to relaΔ/Δ mice, we detected late NF-κB/Rel activation in relaΔ/Δ mice (data not shown). Among other factors, prolonged activation of NF-κB/Rel in inflammatory cells might be responsible for enhanced systemic complications during AP in relaΔ/Δ mice (11).

Although lentivirus-mediated expression of PAP1 in relaΔ/Δ mice slightly decreased the characteristic lung inflammation as evaluated morphologically, this might be simply a manifestation of the more severe pancreatitis in relaΔ/Δ mice, although it has previously been shown that PAP1 administration enhances pancreatitis-associated lung inflammation in rats (55). In general, our data support a beneficial role for PAP in AP, as previously suggested in studies of rats (56). First, siRNA-mediated knockdown of PAP1 resulted in increased infiltration and necrosis in nontransgenic wild-type mice. Second, lentivirus-mediated complementation of PAP1 in relaΔ/Δ mice significantly reduced the extent of necrosis, while it had no significant effect on apoptosis. Therefore, other RelA/p65-dependent mechanisms involved in mediating apoptosis might contribute to increased programmed cell death. Indeed, impaired upregulation of Bcl-XL in the pancreas with functional inactive

Figure 9
Pancreas-specific ablation of RelA/p65 promotes systemic complications and multiorgan dysfunction syndrome. (A) Expression of RelA/p65 in lung tissue of relaΔ/Δ and relaΔ/Δ mice was assessed by subjecting 20 μg of lung whole cell lysates to Western blot analysis. (B–G) At the indicated times after cerulein injection, lung tissue was removed, embedded in paraffin, and stained with H&E. Higher magnification of representative H&E stains reveal marked hemorrhage and alveolar collapse in relaΔ/Δ mice compared with relaΔ/Δ mice. Original magnification, ×100 (B–E); ×200 (F and G). (H) Lung tissue of relaΔ/Δ and relaΔ/Δ mice was removed at the indicated time points and used to determine MPO enzyme activity. (I) Serum concentrations of IL-6 were determined at the time points indicated. Data represent the mean ± SD of 5 animals. *P < 0.05 versus relaΔ/Δ/Δ.

RelA/p65 might contribute to increased apoptosis (our unpublished observations), a mechanism that has recently been proposed in IKK2-deficient enterocytes (57). In a further study with a model of gut ischemia/reperfusion in mice with enterocyte-specific deletion of IKK2 resulted in increased apoptosis and less systemic complications (58). Although these results might be paradoxical at the first glance, they support the hypothesis that necrotic cell death, but not apoptosis, is critical for SIRS during acute inflammation. Therefore, these data confirm that PAPI protects acinar cells from necrosis rather than from apoptosis. Moreover, truncation of RelA/p65 might be responsible for still observable increased programmed cell death.

In summary, our data suggest that deletion of IKK and NF-κB/Rel signaling exerts organ-specific effects. Although antinflammatory effects have been attributed to NF-κB/Rel inhibitors, our data underline that caution should be exercised in using them during severe inflammatory processes in the pancreas. The contribution of NF-κB/Rel activity in other compartments during acute pancreatitis has to be elucidated in further studies using genetic approaches.

Methods
Generation of Ptf1a-creΔ/Δ and relaΔ/Δ mice. The relaΔ/Δ mice were generated according to a previously described 2-step procedure (59). A targeting vector was constructed to insert a modified loxP site (60) and the selection marker genes PGK-neo and TK into the relaΔ/ΔΔ gene. The mouse relaΔ/Δ contains exons 7–10, which code a part of the Rel homology domain and the nuclear localization site. After electroporation, the transfected ES cells were selected with G418 (0.2 mg/ml), and recombinants were identified by PCR analysis. One recombinant was further cultured and then transfected with a Cre recombinase expression vector to delete the selection marker genes and to generate relaΔ/ΔΔ ES cells. The relaΔ/ΔΔ ES cells were screened by Southern blotting using a probe external to the S’ end of the targeting construct. Two relaΔ/ΔΔ ES clones were injected into C57BL/6 blastocysts. The progeny with the highest degree of chimerism were crossed with C57BL/6 mice to derive the experimental lines.

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Model for RelA/p65 function in AP. During inflammation in the pancreas, acinar cells undergo apoptotic and necrotic cell death. Cellular constituents are released from injured necrotic acinar cells following stimulation with cerulein. This feature is enhanced when functional RelA/p65 is lacking. Increased acinar cell necrosis causes infiltration by mononuclear cells, which are activated to produce cytokines such as IL-6 and TNF-α that cause systemic effects including lung inflammation.
filtration system, and concentrated by ultracentrifugation. Lentiviral vector titers were determined by HIV-1 p24 ELISA (PerkinElmer). Mice were infected with equal viral concentrations every third day (3 injections total) and followed 7 days before the initiation of pancreatitis.

**Histology, immunohistochemistry, and TUNEL.** For morphological analyses, pancreatic and lung tissues were removed, immediately immersed in 4% neutral phosphate-buffered paraformaldehyde for 12 hours, embedded in paraffin, and sectioned (5 μm). Sections were processed for immunohistochemistry as described previously (14). Frozen pieces of pancreas were embedded in Tissue-Tek (Sakura), and 10-μm sections were processed for immunohistochemistry. The TUNEL assay was performed using the in situ cell death kit (POD; Roche Diagnostics) according to the manufacturer’s instructions. Antibody against TNF-α was purchased from Santa Cruz Biotechnology Inc., antibody against β-galactosidase was purchased from Sigma-Aldrich, and the Gr-1 antibody was purchased from BD Biosciences—Pharmingen.

**Quantification of necrosis.** Pancreatic tissue sections were stained with H&E. Necrotic cells with swollen cytoplasm, loss of plasma membrane integrity, and leakage of organelles into the interstitium were counted and evaluated by 2 researchers in a blinded manner. Necrosis was expressed as the percentage of examined pancreatic parenchyma.

**Histopathologic score.** For evaluation of lung inflammation during pancreatitis we randomly chose 10 microscopic fields for each mouse (n = 4). Alveolar wall thickening was scored by 2 researchers in a blinded manner as follows: 0, no lung involvement; 1, mild (<25%) lung involvement; 2, moderate (25%–50%) lung involvement; 3, severe (50%–75%) lung involvement.

**Microarray analysis of gene expression.** Total pancreatic RNA (8 μg) was labeled and hybridized to Affymetrix MOE430A GeneChips according to the manufacturer’s instructions. Two biological replicates per mouse line were collected with 80 μl of salmon sperm–saturated protein A agarose (QIAGEN) and for immunohistochemistry as described previously (14). Transcribed cDNA was further analyzed by real-time PCR as described by Chen et al. (58). The following primer sequences were used: GAPII mRNA, forward 5′-CTCTGCTTGTACGGGTTCT-3′, reverse 5′-TTGT-TACTCCACTCCCATTCC-3′; cyclophilin cDNA, forward 5′-ATGTGCAACACCCCCACGTG-3′, reverse 5′-TTCCTGCTTGTACGGGTTCTT-3′. All values were normalized to the level of cyclophilin cDNA.

**Statistics.** Data are expressed as mean ± SD. Differences were analyzed by Student’s t test. In all cases, sample sizes were chosen to produce statistically unambiguous results. A P value of 0.05 or less was considered significant.

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