ANNALS OF SURGERY
Vol. 223, No. 2, 163–169

Active Interleukin-1 Receptor Required for Maximal Progression of Acute Pancreatitis

James G. Norman, M.D.,* Gregory Fink, M.D.,* Michael Franz, M.D.,* Jean Guffey, M.D.,†
Gay Carter, B.S.,* Barry Davison, Ph.D.,‡ Cheryl Sexton, B.S.,* and Moira Glaccum, M.S.‡

From the Departments of Surgery and Surgical Pathology,† University of South Florida, Tampa, Florida; and Immunex Corporation,‡ Seattle, Washington*

Objective
The authors' aim was to determine the requirement for an active interleukin (IL)-1 receptor during the development and progression of acute pancreatitis.

Summary of Background Data
Interleukin-1 is a pro-inflammatory cytokine that has been shown to be produced during acute pancreatitis. Earlier animal studies of moderate and severe pancreatitis have shown that blockade of this powerful mediator is associated with attenuated pancreatic destruction and dramatic increases in survival. The exact role played by IL-1 and the requirement for activation of its receptor in the initiation and progression of pancreatitis is unknown.

Methods
Conventional and IL-1 receptor "knockout" animals were used in parallel experiments of acute pancreatitis induced by intraperitoneal injection of cerulean (50 μg/kg every 1 hour × 4). The conventional mouse strain had the IL-1 receptor blocked prophylactically by means of a recombinant IL-1 receptor antagonist (10 mg/kg injected intraperitoneally every 2 hours). The second mouse strain was genetically engineered by means of gene targeting in murine embryonic stem cells to be devoid of type 1 IL-1 receptor (IL-1 receptor knockout). Animals were killed at 0, 0.5, 1, 2, 4, and 8 hours, with the severity of pancreatitis determined by serum amylase, lipase, and IL-6 levels and blind histologic grading. Strain-specific controls were used for comparison.

Results
The genetic absence of the IL-1 receptor or its pharmacologic blockade resulted in significantly attenuated pancreatic vacuolization, edema, necrosis, inflammation, and enzyme release. Serum IL-6, a marker of inflammation severity, was dramatically decreased in both groups.

Conclusions
Activation of the IL-1 receptor is not required for the development of pancreatitis but apparently is necessary for the maximal propagation of pancreatic injury and its associated inflammation.

Interleukin (IL)-1 is an inflammatory cytokine produced during acute and chronic inflammatory conditions and is believed to be primarily responsible for many of the signs and symptoms of clinical sepsis.[1-3] More recently, IL-1 has been implicated in the progression of acute pancreatitis in human disease and in several animal models. Clinical pancreatitis is associated with high circulating levels of this proximal inflammatory mediator, with serum concentrations correlating with morbidity and mortality rates.[4,5] Similarly, several animal models of acute pancreatitis examined have shown rapid rises in serum levels of IL-1, which correspond to the severity of pancreatic damage.[6-8] Earlier studies from our laboratory have shown IL-1 to be produced within the pancreatic parenchyma during acute pancreatitis, with concentrations several-fold higher than those of corresponding serum levels.[6] More recently, we have observed the rapid induction of IL-1 mRNA within the pancreas during the course of pancreatitis followed by the appearance of its active protein product, thereby establishing the production of this cytokine within the pancreas.[9] Further support that IL-1 is important in the pathogenesis of acute pancreatitis is the protective effect that specific receptor antagonist provided in a lethal model of acute hemorrhagic necrotizing pancreatitis.[7]

There is a limited understanding of the many *in vivo* functions attributable to IL-1 during pancreatitis and the means by which its antagonism elicits protective effects. The role that this pro-inflammatory cytokine plays in the progression of pancreatic destruction, enzyme release, autodigestion, and eventual development of distant organ dysfunction remains unclear, in part due to the complex interactions among cytokines and their inherent redundancy and pleiotropy. The lack of good models possessing the ability to isolate the actions of individual cytokines has also contributed to this deficiency. We specifically designed the current study to isolate and identify the extent to which IL-1 contributes to the *in vivo* propagation of acute pancreatitis and intrinsic pancreatic damage.

METHODS

Experimental Design

Two strains of mice were used in a single model of acute pancreatitis. The first was a conventional NIH Swiss mouse strain that underwent IL-1 receptor blockade by the administration of a specific recombinant IL-1 receptor antagonist (IL-1ra). The second strain used involved C57BL mice that had been genetically engineered for a homozygous deletion, or "knockout," of the type 1 IL-1 receptor. These transgenic animals are unable to recognize or respond to IL-1. Separate strain-specific control groups (discussed below) were used for each series of experiments so that comparisons could be made between animals of the same strain.

Acute Pancreatitis

Acute pancreatitis was induced in all animals with the intraperitoneal administration of cerulean (Bachem, Torrence, CA) (50 μg/kg every 1 hour × 4). A minimum of 12 animals from each group were killed at 0, 0.5, 1, 2, 4, and 8 hours by cardiocentesis. The severity of pancreatitis was determined by serum amylase, lipase, and IL-6 levels and histologic grading.

Histologic Grading

All pancreata were excised rapidly, fixed in 10% buffered formalin, and stained with hematoxylin and eosin for light microscopy. Pancreatic sections were graded to establish the severity of pancreatitis (0–4, normal to severe, respectively) for degree of edema, necrosis, vacuolization, and inflammation, as described.[10] Six high-power fields of each specimen were graded on three separate occasions (18 readings per pancreas). Histologic grading was performed by a single pathologist (J.G.) who was blinded to experimental groups. Slides were relabeled before each subsequent reading.

Interleukin-1 Antagonism

Recombinant IL-1ra (Synergen, Boulder, CO) (10 mg/kg administered intraperitoneally) was administered to conventional adult male NIH Swiss mice (33.2 ± 0.4 g) 1 hour before administration of the first dose of cerulean and repeated every 2 hours. Comparisons were made with identical control animals that received intraperitoneal vehicle (normal saline) injections instead of IL-1ra.

Knockout Animals

Mice deficient for the expression of the type 1 IL-1 receptor were generated by the process of gene targeting in murine embryonic stem cells.[11-14] Briefly, a DNA targeting vector containing portions of a deleted type 1 IL-1 (P80) receptor gene (nonsense construct) was introduced into 129/SV-derived embryonic stem cells by electroporation. Neomycin-resistant colonies were selected

Presented at the 29th Annual Meeting of the Pancreas Club; May 14, 1995; San Diego, California.

Address reprint requests to James G. Norman, M.D., University of South Florida, Department of Surgery 112, 13000 Bruce B. Downs Boulevard, Tampa, FL 33612.

Accepted for publication June 1, 1995.

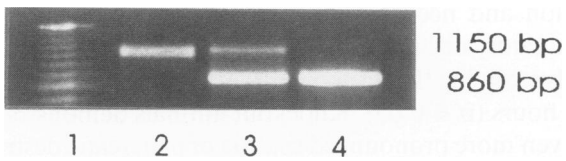

Figure 1. Mouse type 1 IL-1 receptor genotypes. The PCR products of normal and transgenic C57BL/129J mice run in 1% agarose gels and stained with ethidium bromide are shown. Mice expressing the homozygous allele for the normal wild-type IL-1 receptor are shown in lane 2 (control animals). Lane 3 demonstrates the genotypic banding pattern of animals that were heterozygous for both the wild-type and nonsense IL-1 receptor genes. Transgenic knockout animals used in these experiments are shown in lane 4 and were homozygous for the nonsense allele and have had the wild-type IL-1 receptor gene knocked out. Lane 1 portrays a DNA ladder.

as described previously[15] and screened for homologous recombination events by means of the polymerase chain reaction (PCR) with diagnostic primers. Clones harboring specific gene targeting events were further characterized by Southern blotting to confirm that they were rendered heterozygous for the type 1 IL-1 receptor gene. The mutated embryonic stem cells were injected into 3-day-old blastocysts (C57BL/6J) and then transferred to the uteri of pseudopregnant recipient females to generate chimeras. Initially, chimeric animals were bred to normal C57BL/6J animals for germ-line transmission of the mutant allele. Pairs of the resulting heterozygous animals were then bred to produce mice with the null (−/−) genotype. Figure 1 shows the PCR products for C57BL/129J hybrid control mice (wild type for the normal type 1 IL-1 receptor alleles; used as control animals for all knockout experiments) as well as for those animals that were heterozygous or homozygous for the nonsense gene. All transgenic knockout animals used in these experiments had been shown to be homozygous (−/−) for the knockout event. Subsequent screening for type 1 IL-1 receptor mRNA by reverse transcription (RT)-PCR has shown that no message is expressed (manuscript in preparation). Similarly, Western blot analysis has shown these animals to produce no detectable IL-1 receptor protein.

Serum Amylase and Lipase Levels

Serum amyiase and lipase levels were determined by means of a Kodak Ektachem 700 automated analyzer (Kodak, Rochester, NY) standardized for these murine proteins. All samples were assayed in triplicate and averaged.

Serum Interleukin-6 Concentration

Serum concentrations of IL-6 were measured with the enzyme-linked immunosorbent assay utilizing murine-specific primary and biotinylated secondary antibodies (rat anti-murine IL-6, PharMingen, San Diego, CA). A standard curve was prepared with the use of recombinant murine IL-6 (PharMingen) run in conjunction with the serum samples. Optical density was determined at 405 nm with use of an automated plate reader and analyzed with the Immunosoft software package (Dynatech Laboratories, Chantilly, VA).

Quantitative Immunohistochemistry

Immunohistochemistry was performed for the macrophage-specific CD14 myeloid differentiation antigen on serially prepared sections of pancreata as described previously[16] and was then analyzed with digital optical scanning.[17] Briefly, pancreata were immediately snap frozen in liquid nitrogen and stored at −80 C. until use. Cryostat sections (7 μm) were acetone fixed at 4 C. and then blocked with 4% normal rabbit serum. Subsequently, fluoroisothiocyanate-labeled anti-CD14 antibodies (1:500 vol/vol in phosphate-buffered saline, PharMingen) were applied for 2 hours, washed, dried, and then photographed digitally and stored on computer disk. The number of macrophages infiltrating the pancreas were counted on ten or more representative high-power fields (at 100× magnification) from each specimen by a blinded microscopist. Optical densities of these same sections were also obtained and compared statistically with use of Sigma Scan software (Jandel Scientific, San Rafael, CA).

Statistical Analysis

Results are expressed as mean ± SEM and were analyzed with use of an EPISTAT statistical program (Epistat Services, Richardson, TX) applying a Wilcoxon test for comparison of serum proteins. For comparison of histologic grading the two-tailed Student's t test was used.

RESULTS

Serum Amylase and Lipase Levels

Serum levels of amylase and lipase rose rapidly in the two control groups after induction of pancreatitis ($p < 0.001$ compared to time 0) (Figs. 2 and 3, respectively). There was no statistical difference found in maximal enzyme release between the two strains of control animals. The prophylactic administration of IL-1ra blunted the rise in amylase and lipase levels. Likewise, serum amylase and lipase levels were significantly attenuated in those animals genetically devoid of IL-1 receptors (both groups, $p < 0.01$ compared with their respective con-

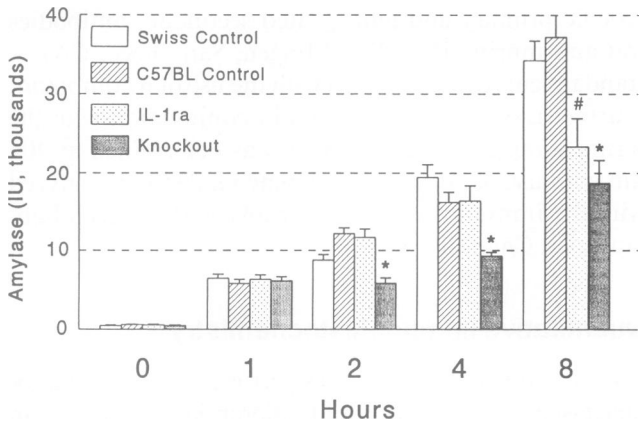

Figure 2. Serum amylase progression during acute pancreatitis. Maximal serum amylase levels were observed in all animals at 8 hours (all, $p < 0.001$ *vs.* time 0). The IL-1ra treated animals as well as the knockout animals demonstrated decreased serum amylase levels compared with their respective controls (# = IL-1ra $p < 0.01$ *vs.* Swiss control; * = knockout $p < 0.01$ *vs.* C57BL control). The animals devoid of IL-1 receptors showed attenuated levels throughout the study period, whereas the IL-1ra group showed only an attenuation of maximal values.

trols). In general, receptor antagonism was as effective in suppressing the release of amylase into the serum as was the genetic knockout of the IL-1 receptor. The knockout animals, however, did show significantly less lipase release at 4 and 8 hours (knockout vs. IL-1ra, $p < 0.05$).

Serum Interleukin-6 Concentration

Serum IL-6 concentrations rose progressively in both groups of control animals as pancreatitis evolved, reaching maximal values at 8 hours (both $p < 0.001$ compared to time 0) (Fig. 4). The prophylactic administration of IL-1ra was shown to decrease this response at all later time points ($p < 0.01$). All knockout animals demonstrated a similar decrease in IL-6 production ($p < 0.01$), which was not significantly different from that of the antagonist-treated animals.

Histologic Grading

Animals receiving cerulean developed a characteristic, progressive necrotizing pancreatitis that reached maximal intensity at 8 hours. Table 1 lists the maximal (8-hour) blind histologic grading for all groups of animals after the induction of pancreatitis. There was a significant ($p < 0.05$) increase in edema, acinar vacuolization, necrosis, and inflammatory cell infiltrate in both groups of control animals when compared with baseline (baseline normal values were defined as 0 for all four parameters). Maximal histologic scores did not differ between the two strains of control animals. The administration of IL-1ra significantly decreased the degree of edema formation and necrosis (both $p < 0.05$ *vs.* control). Inflammatory cell infiltrate was also decreased in most animals receiving IL-1ra and reached statistical significance at 8 hours ($p < 0.05$). Knockout animals demonstrated an even more pronounced sparing of pancreatic destruction. It is important to note that these animals demonstrated an appreciable attenuation of each of the four histologic criteria used to grade the severity of pancreatitis and pancreatic damage (all $p < 0.05$ *vs.* control).

Macrophage Infiltration

All groups of animals failed to show the presence of CD14-positive cells within the pancreatic parenchyma before the initiation of pancreatitis. Both control groups subsequently demonstrated a progressive infiltration of these leukocytes into the inflamed pancreas, which reached a maximum level at 8 hours (Swiss = 30.9 ± 0.7/high-power field; C57BL/129J = 33.9 ± 0.6/high-power field, both $p < 0.001$ compared to baseline). There was significant attenuation of macrophage infiltration in the receptor antagonist and knockout animals (IL-1ra = 4.5 ± 0.4, knockout = 6.2 ± 0.3, both $p < 0.05$ *vs.* controls). Blockade of the receptor using IL-1ra was as effective in preventing macrophage infiltration as was absence of the receptor (IL-1ra *vs.* knockout, $p = 0.19$).

DISCUSSION

Interleukin-1 elicits a wide range of physiologic responses of great importance to immune regulation and inflammation. Although IL-1 is produced by many

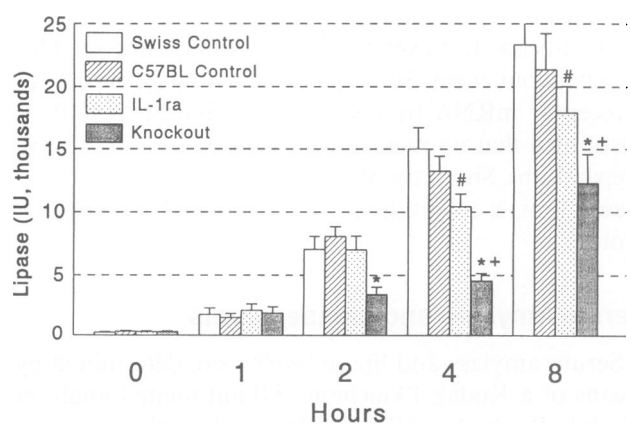

Figure 3. Serum lipase progression during acute pancreatitis. Maximal serum lipase was seen in all animals at 8 hours (all, $p < 0.001$ *vs.* time 0). Both the IL-1ra treated and the knockout animals demonstrated decreased serum lipase levels compared with their respective controls (# = IL-1ra, $p < 0.05$ *vs.* Swiss control; * = knockout, $p < 0.01$ *vs.* C57BL control). Knockout animals demonstrated significantly lower serum lipase levels than did the antagonist-treated animals at the 4- and 8-hour time points (+ = knockout, $p < 0.05$ *vs.* IL-1ra).

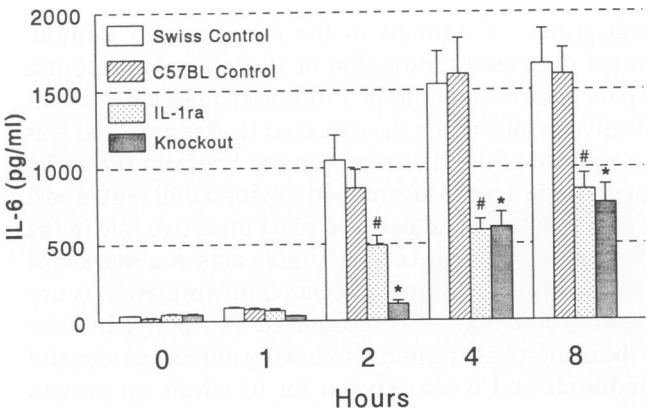

Figure 4. Serum IL-6 concentration during acute pancreatitis. The maximal IL-6 concentration was seen in both control groups at 8 hours (both, $p < 0.001$ *vs.* time 0). Antagonism or genetic loss of the IL-1 receptor resulted in a significant decrease in circulating IL-6 during the course of pancreatitis (# = IL-1ra, $p < 0.01$ *vs.* Swiss control; * = knockout, $p < 0.01$ *vs.* C57BL control).

different cell types, including nearly all cells of the immune system, the macrophage is the major producer during antigen stimulation.[18] Several investigators have shown that acute inflammation of the pancreas is associated with the rapid production of IL-1 (and other proinflammatory cytokines) in the absence of bacteria, endotoxin, and other foreign antigens.[6–8,19–23] Neither IL-1 nor its associated mRNA is found in the normal pancreatic parenchyma, but both are rapidly produced during the course of pancreatitis, with concentrations directly correlating with the degree of inflammation and acinar damage.[9] Serum levels of this powerful mediator have also been shown to correlate with clinical outcome during severe pancreatitis and are predictive of death in several animal models of pancreatitis.[5–8]

The degree to which cytokines are responsible for intrinsic pancreatic damage and the eventual involvement of distant unrelated organs into a systemic inflammatory response remains undefined. We have previously demonstrated that prophylactic or delayed administration of a specific IL-1 receptor antagonist in a model of lethal hemorrhagic necrotizing pancreatitis resulted in decreased mortality rates of 40% and 31%, respectively.[7] The mechanisms by which IL-1 antagonism provided this benefit was unclear, however, and seem to be multifactorial. We designed the current study, therefore, to specifically examine the *in vivo* role of IL-1 in pancreatic damage induced during a nonlethal form of acute pancreatitis. The use of transgenic knockout mice devoid of the type 1 IL-1 receptor (P80) provided us with the unique opportunity of independently scrutinizing the effects of IL-1 on all potentially effected cells of the animal[18,24–28] without the addition of exogenous agents or the concern for incomplete blockade.

We demonstrated in the current study that activity of the IL-1 receptor is required for maximal progression of cerulean-induced acute pancreatitis and suggests that IL-1 plays a major role in the pathogenesis of pancreatic destruction. Because pancreatitis still develops when the receptor is prophylactically blocked or genetically eliminated, it seems apparent that IL-1 is not required for the initiation of the intracellular events associated with vacuole formation and the actual autodigestion of pancreatic acinar cells.[28] A more likely role for IL-1 is to propagate and enhance these processes once they have been initiated. The absence of IL-1 activity was associated with decreased inflammation and edema formation, which may be directly attributable to the loss of the chemoattractant and vasopermeability properties of this cytokine. Interleukin-1 is known to enhance or induce the production of other inflammatory cytokines, including tumor necrosis factor, IL-6, and itself.[3,29,30] The inability of IL-1 to augment and amplify the normal acute inflammatory response in our experiments is believed to play a major role in attenuating the intrapancreatic processes involved in the progression of pancreatitis.

Although acinar cell vacuolization was not statistically reduced in the antagonist group, there was a trend toward this, as was demonstrated previously by our group with IL-1 antagonism during the first several days of the

Table 1. BLIND HISTOLOGIC SCORING OF PANCREATA

	Edema	Necrosis	Vacuolization	Inflammation
NIH Swiss control	3.6 ± 0.1	3.4 ± 0.1	3.8 ± 0.0	3.9 ± 0.0
C57 BL/129 J control	3.5 ± 0.1	3.6 ± 0.2	3.7 ± 0.1	3.8 ± 0.0
IL-1ra	2.1 ± 0.2*	1.8 ± 0.1*	3.5 ± 0.2	3.3 ± 0.1*
IL-1R knockout	2.0 ± 0.1†	2.1 ± 0.1†	1.5 ± 0.2†	2.8 ± 0.1†

NIH = National Institutes of Health; IL-1ra = interleukin-1 receptor antagonist; IL-1R = interleukin-1 receptor.

* Measures are maximal scores obtained (8 hrs). Baseline (time 0) scores are defined as 0 and are not shown. Comparisons are made between experimental groups and their respective strain-specific controls (*IL-1ra $p < 0.05$ *vs.* Swiss control; †knockout $p < 0.05$ *vs.* C57BL/129J control).

more severe choline-deficient, ethionine-supplemented diet model of pancreatitis.[7] The decreased vacuolization seen at all time points in the knockout animals, however, provides further evidence that IL-1 induces or amplifies intracellular events during pancreatitis, which are important to the formation of vacuoles. Similarly, acinar cell necrosis was reduced when IL-1 was prevented from having its effects within the pancreas and is presumed to be a direct result of lessened vacuolization and autodigestion. The mechanism by which IL-1 attenuates vacuolization formation and subsequent necrosis remains poorly defined. Possibly, IL-1 directly stimulates enzyme production, packaging, and/or release, although this is unknown. Additionally, the direct effects of IL-1 on intracellular processes, such as co-localization ongoing within the acinar cell during acute pancreatitis, is unknown. Studies of isolated pancreatic acinar cells are underway to examine these questions more closely.

We have previously shown that acute pancreatitis is associated with the infiltration of leukocytes into the pancreatic parenchyma, which are subsequently responsible for the majority of total intrapancreatic cytokine production.[31] Immunohistochemical staining has demonstrated that the vast majority of these infiltrating cytokine producers are macrophages. Total macrophage infiltration, therefore, correlates well with the degree of inflammation within the gland as well as with true intrapancreatic inflammatory cytokine levels. The current study demonstrated that IL-1 plays a major role in the recruitment of macrophages into the inflamed pancreas. Interleukin-1 is known to have specific and direct effects on leukocyte migration into damaged or inflamed tissues.[1-3] It can also effect leukocyte migration through the upregulation of L-selectin and ICAM-1 receptors.[32-35] The loss of this inflammation-inducing property of IL-1 as well as its direct chemoattractant properties are likely mechanisms by which our experimental animals failed to show maximal inflammation. It is also likely that direct pancreatic damage and necrosis secondary to the effects of the products of activated leukocytes is lessened in these animals simply because fewer activated cells are present.

The redundancy of the cytokine cascade and the ability of IL-1 to induce the production of IL-6 may also be of importance in determining the mechanisms by which inactivation of the IL-1 receptor results in attenuation of pancreatitis. Interleukin-6 has been shown to be generated during the course of pancreatitis, with serum levels correlating with outcome in several clinical studies.[5,19] Similar to the findings in our previous reports regarding IL-1 induction during pancreatitis, we have also shown that IL-6 is generated within the pancreas, with intrapancreatic and serum levels being directly associated with the degree of inflammation and parenchymal damage.[6] Both groups of animals in the current study demonstrated decreased production of IL-6 during the course of pancreatitis. Interleukin-1 receptor antagonism was as effective in alleviating the expected IL-6 response as was the absence of the IL-1 receptor and has been shown by our group in a previous study involving a different model of pancreatitis.[7] Whether IL-6 plays an active role in the destruction of acinar cells or simply acts as a marker of pancreatitis severity and associated inflammation is unknown. Interleukin-6 is recognized as a major inducer of the acute-phase response following numerous stressful conditions and is also known for its effects on protein production within various cell types *in vivo* and *in vitro*. The specific effects of this cytokine within the pancreas and on the intracellular processes within acinar cells are unknown.

It is apparent that IL-1 has specific although incompletely defined effects within the inflamed pancreas. Interleukin-1 activity is not necessary for the formation of vacuoles, the release of massive amounts of pancreatic enzymes into the serum, or the eventual lysis of acinar cells. This cytokine, however, does modulate these processes during acute pancreatitis and appears to be necessary for maximal experimental pancreatitis to become manifest. The use of transgenic knockout mice provides a unique model with which to investigate these properties *in vivo* and confirms our previous findings of the importance of IL-1 in a more severe model of pancreatitis. Further *in vivo* and *in vitro* studies are needed to more specifically identify those processes affected by successful activation and signal transduction of the IL-1 receptor.

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