Pancreatic and lung inflammation during acute pancreatitis is a poorly understood, but clinically important, phenomenon. The proto-oncogene Tpl2 (tumor progression locus-2) has recently been shown to have important immunomodulatory effects on some inflammatory processes, but its importance to pancreatitis has not been previously examined. Our studies were designed to (a) define the effects of Tpl2 on pancreatic and lung inflammation during pancreatitis and (b) identify mechanisms and cell types responsible for those effects. We examined pancreatitis-associated Tpl2 effects in wild type and Tpl2−/− mice subjected to either secretagogue-induced or bile salt-induced pancreatitis. To determine the myeloid or non-myeloid lineage of cells responsible for the Tpl2 effects, we used Tpl2−/− chimeric mice generated by lethal irradiation followed by bone marrow transplantation. Mechanisms responsible for the effects of Tpl2 ablation on caerulein-induced proinflammatory events were evaluated under in vivo and in vitro conditions using the techniques of electrophoretic mobility shift assay, immunoblot analysis, and quantitative reverse transcription-PCR. We found that Tpl2 ablation markedly reduced pancreatic and lung inflammation in these two dissimilar models of pancreatitis, but it did not alter pancreatic injury/necrosis in either model. The reduction in caerulein-induced pancreatic inflammation is dependent upon Tpl2 ablation in non-myeloid cells and is associated with both in vivo and in vitro inhibition of MEK, JNK, and AP-1 activation and the expression of MCP-1, MIP-2, and interleukin-6. Non-myeloid cell expression of Tpl2 regulates pancreatic inflammation during pancreatitis by mediating proinflammatory signals and the generation of neutrophil chemoattracting factors.

Severe acute pancreatitis is a devastating disease associated with considerable morbidity and with mortality rates that can reach or exceed 20%. Most of the early deaths during severe pancreatitis are the result of inflammation-related complications including the systemic immune response syndrome and/or pancreatitis-associated lung injury, which clinically presents as the adult respiratory distress syndrome. The events responsible for regulating the inflammatory response in acute pancreatitis, and for coupling pancreatic injury with lung injury, are poorly understood but are of considerable clinical importance because interventions that prevent or limit those responses are likely to reduce the morbidity and mortality of the disease.

Tpl2 is a proto-oncogene that has been shown to function as a MAP3K (mitogen-activated protein kinase kinase kinase) and, in this way, to play critical roles in certain inflammatory conditions. We now report the first studies that have examined the possible immunomodulatory effects of Tpl2 in acute pancreatitis. The results of our studies lead us to conclude that Tpl2 acts as a critical regulator of pancreatitis-associated inflammation by mediating the induction of chemoattracting chemokines and that it is Tpl2 expressed by a non-myeloid-derived pancreatic cell type (possibly the pancreatic acinar cell) that exerts this effect. Our findings also indicate that pancreatic inflammation and acinar cell injury/necrosis during pancreatitis are separable events that are not interdependent, as believed previously, and that the extent of lung inflammation as well as the extent of lung injury during pancreatitis correlates better with the severity of pancreatic inflammation than the extent of pancreatic injury/necrosis.

**Experimental Procedures**

All experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of the Tufts-New England Medical Center. Tpl2-deficient (Tpl2−/−) mice were generated and their identity confirmed by PCR analysis as described previously (1). Animals were housed in standard shoebox cages in a climate-controlled room with an ambient temperature of 23 ± 2 °C and a 12-h light/12-h dark cycle. They were fed standard laboratory chow, given water ad libitum, and randomly assigned to experimental groups. Tpl2−/− mice were back-crossed 10 times into a C57B6 background, and C57B6 mice served as the wild type control for each experiment. FD-4 (fluorescein isothiocyanate-labeled dextran with an average mass of 4 kDa) was purchased from
Sigma. Rabbit antibodies against pJNK,2 JNK, pERK1/2, ERK1/2, pMEK1/2, and MEK1 were purchased from Cell Signaling (Beverly, MA). Rabbit antibodies againstTpl2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant MEK1 was purchased from Upstate (Temecula, CA). Caerulein (the decapeptide analog of cholecystokinin) and all other reagents were purchased from Sigma unless stated otherwise.

Elicitation of Tpl2 Expression and Activation in the Pancreas during Secretagogue-induced Pancreatitis—The expression of Tpl2 in pancreas samples was evaluated by immunoblot analysis. To examine activation of Tpl2 we used a kinase assay employing recombinant MEK as a substrate (2). Mouse pancreatic acini were pacified by incubating at 37 °C for 2 h to allow any signal transduction pathways activated during the process of preparation to return to baseline. They were incubated with 100 nM caerulein and after 5 min were washed with ice-cold phosphate-buffered saline containing Complete® protease inhibitors (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (Active Motif, Carlsbad, CA). The cells were then lysed, the Tpl2 was immunoprecipitated, and its ability to phosphorylate exogenous MEK was determined by immunoblot analysis as described earlier (2).

Elicitation and Evaluation of Secretagogue-induced Experimental Pancreatitis—Secretagogue-induced pancreatitis (3) was elicited by administration of 12 hourly intraperitoneal injections of caerulein (50 μg/kg). At either 12 or 24 h after the initial caerulein injection, the animals were sacrificed by CO₂ inhalation, and samples of pancreas, lung, and blood were rapidly harvested. Serum amylase activity was determined using 4,6-ethylidene (G₆p)-p-nitrophenyl (G₁p)-α,α-d-maltoheptoside as the substrate as described previously (4). Pancreatic inflammation (i.e. neutrophil sequestration within the pancreas) was quantitated by measurement of pancreatic myeloperoxidase activity as described by Haqqani et al. (6) by determining the bromide-enhanced chemiluminescence using a Lumimark microplate reader (Bio-Rad). Acinar cell apoptosis was quantitated using the terminal transferase-mediated dUTP nick end labeling (TUNEL) technique (7). For this purpose, samples were counterstained with methyl green to permit counting of all nuclei, and apoptosis was expressed as the percent of total acinar cells showing positive staining.

Elicitation and Evaluation of Duct Infusion-induced Experimental Pancreatitis—To elicit duct infusion-induced pancreatitis, the biliopancreatic duct of mice was cannulated with a 22-gauge blunted needle connected to a Harvard infusion apparatus, and the bile duct was clamped at the liver hilus. The pancreatic duct was then infused with sodium taurocholate (5% w/v, 10 μl) in methylene blue-containing saline at a rate of 5 μl/min (8). Twenty-four hours later samples of blood and pancreas were rapidly removed and used for quantitation of serum amylose activity, pancreatic edema, acinar cell injury/necrosis, and pancreatic inflammation employing techniques identical to those utilized with the secretagogue-induced model.

Quantitation of Secretagogue-induced Pancreatitis-associated Lung Inflammation and Injury—Lung inflammation during secretagogue-induced pancreatitis was quantitated by measuring lung myeloperoxidase activity using the technique described above for measurement of pancreas myeloperoxidase activity. In this case, however, the lungs were first rinsed in situ by perfusion with 3 ml of saline instilled through a cannula placed into the pulmonary artery via the right ventricle. Lung injury during secretagogue-induced pancreatitis was evaluated by quantitating the leakage of intravenously administered FD-4 into the bronchoalveolar space (9). Fifteen minutes before sacrifice, mice used for evaluation of pulmonary microvascular permeability were given 10 mg/kg FD-4 in 200 μl of phosphate-buffered saline by tail vein injection. After sacrifice, a 400-μl sample of blood was obtained by cardiac puncture with a heparinized needle, and all animals underwent a bronchoalveolar lavage. For this, the trachea was exposed, and a PE-240 cannula was inserted for a distance of 0.5 cm into the trachea via a small incision. With constant flow (0.1 ml/s), the lungs were lavaged twice with the same 1 ml of phosphate-buffered saline (37 °C). This wash process was repeated twice, and the combined wash fluid (BAL) (~2.6 ml recovered) was kept on ice. The blood sample was centrifuged for 5 min at 2000 × g, and the plasma was removed. The presence of FD-4 in the BAL and the plasma (1% dilution) was measured using a Hitachi fluorimeter with excitation at 494 nm and emission at 518 nm. Background levels of fluorescence, determined in each group by measuring fluorescence in blood and BAL from animals that did not receive FD-4, were subtracted from the actual FD-4 readings. Lung microvascular permeability (i.e. injury) was expressed as the ratio of fluorescence units in BAL to fluorescence units in 1% plasma.

Generation of Bone Marrow Chimeric Animals—Bone marrow donor animals were prepared by pretreating wild type and Tpl2⁻/⁻ mice with 5-fluorouracil (160 mg/kg intravenously). Five days later, the animals were sacrificed, and bone marrow was collected from their harvested tibias and femurs as described (10). Other wild type and Tpl2⁻/⁻ mice destined to serve as bone marrow recipients were placed in sterile cages and given sterile food and water for 1 week prior to and 1 week following bone marrow transplantation. These recipient mice were then exposed to a total of 900–1000 cGy irradiation delivered in two treatment sessions separated by 4 h (i.e. lethal irradiation). Two hours later, a suspension of donor-derived bone marrow cells (200 μl) with added penicillin (100,000 units/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml) was administered by tail vein injection, and the animals were observed for either 2 weeks or 120 days to allow for bone marrow engraftment and restitution prior to subsequent experimentation. During that period of observation, animals (<5% of each group) failing to maintain and gain weight normally and
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those appearing unhealthy were euthanized. The adequacy of bone marrow restitution in the remaining animals was evaluated by measuring circulating blood hematocrit and quantitating the circulating leukocytes prior to induction of pancreatitis. Only animals with normal hematocrit and leukocyte counts were used in these subsequent experiments. Based upon studies performed by others (11), this lethal irradiation/bone marrow transplantation protocol would be expected to yield the following four groups of mice: (a) global wild type mice (i.e. mice with wild type myeloid and non-myeloid cells); (b) global Tpl2−/− mice (i.e. mice with Tpl2−/− myeloid and non-myeloid cells); (c) chimeric mice with wild type myeloid and Tpl2−/− non-myeloid cells; and (d) chimeric mice with Tpl2−/− myeloid and wild type non-myeloid cells. To confirm that, indeed, the appropriate chimeric animals had been generated, DNA was isolated from circulating leukocytes using the Qiagen kit (Qiagen, Studio City, CA) and PCR analysis of that DNA was performed using the Tpl2 forward (cttcagtcatcttaacactcaggc), and common reverse (ctgcttgatgagtcagccggaa-3) probe (Promega, Madison, WI) and reverse, 499gcttgaggtggtgtggaaaa (318 bp); MIP-2 forward, 171acacccacaggctacagg, and reverse, 461cagacgaggcatcagg (345 bp); IL-6 forward, 33attgaccttgctgactagt, and reverse, 421tctgcctgtttcttctgt (389 bp).

Activation of Proinflammatory Signaling Cascades—Nuclear and cytoplasmic protein extracts from pancreatic fragments were prepared as described (14). JNK and MEK activation was evaluated by immunoblot analysis using the cytoplasmic protein extracts and antibodies raised against the phosphorylated forms of JNK (Thr183/Tyr185) and MEK1/2 (Ser217/Ser221) (Cell Signaling). After incubation with horseradish peroxidase-conjugated secondary antibodies, immunoreactive bands were evaluated using Boc-Gln-Ala-Arg-MCA (methyl-coumaryl-7-amide) as the substrate for trypsin (5).

Results

Tpl2 Is Expressed in Mouse Pancreas and Tpl2 Is Activated after Supramaximal Secretagogue Stimulation—As shown in Fig. 1A, expression of Tpl2 could be detected when pancreas homogenates from wild type but not Tpl2−/− mice were subjected to immunoblotting using anti-Tpl2 antibodies. Because there are no good phosphospecific antibodies for Tpl2, we were not able to monitor Tpl2 activation by probing for phospho-Tpl2 during caerulein-induced pancreatitis. Tpl2 activation during pancreatitis, was therefore monitored via an in vitro kinase assay that employs the physiologic substrate for Tpl2 (MEK1) (3). Tpl2 was first immunoprecipitated from acini that had been exposed to either buffer alone or to buffer containing 100 nm caerulein for 5 min. The immunoprecipitated Tpl2 was then incubated with recombinant MEK-1 and ATP for 10 min at room temperature, and the products of the kinase reaction were immunoblotted and probed with a phospho-MEK-specific antibody. We found that Tpl2 immunoprecipitated from acini incubated with caerulein phosphorylated recombinant MEK in the in vitro kinase assay but that Tpl2 immunoprecipitated from non-caerulein-stimulated acini was not able to phosphorylate MEK1 (Fig. 1B). Isolated acini from wild type and Tpl2−/− mice, however, did not differ in their responses to caerulein. Both types of acini secreted amylase in a biphasic manner, and when stimulated with supramaximal caerulein concentrations (>0.1 nm), intracellular trypsin activity was observed (Fig. 1, C and D).

Genetic Ablation of Tpl2 Reduces Pancreatic Inflammation without Altering the Magnitude of Hyperamylasemia, Edema, or Acinar Cell Injury/Necrosis during Secretagogue-induced...
Pancreatitis—Pancreatic edema (Fig. 2A), acinar cell injury/necrosis (Fig. 2B), and pancreatic myeloperoxidase activity (i.e. inflammation) (Fig. 2C) were all increased 12 and 24 h after the start of supramaximal secretagogue stimulation in wild type animals and, under these conditions, serum amylase activity was also increased (data not shown). Pancreatic edema (Fig. 2A), acinar cell injury/necrosis (Fig. 2B), and serum amylase activity (not shown) were also similarly increased 12 and 24 h after the start of caerulein administration to Tpl2−/− mice. In contrast, no caerulein-induced increase in pancreatic myeloperoxidase activity was observed 12 h after the start of caerulein administration to Tpl2−/− mice, and the increased myeloperoxidase activity observed 24 h after the start of caerulein administration to wild type animals was markedly attenuated when caerulein was administered to Tpl2−/− animals (Fig. 2C). Representative photomicrographs of pancreas sections obtained from both wild type and Tpl2−/− mice 24 h after the start of caerulein administration also indicate that Tpl2 ablation reduces caerulein-induced neutrophil sequestration within the pancreas without altering pancreatic edema or the extent of acinar cell injury/necrosis (Fig. 2D).

Genetic Ablation of Tpl2 Does Not Alter the Mode of Acinar Cell Death during Secretagogue-induced Pancreatitis—On purely morphological grounds, the histological distinction between necrosis and apoptosis is imprecise, particularly when the inflammatory reaction has been altered. To determine whether Tpl2 ablation changes the mode of cell death during pancreatitis, studies were performed using the TUNEL staining technique (7) to quantitate acinar cell apoptosis during secretagogue-induced pancreatitis in wild type and Tpl2−/− mice. For these studies, the mice were given six, rather than 12, hourly injections of caerulein, because the extensive tissue injury noted at the later time made accurate quantitation of apoptosis using the TUNEL technique impossible. As shown in Fig. 2E, after six hourly injections of caerulein the fraction of TUNEL-positive acinar cells was not altered by Tpl2 ablation. Roughly 3–4% of acinar cells were TUNEL-positive in both Tpl2+/+ and Tpl2−/− mice.

Genetic Ablation of Tpl2 Reduces Pancreatic Inflammation without Altering the Severity of Pancreatic Edema or Acinar Cell Injury/Necrosis during Duct Infusion-induced Pancreatitis—To exclude the possibility that the effects of Tpl2 ablation on pancreatitis might be model-specific and limited to the secretagogue-induced model, we also induced pancreatitis in wild type and Tpl2−/− mice by retrograde infusion of sodium taurocholate into the pancreatic duct. As shown in Fig. 3, F–H, the extent of pancreatic edema and acinar cell injury/necrosis is comparably increased when either wild type or Tpl2−/− mice are infused with sodium taurocholate, and a marked rise in pancreatic myeloperoxidase activity is observed when wild type mice are infused with sodium taurocholate. That duct infusion-induced rise in myeloperoxidase activity, however, is significantly blunted by Tpl2 ablation (Fig. 3H). Representative photomicrographs of samples taken from wild type and Tpl2−/− mice infused with sodium taurocholate also demonstrate that acinar cell injury/necrosis is similar in both groups but that sequestration of neutrophils within the pancreas is markedly reduced in Tpl2−/− mice (Fig. 2, I and J).

Genetic Ablation of Tpl2 Reduces Lung Inflammation and Injury during Secretagogue-induced Pancreatitis—Previously reported studies indicate that intrapulmonary sequestration of neutrophils (i.e. lung inflammation) and an increase in pulmonary microvascular permeability (i.e. lung injury) are evident after 12 hourly injections of caerulein (18). To evaluate the role of Tpl2 in coupling pancreatitis to lung inflammation and lung injury during secretagogue-induced pancreatitis, the effect of Tpl2 ablation on these phenomena were evaluated 12 and 24 h after the first of 12 caerulein injections. As shown in Fig. 3A, lung myeloperoxidase activity is increased 12 h after the first
FIGURE 2. Effects of Tpl2 ablation on secretagogue- and duct infusion-induced acute pancreatitis. Secretagogue-induced pancreatitis was induced as described under “Experimental Procedures,” and animals were sacrificed 12 or 24 h (A–D) or 6 h (E) after the start of caerulein administration. Data quantitating pancreatic edema (i.e. water content), acinar cell injury/necrosis, and pancreatic inflammation (i.e. myeloperoxidase activity) are shown in A–C, respectively, and D shows representative photomicrographs of pancreatic tissue samples 24 h after the first of 12 hourly caerulein injections. E shows results of TUNEL studies quantitating acinar cell apoptosis. Duct infusion-induced pancreatitis was elicited as described under “Experimental Procedures,” and animals were sacrificed 24 h later (F–J). Data quantitating pancreatic edema, acinar cell injury/necrosis, and pancreatic inflammation are shown in F–H, respectively, and the representative photomicrographs are shown in I and J. Shown are the averages from 8 animals/group (A–E) and 4 animals/group (F–H). Bracketed columns with an asterisk shown in C and H denote significantly differing values (p < 0.05).
administration of caerulein to wild type animals and even further increased 24 h after the start of caerulein administration, but that increase in lung myeloperoxidase activity is significantly attenuated by genetic ablation of Tpl2. The leakage rate of intravenously administered FD-4 into the bronchoalveolar space was employed as a measure of pulmonary microvascular permeability (9) and as shown in Fig. 3B, the rate of FD-4 leakage is significantly increased 12 h after the final administration of caerulein to wild type animals. In contrast, no significant increase in pulmonary microvascular permeability is noted in Tpl2−/− mice evaluated at the same time. Leakage of FD-4 is also increased 24 h after the start of caerulein administration to wild type mice but, again, no increase in Tpl2−/− mice is observed at that time. These observations indicate that Tpl2 ablation reduces both lung inflammation and lung injury during secretagogue-induced pancreatitis.

Anti-inflammatory Effect of Tpl2 Ablation Is Mediated by Tpl2 Expression in Non-myeloid Cells—To determine whether the protection of the Tpl2−/− mice from pancreatic inflammation was due to the Tpl2 ablation in hematopoietic or pancreatic epithelial cells, we transplanted wild type and Tpl2−/− bone marrow into both wild type and Tpl2−/− recipient mice. This allowed us to generate chimeric animals with Tpl2 ablation confined to either myeloid or non-myeloid cells (Fig. 4A). As shown in Fig. 4, B and C, pancreatic inflammation during secretagogue-induced pancreatitis is markedly reduced in reconstituted global Tpl2−/− mice and in mice with Tpl2 ablation confined to non-myeloid cells, but it is not reduced in mice with Tpl2 ablation confined to myeloid cells. Similar results are observed when the Tpl2 chimeric animals were treated with caerulein either 2 weeks (Fig. 4C) or 120 days (Fig. 4D) after bone marrow transplantation.

Genetic Ablation of Tpl2 Interferes with Caerulein-induced Activation of Proinflammatory Transcription Factors and Proinflammatory Kinases and with the Expression of Neutrophil Chemoattractants in the Pancreas—Our finding that Tpl2 ablation in a non-myeloid cell type reduces inflammatory responses during pancreatitis led us to hypothesize that, in wild type mice, Tpl2 may play a critical proinflammatory role in pancreatitis by regulating events leading to chemoattraction and activation of inflammatory cells to sites of pancreatic injury. To explore this possibility, we examined the effects of Tpl2 ablation on a variety of caerulein-induced pancreatic proinflammatory events under both in vivo and in vitro conditions. We found that genetic ablation of Tpl2 markedly inhibits caerulein-induced activation of pancreatic MEK-1/2, ERK1/2, and JNK and activation of AP-1, but not NF-κB, as judged by nuclear translocation of p65 or degradation of IκB-α (Fig. 5). At the same time, Tpl2 ablation inhibits expression of the neutrophil chemoattracting chemokines MCP-1, MIP-2, and IL-6 (Fig. 6). These effects of Tpl2 ablation are observed in the in situ pancreas under in vivo conditions within 2 h of caerulein administration, i.e. prior to inflammatory cell sequestration within the pancreas during pancreatitis (Figs. 5A and 6A). These effects of Tpl2 ablation are also observed in pancreas fragments studied in vitro, i.e. under conditions that preclude chemoattraction of inflammatory cells to the pancreas (Figs. 5B and 6B).

**DISCUSSION**

The current communication focuses on the potential importance of Tpl2 as a regulator of injury and inflammation in the pancreas and lungs during acute pancreatitis. Tpl2, also known as Cot (cancer Osaka thyroid) is a 52- and 58-kDa serine-threonine protein kinase that is activated by provirus integration in Moloney murine leukemia virus-induced rodent lymphomas and murine mammary tumor virus-induced mammary adenocarcinomas (19). Overexpression of Tpl2 in a variety of cell types has been shown to activate all of the MAPK pathways, NFAT, and NF-κB and to promote cell proliferation as well as cell transformation in culture and oncogenesis in animals (1, 2). Recently, Tpl2 was found to transduce Toll-like receptors and death receptor signals that activate different combinations of MAPK pathways and NF-κB in different cell types and to promote inflammation. As a result Tpl2 ablation interferes with the induction of inflammatory cytokines in response to lipopolysaccharide and TNF-α signals and inhibits lipopolysaccharide-δ-galactosamine-induced endotoxic shock and TNF-α-induced inflammatory bowel disease (1, 20, 21).

In this communication, we report the results of the first studies to have examined the role of Tpl2 in pancreatic inflammatory disease. We have shown that Tpl2 is expressed in the mouse pancreas (Fig. 1A) and that mouse pancreatic Tpl2 is rapidly activated by supramaximal stimulation with the secretagogue caerulein (Fig. 1B). We also found that genetic ablation of Tpl2 markedly reduces the extent of pancreatic inflammation in two dissimilar experimental models of acute pancreatitis in mice, the secretagogue-induced (caerulein) model and a model in which acute pancreatitis is elicited by retrograde pancreatic duct infusion with sodium taurocholate (Fig. 2). This effect of Tpl2 ablation is limited to inflammatory cell infiltration because Tpl2 ablation did not reduce the hyperamylasemia, pancreatic edema, or acinar cell injury/necrosis...
observed in both of these models. Moreover it did not affect either the high dose inhibition of secretion or the intrapancreatic activation of digestivezymogens that occur when animals or pancreatic tissue are exposed to supramaximally stimulating concentrations of caerulein (Fig. 1). Our finding that pancreatic infiltration with inflammatory cells can be virtually eliminated by Tpl2 ablation, without altering the extent of pancreatic injury during pancreatitis, clearly challenges the current widely held belief that the severity of pancreatic injury during pancreatitis is determined by the severity of the intrapancreatic inflammatory process. Rather, the two manifestations of pancreatitis appear to be regulated individually.

We considered the possibility that Tpl2 might modulate pancreatic inflammation by altering the mode of acinar cell death during pancreatitis, i.e. by promoting acinar cell apoptosis while preventing acinar cell necrosis, because apoptosis is often the mode of cell death that is observed in the absence of inflammation, whereas necrosis is generally believed to trigger an inflammatory reaction. However, our studies examining this issue using the TUNEL technique to quantitate acinar cell apoptosis have shown that Tpl2 ablation does not alter the extent of apoptosis, at least not in the secretagogue-induced model (Fig. 2E).

Secretagogue-induced pancreatitis, like its clinical counterpart, is associated with significant acute lung injury, and that lung injury is believed to be mediated, to a great extent, by the sequestration of activated inflammatory cells within the pulmonary microcirculation. We found that the sequestration of neutrophils within the pulmonary microvascular compartment is reduced during caerulein-induced pancreatitis in animals that lack Tpl2 and that that reduction in neutrophil sequestration is associated with reduced leakage of the intravenously administered FD-4 (Fig. 3) into the bronchoalveolar space. These observations indicate that ablation of Tpl2 protects animals from both pancreatitis-associated lung inflammation and lung injury, and they lead us to conclude that Tpl2 regulates events that are critical to the coupling of acute pancreatitis to pancreatitis-associated acute lung injury. The fact that Tpl2 ablation prevents both pancreatic inflammation and lung injury without altering pancreatic injury/necrosis suggests that the severity of the acute lung injury of pancreatitis is determined primarily by the extent of pancreatic inflammation and not, as
widely believed, by the extent of pancreatic injury during pancreatitis. Whether this is the case for other manifestations of the systemic immune response syndrome besides acute lung injury/inflammation could not be determined by our studies. The mechanisms by which Tpl2 might promote pancreatic inflammation were also addressed in our studies. Although Tpl2 is widely expressed, most of the previous studies evaluating the proinflammatory function of Tpl2 have been focused on the role of Tpl2 in hematopoietic cells, primarily macrophages in which Tpl2 has been shown to play a proinflammatory role (2). Based on this knowledge, it would be reasonable to assume that the failure of inflammatory cells to be sequestered within the pancreas during pancreatitis in Tpl2−/− mice reflects the inability of circulating Tpl2−/− inflammatory cells to respond to proinflammatory signals arising from the pancreas during pancreatitis. However, the possibility that genetic ablation of Tpl2 in pancreatic cells interferes with the generation of those proinflammatory signals must also be considered as an additional or perhaps alternative explanation for our observations. An unambiguous distinction between these two mechanisms would require either the use of animals with Tpl2 ablation limited to specific cell types or the creation of bone marrow chimeric animals with Tpl2 ablation confined to either myeloid or non-myeloid derived cells. Our findings, based on studies employing chimeric animals, are described in this report. Wild type and Tpl2−/− recipient mice were lethally irradiated with 900–1000 cGy (11). They were then rescued by bone marrow transplantation using marrow harvested from either wild type or Tpl2−/− mice. Bone marrow engraftment would be expected to yield four groups of animals: (a) reconstituted global wild type mice, (b) reconstituted global Tpl2−/− mice, (c) chimeric mice with wild type myeloid cells and Tpl2−/− non-myeloid cells, and (d) chimeric mice with Tpl2−/− myeloid cells and wild type non-myeloid cells. Studies reported by others using green fluorescent protein-labeled bone marrow cells for transplantation after exposure to 900 cGy have indicated that complete replacement of myeloid elements with donor-derived cells occurs ~60 days after bone marrow transplantation and
Tpl2 Regulates Inflammation in Pancreatitis

FIGURE 6. Effects of Tpl2 ablation on neutrophil chemoattractant chemokine expression in response to supramaximal caerulein stimulation. RNA was prepared from wild type (wt) or Tpl2−/− animals 2 h after the start of an hourly saline or caerulein (50 μg/kg) injection (A) or from pancreas fragments obtained from wild type or Tpl2−/− animals that had been incubated with buffer alone or buffer containing caerulein (10 nm) for 2 h (B). Expression of MCP-1, MIP-2, and IL-6 was measured by quantitative reverse transcription-PCR as described under “Experimental Procedures.” Results shown are the average of 4 mice/group tested in duplicate. Bracketed columns with an asterisk denote significantly differing values (p < 0.05).

that, by 90 days, even slowly turning over cells, such as the alveolar macrophages are of donor origin (11). In the experiments described here, transplanted mice were used at 2 weeks and 120 days after transplantation. The results were identical at both time points. First, the reconstituted global wild type and Tpl2−/− mice displayed their expected pancreatitis phenotypes, confirming that bone marrow transplantation had been successful and that complete bone marrow engraftment had been achieved. Indeed, circulating leukocytes harvested from our chimeric animals at the later time point were found to display the expected genotype (Fig. 4A).

Surprisingly, we found that the pancreatitis phenotype of global Tpl2−/− mice was faithfully replicated in chimeric mice with ablation confined to non-myeloid cells but not in chimeric mice with Tpl2 ablation confined to myeloid cells (Fig. 4). This observation led us to the unanticipated conclusion that the reduction in pancreatic inflammation observed in global Tpl2−/− mice reflects the effect of Tpl2 ablation in non-hematopoietic cells. Based on this finding, we speculate that the cell type initiating the process is the pancreatic acinar cell. Our findings also led us to hypothesize that Tpl2 ablation might reduce pancreatic inflammation during pancreatitis by interrupting proinflammatory signals emanating from the pancreas, which are responsible for the activation and chemoattraction of inflammatory cells. To explore this possibility, we examined the activation of MEK1, ERK1/2, JNK, AP-1, and NF-κB in vivo and in vitro following treatment with caerulein. The in vivo studies were performed 2 h after the start of caerulein administration so that observations would be reflective of changes occurring within pancreatic cells and not within inflammatory cells, which are chemoattracted to the pancreas at later times. The in vitro studies were performed 30 min after exposure to caerulein so that changes evolving more rapidly could be examined. As shown in Fig. 5, these studies clearly indicate that Tpl2 ablation interferes with caerulein-induced activation of MEK-1, ERK1/2, and JNK and activation of AP-1 but not NF-κB. Moreover, Tpl2 ablation interferes with caerulein-induced up-regulated expression of neutrophil chemoattracting chemokines including MCP-1, MIP-2, and IL-6 (Fig. 6). These observations add further support to our hypothesis that Tpl2 ablation reduces pancreatic inflammation by interfering with the pancreatic cell generation of proinflammatory factors.

Although it is tempting to speculate that the mechanisms responsible for the effect of Tpl2 ablation on lung inflammation and injury during pancreatitis are identical to those responsible for the effect of Tpl2 ablation on pancreatic inflammation, our studies did not directly address this issue, and alternate explanations should be considered. Thus, the effect of Tpl2 ablation on lung inflammation and injury may involve cells other than pancreatic acinar cells (e.g. macrophages, T-cells) and/or other mediators (e.g. proteases released from the pancreas).

We were surprised to note that ablation of Tpl2 did not prevent caerulein-induced activation of pancreatic NF-κB (Fig. 5), because in other systems Tpl2 has been shown to play an important role in regulating NF-κB activation (2, 22, 23). This is reminiscent of earlier findings showing that TNF-α-induced NF-κB activation in mouse embryonic fibroblasts is Tpl2-dependent, whereas in macrophages, it is not (2). The role of NF-κB in pancreatitis is currently a subject of considerable controversy. Although it is generally believed that NF-κB activation in the pancreas triggers proinflammatory events (24, 25), some studies (26, 27) have suggested that NF-κB activation may trigger anti-inflammatory responses. Our observation (a) that Tpl2 ablation interferes with pancreatic inflammation but not pancreatic injury/necrosis and (b) that Tpl2 ablation does not alter secretagogue-induced NF-κB translocation to the nucleus during pancreatitis might suggest that NF-κB functions primarily as a mediator of events leading to pancreatic injury/necrosis rather than inflammation. In addition our findings do not exclude the possibility that Tpl2 might regulate NF-κB activation during pancreatitis via mechanisms that are not affected by changes in nuclear translocation.

Pancreatitis-associated lung injury and the late inflammation-related peripancreatic complications of pancreatitis such as pancreatic abscess formation account for most of the morbidity and mortality of severe clinical pancreatitis. The current studies suggest that inhibition of proinflammatory cytokine expression in the pancreas, as a result of Tpl2 ablation, decreases lung neutrophil infiltration and injury despite exten-
sive pancreatic injury. The observations reported in this communication indicate that Tpl2 plays an important role in regulating inflammation during pancreatitis, and they suggest that interventions that modify Tpl2-dependent events might prove beneficial in the treatment and/or prevention of severe acute pancreatitis.

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