

Targeted Disruption of the β -Chemokine Receptor CCR1 Protects against Pancreatitis-associated Lung Injury

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Abstract

β -Chemokines and their receptors mediate the trafficking and activation of a variety of leukocytes including the lymphocyte and macrophage. An array of no less than eight β -chemokine receptors has been identified, four of which are capable of recognizing the chemokines MIP1 α and RANTES. Genetic deletion of one of the MIP1 α and RANTES receptors, CCR5, is associated with protection from infection with HIV-1 in humans, while deletion of the ligand MIP1 α protects against Coxsackie virus-associated myocarditis. In this report we show that the deletion of another receptor for MIP1 α and RANTES, the CCR1 receptor, is associated with protection from pulmonary inflammation secondary to acute pancreatitis in the mouse. The protection from lung injury is associated with decreased levels of TNF- α in a temporal sequence indicating that the activation of the CCR1 receptor is an early event in the systemic inflammatory response syndrome. (*J. Clin. Invest.* 1997. 100:2022–2027.) Key words: MIP1 α /RANTES receptor • caerulein • gene deletion • respiratory distress • systemic immune response syndrome

Introduction

The activation and trafficking of inflammatory cells involves a multigene family of chemoattractant cytokines known as chemokines (1, 2). The chemokine system is characterized by apparent redundancies of ligands and receptors, which complicates investigation of chemokine-regulated events. One approach that has been fruitful in exploring the actions of the chemokines and their receptors involves gene targeting techniques (3–5). Mice deficient in the β -chemokine MIP1 α have been demonstrated to be defective in clearance of Coxsackie and influenza viruses, with attendant consequences on immune-mediated injuries (5). However, to date there are at least four MIP1 α receptors identified (2). To address the indi-

vidual contributions of the β -chemokine receptors (CCR)¹ in leukocyte generation, trafficking, host defense, and inflammation, we undertook a gene targeting approach.

Acute lung injury and the adult respiratory distress syndrome complicate many disease states and are central components of the systemic immune response syndrome (6). The mechanisms underlying this syndrome are unresolved, but the uniform pathologic features of adult respiratory distress syndrome involve sequestration of activated inflammatory cells within the lung, pulmonary microvascular injury, and leakage of intravascular fluid into the tissue spaces. A linkage between the cytokine (e.g., TNF- α) and chemokine systems in the genesis of these syndromes may be postulated. In rodents, a model of these processes dependent on the initiation of acute pancreatitis has been well described (7, 8). After overstimulation of pancreatic exocrine acinar cells with the cholecystokinin analogue caerulein, pancreatic injury occurs that is characterized by edema, leukocyte influx into the pancreas, and elevated levels of pancreatic enzymes in the serum (9). The pancreatic insult precedes the development of pulmonary leukosequestration. Guice et al. have reported that complement and PMN neutrophils are important for pancreatitis-associated lung injury (10). Our results show a dissociation between pancreatic injury and secondary lung injury in a model of acute pancreatitis produced in mice lacking the β -chemokine receptor CCR1.

Methods

CCR1 gene targeting. The murine homologue of human CCR1 has been identified previously to exist on macrophages and eosinophils (11). The gene encoding CCR1 was cloned from a mouse 129Sv genomic library. A 12-kb genomic fragment from lambda EMBL3 was subcloned into pBluescript. A 0.9-kb Apal fragment containing the COOH-terminal 660 bp of the coding sequence for the receptor was deleted. A neomycin resistance cassette under the control of a phosphoglucokinase promoter was cloned into an internal Clal site. The \sim 13-kb targeting construct was cloned into pPNT vector for +/– selection with G418 and ganciclovir as previously described (12).

The pPNT-CCR1 construct was linearized with NotI at a unique vector site, and electroporated into J1 ES cells. Clones receiving the transfected DNA were selected with G418 and ganciclovir. 30 resistant clones were identified as correctly targeted, and one of these was injected into blastocysts derived from C57Black/6 mice. Chimeric animals were bred to effect transmission of the targeted allele through the germ line. Southern analysis of tail snip DNA after digestion with BamHI allowed identification of the mutant allele using a flanking probe.

Northern and Southern analyses. Genomic DNA was isolated from tails of weanling mice as previously described (12) and was digested with BamHI. After electrophoresis through 0.9% agarose, the DNA was transferred to GeneScreen Plus membranes (NEN Life

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Received for publication 20 May 1997 and accepted in revised form 22 August 1997.

J. Clin. Invest.

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0021-9738/97/10/2022/06 \$2.00

Volume 100, Number 8, October 1997, 2022–2027
<http://www.jci.org>

1. **Abbreviations used in this paper:** BAL, bronchoalveolar lavage; CCR, chemokine receptor; MPO, myeloperoxidase.

Sciences, Boston, MA) by capillary action, hybridized at 42°C in 50% formamide, containing 2× SSC, 1% SDS, and 10% dextran sulfate, with [³²P]dCTP-labeled flanking probe (probe A, see Fig. 1), prepared by random priming (Boehringer Mannheim, Indianapolis, IN), and washed according to the manufacturer's instructions.

mRNA was purified from the bone marrow of wild-type, heterozygous, and homozygous CCR1 deleted littermates and Northern blotted as described previously (12). Blots were hybridized with ³²P-labeled cDNA probe, stripped, and rehybridized for β-actin.

Animal studies. All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committees of the Beth Israel Deaconess Medical Center and Children's Hospital, Boston, MA.

Caerulein-induced pancreatitis. CCR1 receptor deficient and wild-type littermate mice weighing 17–20 g were fasted overnight and then given 12 hourly intraperitoneal injections of caerulein (Sigma Chemical Co., St. Louis, MO) at a dose of 10 μg/kg. Animals were killed with a lethal dose of pentobarbital 1 h after the final caerulein injection and samples of lung, pancreas, and blood were rapidly harvested.

Measurement of pancreas and lung water content. Fragments of lung and pancreas were blotted dry and weighed to determine tissue wet weight. They were then desiccated by overnight incubation at 140°C and reweighed to determine tissue dry weight. Tissue water content

was calculated as the difference between wet and dry weight and expressed as a percentage of wet weight.

Measurement of pulmonary microvascular permeability. 2 h before time of killing (i.e., after the 11th caerulein injection) FITC-labeled BSA (Sigma Chemical Co.) (5 mg/kg) was administered via tail-vein injection. At the time of killing, the trachea was cannulated and the lungs lavaged in situ three times with saline (1 ml per lavage). The bronchoalveolar lavage (BAL) fluid was collected and combined (fluid recovery was 80–85%). FITC fluorescence in serum and lavage fluid was measured using a fluorescence spectrometer with excitation at 494 nm and emission at 520 nm. The fluorescence ratio of lavage fluid-to-blood was calculated and taken as a measure of microvascular permeability in the lung. To facilitate comparisons, all values were normalized to the maximal increase in permeability.

Assays. Serum amylase and lipase enzymatic activity were measured as described previously (13, 14). Myeloperoxidase (MPO) activity in pancreas and lung was measured in homogenates of the respective organs as described previously (8) using samples obtained from animals not given FITC-labeled albumin. TNF-α levels were measured in homogenates of pancreatic tissue and in BAL fluid using an ELISA (Genzyme, Cambridge, MA). Values for pancreas were expressed per microgram of DNA in each sample while those for BAL fluid were expressed per milliliter of BAL fluid.

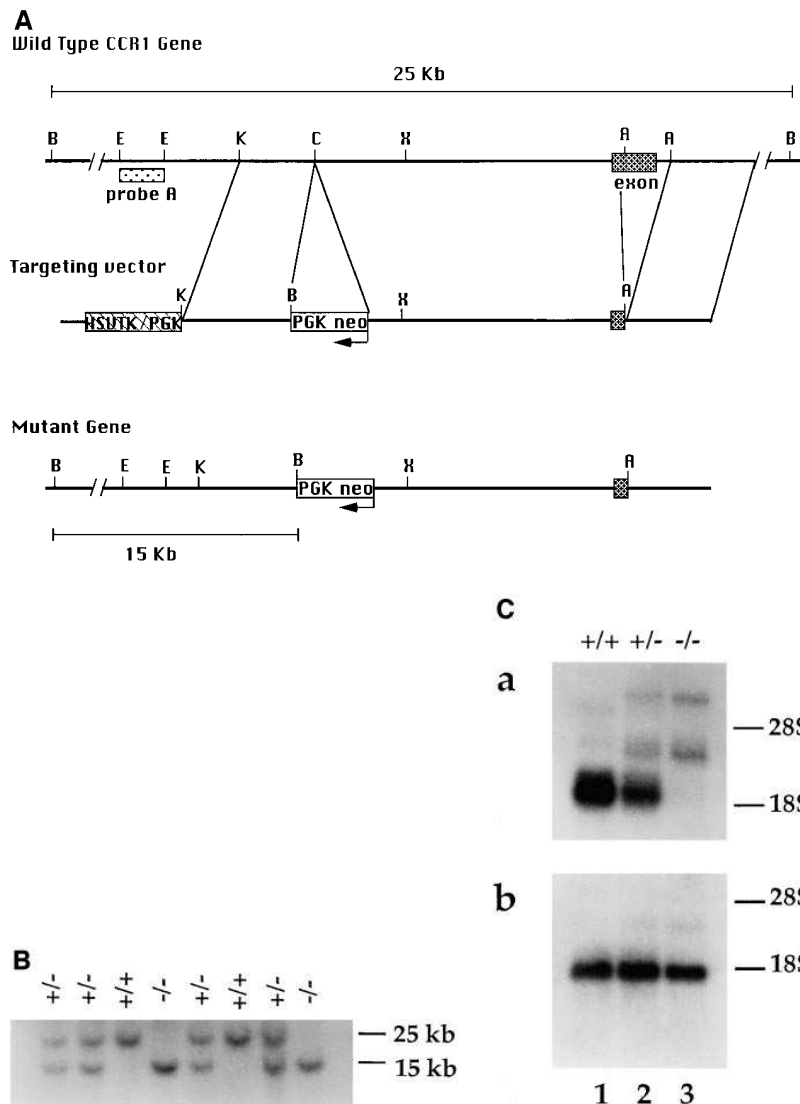


Figure 1. Molecular characterization of CCR1 gene deleted mice. (A) Schematic diagram of wild-type loci, targeting vector, and mutant loci of the mouse CCR1 receptor gene (A, Apa1; B, BamH1; C, Cla1; E, EcoR1; K, Kpn1; X, Xba1). Approximately 25 kb of genomic DNA including the coding sequence of the gene is shown. A 0.9-kb Apa1 fragment including the bulk of the receptor coding sequence was deleted, and a neomycin-resistance cassette, PGK neo, was cloned into a unique Cla1 site. A ganciclovir-resistance gene (HSVTK/PGK) located upstream was used for negative selection. Homologous recombination led to a diagnostic pattern on restriction digests probed with flanking sequence (probe A). (B) Southern blot analysis and genotyping of progeny. Genomic DNA was isolated from littermates, digested with BamH1, and hybridized with probe A. The genotype analysis of a litter is shown. Probe A hybridizes to a 25-kb BamH1 fragment in the wild-type allele and to a 15-kb fragment in the targeted allele. (C) Northern blot analysis of CCR1 expression levels in wild-type (+/+, lane 1), heterozygote (+/–, lane 2), and homozygote (–/–, lane 3) animals. mRNA was prepared from bone marrow cells of each genotype, separated under denaturing agarose gel electrophoresis hybridized with a ³²P-labeled, 1.5-kb CCR1 cDNA fragment. The blot was then stripped and reprobed with β-actin probe.

Morphology. Conventional light microscopic examination of lung and pancreas was performed using paraffin-embedded samples. 5- μ m sections were stained with hematoxylin and eosin and assessed in blinded fashion. Morphometric analyses of pancreas samples to evaluate the extent of acinar cell necrosis were performed with a computerized imaging video unit (CCD-72; DAGEMTI, Michigan City, IN) equipped with the NIH 1200 image analysis software as previously described (15).

Analysis of data. Results are expressed as mean \pm SEM values. Differences between groups were analyzed for significance using ANOVA with Tukey's studentized range test. Significant differences were defined as those associated with a P value ≤ 0.05 . Vertical bars in figures represent \pm SEM and the absence of such bars indicates that the SEM value was too small to display.

Results and Discussion

Mice with a targeted disruption of the CCR1 allele were identified through Southern analyses of F1 littermates from chimeric breeders. The targeting vector and map, Southern, and Northern analyses are presented in Fig. 1. Homozygous CCR1 deficient animals were identified in the F2 generations. Mice maintained under barrier isolation were healthy, bred according to Mendelian inheritance patterns, and were phenotypically indistinguishable from wild-type mice. The lack of basal abnormalities in the CCR1 receptor gene deleted mice presumably relates to redundant basal pathways of leukocyte trafficking and development. However, less apparent differences may occur as has been appreciated in murine IL-8 receptor homolog deficient mice bred under gnotobiotic versus barrier conditions (16).

To evaluate the role of the CCR1 receptor in a model of secondary lung injury, we administered 12 hourly injections of

the cholecystokinin analogue caerulein. The dose chosen was in excess of that required to stimulate a maximal rate of digestive enzyme secretion from the pancreas. Supramaximal secretagogue stimulation of the pancreas with caerulein is known to cause acute pancreatitis characterized by initial pancreatic inflammation and acinar cell necrosis (9) followed by a secondary respiratory distress syndrome associated with hypoxemia, hypercarbia, and PMN sequestration in the lung (7, 8). Previous studies have suggested a role for complement, neutrophils, and TNF in this response (10). Additionally, we have demonstrated previously that PGE₁ can ameliorate the lung injury, but not the pancreatic necrosis in this model (8).

As shown in Figs. 2 and 3, supramaximal stimulation of wild-type mice with caerulein results in pancreatitis as manifested by a rise in serum amylase and lipase activity, the appearance of pancreatic edema, the development of acinar cell necrosis, an increase in pancreatic MPO activity, and morphological changes of acute inflammation in the pancreas. Similar changes are observed in CCR1 receptor deficient mice although, in this group, the rise in pancreatic MPO activity is blunted. Taken together, these observations indicate that deletion of the CCR1 receptor causes little or no change in the severity of secretagogue-induced pancreatitis although it does appear to reduce intrapancreatic sequestration of neutrophils. This finding is significant in that previous investigators have demonstrated a role for the neutrophil in this model as assessed by blunting of pancreatitis and secondary lung injury after neutrophil depletion (10). In this instance, a significant decrease in neutrophil influx into the pancreas, as reflected by the decreased values of the leukocyte enzyme MPO, is not associated with significant protection from injury.

We next investigated whether caerulein-induced pancreatitis is associated with progression to secondary lung injury in CCR1 deleted animals. This form of injury in wild-type mice is manifested by a thickening of the alveolar membrane (Fig. 3E), and a rise in lung water from 75.0 \pm 0.5% (control) to 81.6 \pm 0.5% (after caerulein) of total lung weight ($P < 0.05$). It is also associated with increased leakage of FITC-labeled albumin in BAL fluid and a rise in lung MPO activity (Fig. 4). In CCR1 receptor deficient mice, the degree of alveolar membrane thickening is markedly reduced (Fig. 3F). Lung water content increased from 75.0 \pm 0.5% of total wet weight in control animals to 78.9 \pm 0.5% of total wet weight after caerulein treatment ($P < 0.05$), but this increase is significantly less ($P < 0.05$) than that noted in the wild-type animals after caerulein administration. In addition, FITC-albumin leakage and lung MPO activity after caerulein administration are markedly reduced in the CCR1 receptor deficient animals (Fig. 4). These observations indicate that genetic deletion of the CCR1 receptor dramatically reduces the severity of caerulein-induced lung injury associated with pancreatitis.

The CCR1 receptor binds the β -chemokines MIP1 α , RANTES, and MCP-3. Engagement of chemokine receptors results in the activation of Gi α and β/γ subunit signal transduction events typically leading to chemotaxis. Various intracellular pathways are activated, including the MAP kinase cascade, protein kinases A and C, and metabolism of phosphatidyl inositol. While it is commonly appreciated that proinflammatory cytokines such as TNF, IL-1, and growth factors are capable of stimulating chemokine mRNA synthesis and expression as immediate early response genes, we questioned whether the reverse effect might occur in vivo. Precedent for

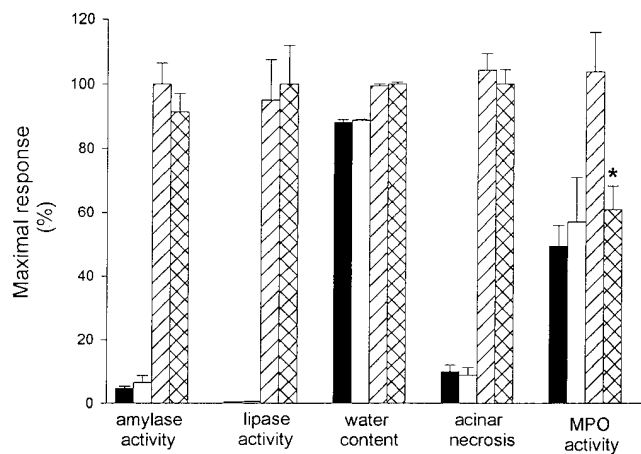


Figure 2. Effects of CCR1 receptor deletion on caerulein-induced pancreatitis. Serum amylase activity, serum lipase activity, pancreatic water content, acinar cell necrosis, and pancreatic MPO activity were measured as described in the text. Solid bars indicate wild-type control animals, open bars indicate CCR1 receptor deficient control animals, single-hatched bars indicate wild-type animals given 12 hourly injections of a supramaximally stimulating dose of caerulein, and double-hatched bars indicate CCR1 receptor deficient animals given 12 hourly injections of caerulein as described in the text. Values are expressed as a percentage of the value obtained for wild-type animals given caerulein. Results shown are mean \pm SEM values for 10 or more animals in each group. * $P < 0.05$ when CCR1 receptor deficient animals were compared with wild-type animals.

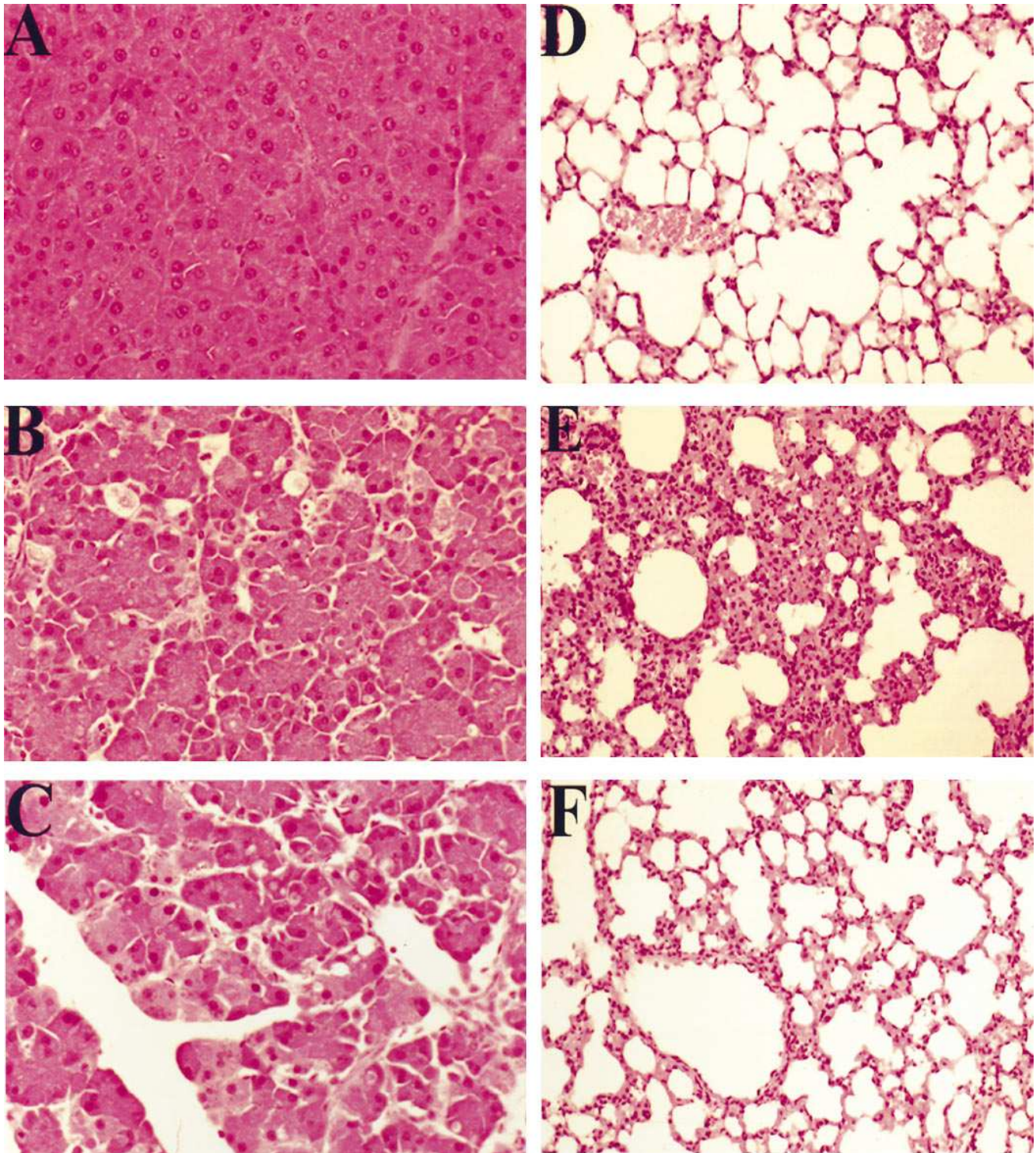


Figure 3. Morphologic changes of pancreatitis and pancreatitis-associated lung injury. Representative hematoxylin and eosin stained sections of pancreas (A–C) and lung (D–F) were examined by light microscopy in normal, control animals not given caerulein (A and D), in wild-type animals given caerulein (B and E), and in CCR1 receptor deficient animals given caerulein (C and F).

this was obtained by Fahey et al. (17) who demonstrated that MIP1 α , but not MIP1 β , could stimulate macrophages to release TNF- α in vitro.

As shown in Fig. 5, caerulein-induced pancreatitis in wild-type animals is associated with a time-dependent rise in pan-

creatic TNF- α levels. A similar but reduced change in pancreatic TNF- α levels is also noted in CCR1 receptor deficient mice. Caerulein-induced pancreatitis-associated lung injury in wild-type animals is also associated with a time-dependent rise in TNF- α levels in BAL fluids. In CCR1 receptor deficient an-

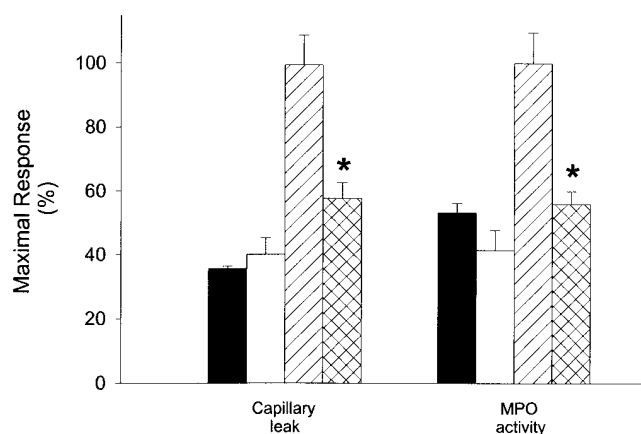


Figure 4. Effects of CCR1 receptor deletion on pancreatitis-associated lung injury. Capillary leakage of FITC-albumin and lung MPO activity were measured as described in the text. Groups are identified as described in the legend to Fig. 2. Values are expressed as a percentage of the value obtained for wild-type animals given caerulein. Results shown are mean \pm SEM values for 10 or more animals in each group. * $P < 0.05$ when CCR1 receptor deficient animals were compared with wild-type animals.

imals no increase in the BAL fluid level of TNF- α was detected after caerulein administration, and this finding correlates with the lack of secondary injury in this organ. A number of possibilities, which are not mutually exclusive, may underlie these findings. For example, CCR1 may be activated on either peritoneal or lung macrophages, leading to an autocrine process whereby increased levels of TNF- α drive further induction of both α - and β -chemokines, resulting in recruitment of inflammatory cells to the pancreas and lung. Another possibility is that pancreatic injury results directly in chemokine synthesis within this organ, which leads to local amplification of cytokine synthesis. Intrapancreatic inflammatory cytokines may then escape to the systemic circulation, activating monocytes and neutrophils in the bloodstream. The lung localization of inflammatory cells could also occur through local and mechanical factors (18). Once inflammatory cells are sequestered in the lung, a second wave of mediator release and synthesis may occur locally in the latter organ. In either circumstance, the CCR1 receptor appears critical in the extension of pancreatic injury to the systemic response.

The utility of animal models in understanding the systemic inflammatory response syndrome has limitations. One major caveat relates to the observation that in rodents no homologue of IL-8 has been described. KC and MIP2 are rodent homologues of Gro proteins, which interact selectively with CXCR-2, the IL-8 receptor B. Similarly, no rodent homologue of CXCR-1, the IL-8 receptor A, has been identified on rodent neutrophils. In contrast, rodents but not humans, appear to contain abundant CCR1 on granulocytes. Additionally, MIP1 α (a potent CCR1 agonist with no activity toward CXCR2) is a powerful granulocyte-activating chemokine (19). Thus, it is tempting to speculate that MIP1 α and CCR1 may subserve in rodents the role of IL-8 and CXCR-1 in humans. In the future, assignment of biological activities to human chemokines and chemokine receptors based on mouse genetics must be made with caution.

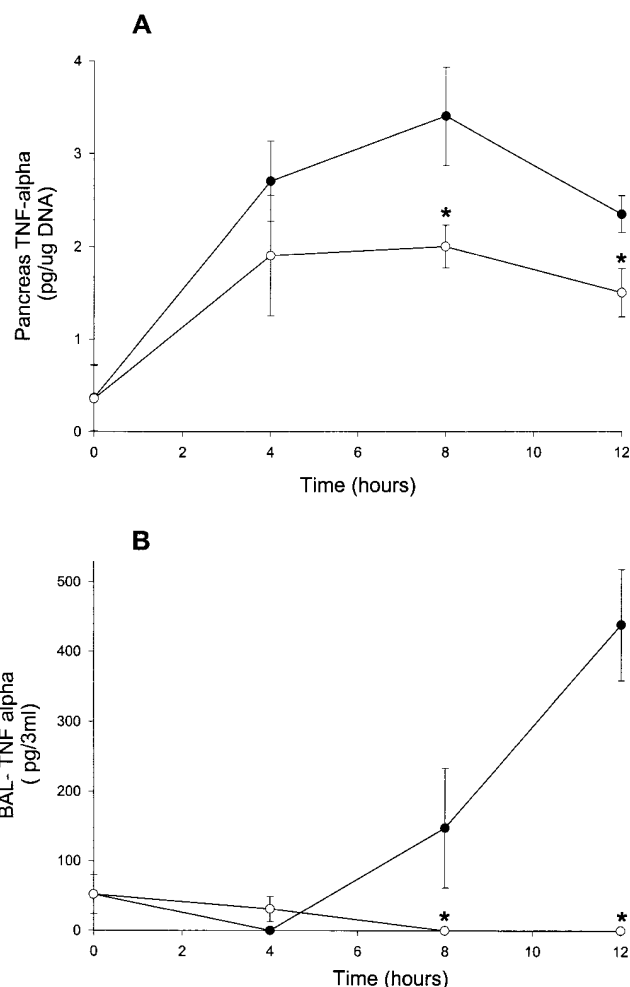


Figure 5. Effects of CCR1 receptor deletion on TNF- α levels in pancreas homogenates (A) and BAL fluid (B). TNF- α levels were measured by ELISA, as described in the text, at varying times after the start of caerulein injection in wild-type (filled circles) and CCR1 receptor deficient (open circles) animals. Results shown are mean \pm SEM values for five or more animals at each time point in each group. * $P < 0.05$ when CCR1 receptor deficient animals were compared with wild-type animals.

Acknowledgments

This work was supported in part by National Institutes of Health grants AI-39759 and HL-52503 to C. Gerard and DK-31396 to M. Steer. J.-L. Frossard is supported by University Hospitals, Geneva, Switzerland.

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