Interleukin-18, together with interleukin-12, induces severe acute pancreatitis in obese but not in nonobese leptin-deficient mice


Departments of *Kinesiology and Nutrition and †Pathology, University of Illinois at Chicago, Chicago, IL 60612; ‡Medizinische Klinik I, Charité Universitätsmedizin Berlin, Campus Benjamin Franklin, Hindenburgdamm 30, 12200 Berlin, Germany; and §Department of Medicine, University of Colorado Health Sciences Center, Denver, CO 80262

Contributed by Charles A. Dinarello, April 29, 2008 (sent for review March 10, 2008)

Obesity is associated with increased severity of acute pancreatitis (AP). The cytokines IL-18 and IL-12 are elevated in patients with AP, and IL-18 levels are high in obesity. We aimed to develop a pathologically relevant model to study obesity-associated severe AP. Lean WT and obese leptin-deficient ob/ob mice received two injections of IL-12 plus IL-18. Survival, pancreatic inflammation, and biochemical markers of AP were measured. Dosing with IL-12 plus IL-18 induced 100% lethality in ob/ob mice; no lethality was observed in WT mice. Disruption of pancreatic exocrine tissue and acinar cell death as well as serum amylase and lipase levels were significantly higher in ob/ob than in WT mice. Edematous AP developed in WT mice, whereas obese ob/ob mice developed necrotizing AP. Adipose tissue necrosis and saponification were present in cytokine-injected ob/ob but not in WT mice. Severe hypocalcemia and elevated acute-phase response developed in ob/ob mice. The cytokine combination induced high levels of regenerating protein 1 and pancreatitis-associated protein expression in the pancreas of WT but not of ob/ob mice. To differentiate the contribution of obesity to that of leptin deficiency, mice received short- and long-term leptin replacement therapy. Short-term leptin reconstitution in the absence of major weight loss did not protect ob/ob mice, whereas leptin deficiency in the absence of obesity resulted in a significant reduction in the severity of the pancreatitis. In conclusion, we developed a pathologically relevant model of AP in which obesity per se is associated with increased severity.

cytokines | inflammation | obesity | pancreas

Acute pancreatitis (AP) is an inflammatory disorder that ranges from interstitial edema to confluent necrosis and hemorrhage. In severe cases, progress to circulatory shock, acute lung injury, renal failure, and eventual death may occur (1). Despite some controversy, numerous studies have demonstrated that obesity is associated with increased risk of the severe form of AP and with development of life-threatening complications (2–8). However, the mechanism by which increased adiposity worsens AP remains unknown.

Leptin, a protein mainly produced by adipocytes, is a critical regulator of appetite (9). Leptin exerts important regulatory effects on inflammation (10). In the context of AP, the role of leptin remains controversial. In patients with AP, a correlation between serum leptin and disease severity has been reported by some investigators but not by others (11, 12). Although in animal models administration of leptin decreases AP severity (13–16), more severe disease is present in leptin-deficient ob/ob and leptin receptor-deficient db/db mice as well as leptin-receptor mutant fa/fa rats (17, 18). However, the differential effect of obesity versus leptin deficiency has not been fully investigated.

IL-12 and IL-18 are two proinflammatory cytokines that drive the Th1 cell response, characterized by high levels of IFN-γ (19). Both cytokines are mainly produced by monocytes/macrophages. Serum and pancreatic levels of IL-12 and IL-18 are elevated in patients with AP and correlate with disease severity (20–25). Elevation of IL-12 and IL-18 is likely responsible for the increased IFN-γ serum levels and expression by CD8+ lymphocytes infiltrating the pancreas in patients with severe AP (26–28). Furthermore, increased levels of IL-18 are observed in obese patients (29, 30). Thus, increased levels of IL-18 are biomarkers of disease severity in obese subjects as well as in patients with AP.

A few studies have reported the responses of mice to multiple doses of the combination of IL-12 plus IL-18. The effects observed included alterations of liver function and intestinal inflammation (31–33). These changes were associated with high levels of IFN-γ and other cytokines. Neutralization of IFN-γ or the use of IFN-γ-deficient mice protected from the toxicity of the IL-12 and IL-18 mixture (32, 33).

In the present study we investigated the effect of dosing mice with two injections of IL-12 plus IL-18 on pancreatic inflammation and the influence of leptin as well as obesity. We demonstrate that obesity associated with leptin deficiency enhances sensitivity to the toxic effects of the IL-12 plus IL-18 combination on the pancreas and that obesity per se rather than leptin deficiency seems to account for the increased severity of AP observed in leptin-deficient ob/ob mice.

Results

Lethality in ob/ob Mice Receiving IL-12 Plus IL-18. Two injections of the combination of IL-12 plus IL-18 induced 100% lethality by 48 h after the second injection in ob/ob mice (zero of 10 surviving mice in two separate experiments); in contrast, no lethality was observed in WT mice up to 2 wk posttreatment (10 of 10 surviving mice in two separate experiments). In agreement with previous studies (32, 33), administration of a 5-fold-higher dose of IL-12 plus IL-18 resulted in 30% lethality in WT mice over the course of 7 d (two of six surviving mice).

Increased Pancreatic Damage in ob/ob Mice Receiving IL-12 Plus IL-18. As shown in Fig. L4, disruption of pancreatic acinar tissue was observed in both WT and ob/ob mice at 24 h. Histologic scoring indicated that pancreatic damage was more pronounced in ob/ob compared to WT mice at both 6 and 24 h (Fig. 1C). TUNEL staining indicated a marked increase in acinar cell death in ob/ob mice receiving the cytokine mixture, whereas TUNEL-positive cells

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The authors declare no conflict of interest.

†To whom correspondence may be addressed. E-mail: cdinarello@mac.com.

‡To whom correspondence may be addressed: Department of Kinesiology and Nutrition, University of Illinois at Chicago, 1919 W. Taylor Street MVC 517, Chicago, IL 60612. E-mail: giamila@uic.edu.

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were observed only sporadically in tissue obtained from cytokine-injected WT mice (Fig. 4A). In both WT and ob/ob mice tissue disruption and cell death was limited to the exocrine tissue.

In WT mice, administration of IL-12 plus IL-18 led to development of significant pancreatic edema at 24 and 48 h (Fig. 2A). In contrast, no pancreatic edema was observed in ob/ob mice at any time point. Compared to vehicle-injected mice, pancreatic wet weight actually decreased by 30% in ob/ob mice receiving IL-12 plus IL-18, in contrast with a 40% weight increase observed in WT mice. These data, together with the observation of massive cell death in the acinar tissue of ob/ob mice, suggest that combination of IL-12 plus IL-18 induced edematous pancreatitis in WT mice whereas necrotizing pancreatitis developed in ob/ob mice.

Development of acute respiratory distress syndrome (ARDS) is one of the first indications of multisystem organ failure in AP (34). Dosing of mice with IL-12 plus IL-18 led to pulmonary edema in both WT and ob/ob mice (Fig. 2B). However, whereas in ob/ob mice significant edema was already observed at 6 and 24 h, in WT mice significant edema was only present at 48 h, a time point at which 100% lethality was observed in ob/ob mice.

**Adipose Tissue Necrosis and Saponification in ob/ob Mice Receiving IL-12 Plus IL-18.** Necrosis and saponification of adipose tissue, particularly in the retroperitoneum, are typically observed in AP patients (35). Macroscopic and histologic observation of adipose tissue obtained from ob/ob mice injected with IL-12 plus IL-18 revealed the presence of numerous loci of necrotic adipose tissue with saponification, which were rarely observed in WT mice (Fig. 1B and H&E staining in Fig. 1A). Adipose tissue necrosis was particularly pronounced in the retropancreatic area (Fig. 1B).

**Serum Amylase, Lipase, and Calcium Levels in Mice Receiving IL-12 Plus IL-18.** Increased circulating levels of the pancreatic enzymes amylase and lipase are useful markers for diagnosis of AP, although levels of these enzymes do not consistently correlate with disease severity (34). Serum amylase (Fig. 2C) and lipase (Fig. 2D) levels significantly increased in both WT and ob/ob mice after administration of IL-12 plus IL-18. However, a significantly greater increase was observed in ob/ob compared to WT mice. The kinetics of serum enzyme levels differed between the two groups: although in WT mice levels peaked at 2 h and then declined to reach baseline levels at 48 h, both amylase and lipase levels continued to increase over time in ob/ob mice. A significant reduction in serum calcium levels was observed in both WT and ob/ob mice injected with the combination of IL-12 plus IL-18 (Fig. 2E). However, only in ob/ob mice did levels fall below the critical level of 7 mg/dl.

**Enhanced Acute-Phase Response in ob/ob Mice Receiving IL-12 Plus IL-18.** Serum levels of IL-6 and the acute-phase proteins C-reactive protein (CRP) and serum amyloid A (SAA) are among the best predictive markers of AP severity in humans (34, 36). Serum IL-6 was markedly elevated in ob/ob mice receiving IL-12 plus IL-18, whereas only a small and transient increase was observed in WT mice (Fig. 3A). In ob/ob mice, IL-6 levels were significantly increased in homogenates of pancreatic tissue but not in homogenates of small intestine or liver (Fig. 3C).

Basal levels of SAA were significantly higher in untreated ob/ob mice compared to their WT littermates (Table 1). Although increased serum SAA levels were observed in both WT and ob/ob mice receiving IL-12 plus IL-18, ob/ob mice exhibited 23-fold higher SAA levels at 24 h compared to WT mice.

**Reduced Induction of Regenerating Protein 1 (Reg1) and Pancreatitis-Associated Protein (Pap) in ob/ob Mice Receiving IL-12 Plus IL-18.** Reduced induction of Reg1 and Pap are highly induced in the presence of AP and are involved in tissue repair and anti-inflammatory functions (37, 38). Lack of Pap induction is associated with severe AP in the cerulein model (39). Administration of IL-12 plus IL-18 induced high levels of Reg1 and Pap expression in WT mice (Fig. 3D and E). In contrast, no significant induction of either Reg1 or Pap was observed in the pancreas of ob/ob mice.

**Role of IFN-γ in Pancreatitis Induced by IL-12 Plus IL-18.** Dosing mice with the cytokine combination induced high levels of serum IFN-γ in both WT and ob/ob mice (Fig. 3B). Significantly lower serum IFN-γ levels were present in ob/ob compared with WT mice at 2 h, whereas no significant differences were observed at later time points.

To verify whether IFN-γ mediates pancreatic damage and acute-phase response induced by IL-12 plus IL-18, IFN-γ activity was neutralized. Blockade of IFN-γ prevented the increase in serum amylase, lipase, and IL-6 levels as well as hypocalcaemia observed in both WT and ob/ob mice (Table 2).
Role of Leptin in the Increased Susceptibility of ob/ob Mice to IL-12 Plus IL-18. To assess whether leptin deficiency or obesity are responsible for the increased susceptibility of ob/ob mice to IL-12 plus IL-18, we used two different approaches: (i) short-term administration of leptin and (ii) generation of “slim” ob/ob mice.

Short-term leptin administration. Leptin was administered for 3 d before start of IL-12 plus IL-18. This schedule of leptin administration led to a 6% decrease in body weight in ob/ob mice and a 10% decrease in WT mice (body weight was 50.0 ± 2.8 vs. 46.9 ± 0.5 g in vehicle- vs. leptin-injected ob/ob mice and 21.0 ± 0.3 vs. 18.8 ± 0.2 g in vehicle- vs. leptin-injected WT mice; n = 5 mice per group, P < 0.05 in vehicle vs. respective leptin-treated group by Student’s t test). Thus, ob/ob mice receiving short-term leptin remained markedly obese, weighing on average 2.5-fold more than WT mice. Despite having only a minor effect on body weight, short-term leptin administration normalized glycemia (glucose was 297 ± 57 vs. 110 ± 6 mg/dl in vehicle- vs. leptin-injected ob/ob mice, respectively; n = 5 mice per group, P < 0.001 by Student’s t test) and restored thymus cellularity in ob/ob mice (thymus cellularity was 12.5 ± 8.5 vs. 50.7 ± 9.4 × 10⁶ in vehicle- vs. leptin-injected ob/ob mice and 52.5 ± 6.7 vs. 54.5 ± 24.7 × 10⁵ in vehicle- vs. leptin-injected WT mice, respectively; n = 3–5 mice per group, P < 0.05 in vehicle ob/ob vs. leptin-treated ob/ob or WT mice by ANOVA). Pretreatment with leptin did not significantly alter the severity of pancreatitis in WT mice and led to a minor enhancement of serum lipase and SAA levels, together with worsening of hypocalcaemia, in ob/ob mice (Fig. 4 A–D). Furthermore, short-term leptin administration did not significantly alter induction of IL-6, while leading to increased levels of serum IFN-γ in both WT and ob/ob mice (Fig. 4 E and F).

P < 0.001 by Student’s t test}
Pancreatitis in "slim" ob/ob mice. Using the schedule of leptin administration and pair-feeding described in Materials and Methods, we attempted to generate "slim" ob/ob mice, i.e., leptin-deficient mice that were not obese. Because of the profound effect of leptin on energy expenditure (9), "slim" ob/ob mice remained intermediate between WT and ob/ob mice in terms of body weight (body weight was 23.3 ± 0.4, 56.3 ± 0.5, and 30.4 ± 0.6 g in WT, ob/ob, and "slim" ob/ob mice, respectively; n = 5–6 mice per group, P < 0.001 for each group vs. each other by ANOVA). Nevertheless, "slim" ob/ob mice weighed on average 46% less than ob/ob mice despite having undetectable serum leptin levels.

Administration of IL-12 plus IL-18 to "slim" ob/ob mice resulted in a reduction in the severity of pancreatitis as well as the acute-phase response, as indicated by significantly improved serum levels of calcium, IL-6, and SAA compared to ob/ob mice (Fig. 5A–C). In particular, serum calcium (A) and IL-6 (B) levels in "slim" ob/ob mice were not significantly different from levels observed in IL-12 plus IL-18-injected WT mice. In contrast, the cytokine combination induced comparable levels of serum amylose and lipase in "slim" ob/ob and ob/ob mice (Fig. 5D and E). Moreover, levels of serum IFN-γ induced by IL-12 plus IL-18 were comparable in the three groups (Fig. 5).

Discussion

Administration of a combination of IL-12 plus IL-18 induced pancreatic damage with features resembling AP in both WT and leptin-deficient ob/ob mice. Because both cytokines are increased in patients with AP and correlate with disease severity (20–25), a pathologically relevant model of AP in mice has been established. The major contributing factor of AP in this model appeared to be IFN-γ, because neutralization of this cytokine’s activity protected mice from pancreatic damage. This finding is not unexpected, because the combination of IL-12 plus IL-18 is well established as inducing IFN-γ. However, whereas neutralization of IFN-γ almost completely reversed hypocalcaemia and induction of IL-6 in ob/ob mice, only a partial effect was observed on amylose and lipase levels, suggesting the possible contribution of IFN-γ-independent mechanisms (32).

Of particular importance was the observation that pancreatic damage induced by the combination of IL-12 plus IL-18 was restricted to exocrine tissue in both WT and ob/ob mice, with islets appearing morphologically normal. The sparing of the insulin-producing islets is consistent with virally induced pancreatitis, which is associated with high levels of intrapancreatic IFN-γ (40). This pattern of inflammation is also reminiscent of the severe pancreatitis with selective exocrine disruption reported in mice injected with IFN-γ plus TNF-α as well as in mice deficient for SOCS1, a critical regulator of IFN-γ activity (41, 42). Although SOCS1 was induced by two injections of IL-12 plus IL-18 in the pancreas of both WT and ob/ob mice (data not shown), it is unclear whether the level of SOCS1 was sufficient to ameliorate the toxic effect of IFN-γ.

Compared to the response of WT mice, the severity of AP was greater in ob/ob mice, which demonstrated massive acinar cell death, adipose tissue saponification, early pulmonary edema, severe hypocalcaemia, a highly enhanced acute-phase response, and lack of induction of proteins involved in tissue regeneration, resulting in 100% lethality. Furthermore, ob/ob mice had a highly increased acute-phase response, which is associated with a more severe outcome in patients with AP (34, 36). Another feature of severe AP in ob/ob mice, which is typically observed in obese AP patients, was the massive necrosis and saponification of adipose tissue, particularly in the retropancreatic area. Although the administration of IL-12 plus IL-18 induced primarily edematous AP in lean WT mice, necrotic AP developed in ob/ob mice receiving the same cytokine dosing. Because this latter form of AP is associated with high mortality in patients, two injections of the combination of IL-12 plus IL-18 reproduces many of the features of the clinical and biochemical changes of severe AP observed in obese patients.

The family of regenerating proteins, including Reg1 and Pap, is highly induced during AP and is involved in tissue repair and anti-inflammatory functions (37, 38). Administration of IL-12 plus IL-18 induced high levels of mRNA expression of Reg1 and Pap in

### Table 1. Induction of SAA in mice receiving IL-12 plus IL-18

<table>
<thead>
<tr>
<th>Time</th>
<th>Control</th>
<th>ob/ob</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 h</td>
<td>1 ± 0.1</td>
<td>8 ± 1*</td>
</tr>
<tr>
<td>6 h</td>
<td>192 ± 28</td>
<td>1,353 ± 169*</td>
</tr>
<tr>
<td>24 h</td>
<td>1,745 ± 637</td>
<td>40,582 ± 2,891*</td>
</tr>
</tbody>
</table>

Mice received two daily injections of IL-12 and IL-18. Data are mean ± SEM of SAA (mg/ml) (n = 5 mice per group).

*p < 0.001 vs. respective WT by Student’s t test.

### Table 2. Neutralization of IFN-γ prevents pancreatic damage and acute-phase response in mice treated with IL-12 plus IL-18

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Amylase, U/L</th>
<th>Lipase, U/L</th>
<th>Calcium, mg/dl</th>
<th>IL-6, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>378 ± 24</td>
<td>71 ± 3</td>
<td>9.8 ± 1.4</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>IL-12 plus IL-18</td>
<td>2,249 ± 550*</td>
<td>1,142 ± 297*</td>
<td>8.0 ± 0.4*</td>
<td>0.8 ± 0.8</td>
</tr>
<tr>
<td>IL-12-IL-18 plus</td>
<td>518 ± 134*</td>
<td>199 ± 84*</td>
<td>9.8 ± 0.7*</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>anti-IFN-γ receptor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ob/ob</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>693 ± 51</td>
<td>74 ± 13</td>
<td>10.3 ± 0.7</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>IL-12 plus IL-18</td>
<td>5,653 ± 540*</td>
<td>3,512 ± 439*</td>
<td>5.6 ± 0.8*</td>
<td>2.9 ± 0.8*</td>
</tr>
<tr>
<td>IL-12-IL-18 plus</td>
<td>1,122 ± 312*</td>
<td>456 ± 212*</td>
<td>10.6 ± 0.2*</td>
<td>0.2 ± 0.1*</td>
</tr>
<tr>
<td>anti-IFN-γ receptor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mice received two daily injections of IL-12 and IL-18, and serum was obtained 24 h after the second injection. In the anti-IFN-γ receptor ab-treated groups, mice received an injection of antibody 1 h before each administration of IL-12 and IL-18. Data are mean ± SEM (n = 5 mice per group).

*p < 0.001 vs. respective control.

p < 0.01 vs. respective control.

p < 0.01 vs. respective IL-12 plus IL-18.

p < 0.001 vs. respective IL-12 plus IL-18.
the pancreas of WT mice, whereas only a small, nonsignificant increase was observed in ob/ob mice. The relative role of leptin and obesity in modulating expression of Reg1 and Pap is not completely clear; whereas leptin induces Pap expression in vitro, this regenerating protein is reduced in vivo in ob/ob mice injected with leptin (43, 44). Despite the current uncertainty as to the causes of disregulation of Reg1 and Pap in ob/ob mice, it is noteworthy that severe AP in these mice was associated with lack of induction of these two protective factors. Therefore, failure to express these anti-inflammatory proteins may contribute to severity of the response in obesity.

Because leptin regulates inflammation and exerts acute protective effects in models of AP (13–15), it was important to differentiate between the direct effects of leptin deficiency and those mediated by massive obesity. Short-term leptin administration did not protect ob/ob mice from AP and actually worsened hypocalcaemia and resulted in increases in lipase, SAA, and IFN-γ levels.

Possible mechanisms for the increased inflammatory response in ob/ob mice receiving short-term leptin include increased calcium mobilization in acinar cells by a leptin-dependent mechanism (45) and/or an effect of leptin in shifting the balance between production pro- and anti-inflammatory cytokines (46).

In contrast, “slim” ob/ob mice, which are leptin-deficient but ~50% leaner than ob/ob mice, responded to the cytokine mixture with a biochemical profile more similar to that of WT mice than of ob/ob mice. Thus, although we cannot exclude that leptin deficiency per se might play a role in the model of AP induced by administration of IL-12 plus IL-18, our data point to a more important role for obesity in worsening the outcome. Future experiments using mice fed a high-fat diet will help clarify this issue.

In conclusion, we have developed a pathologically relevant model of AP, in which massive obesity due to leptin deficiency is associated with a more severe outcome. This model reproduces many of the parameters observed in humans with AP, particularly the increased severity and adipose tissue necrosis associated with obesity and will serve as a useful tool to investigate the link between increased adiposity and heightened AP severity in humans.
Jackson Laboratories. Female mice were used to better compare the results with previously published data (32, 33).

Administration of IL-12 and IL-18. Murine recombinant IL-12 (Peprotech) and IL-18 (R&D Systems) were administered i.p. at 150 ng per mouse and 750 ng per mouse, respectively, at 24-h intervals for a total of two injections. The schedule and dose of cytokine administration were selected based on pilot studies. Mice were killed at 2, 6, 24, or 48 h after the second injection. Control mice received injections of vehicle following the same schedule. For neutralization of IFN-γ activity, 200 μg of a rat-anti-mouse IFN-γ receptor monoclonal antibody (GR-20) was administered i.p. 1 h before each injection of IL-12 plus IL-18.

Administration of Leptin. For short-term leptin administration, mice received i.p. injections twice daily of recombinant murine leptin (R&D Systems) at 5 μg per d, beginning 3 d before administration of IL-12 plus IL-18 and continuing throughout the course of the experiment. For generation of "slim" ob/ob mice, ob/ob mice received s.c. leptin (5 μg/day) for 1 month through Alzet pumps (Durect Corp.). After 1 mo, pumps were removed and mice were pair-fed to WT mice for an additional month to maintain their body weight at the level reached at the time of pump removal. Control ob/ob mice were sham operated and not pair-fed.

Miscellaneous Measurements. IL-6 and IFN-γ were measured by using ELISA kits from BD Pharmingen. Serum SAA was measured by using an ELISA kit from Teco Diagnostics. Blood glucose levels were measured by using a glucometer (Bayer).

Histological Assessment, Immunohistochemistry, and TUNEL. Organs were fixed in 10% buffered formalin, and sections were stained with hematoxylin/eosin for histological scoring by a pathologist (R.J.C.) blinded to the experimental group (47). Briefly, edema was graded as 0 (absent or rare), 1 (in the interlobular space), 2 (in the intralobular space), and 3 (severe edema); inflammation was graded as 0 (absent), 1 (mild), 2 (moderate), and 3 (severe); and parenchymal and intrapancreatic fat necrosis were independently graded as 0 (absent), 1 (focal), 2 (and/or sublobular), and 3 (and/or lobular). To assess for the presence of cell death, a DeadEnd TUNEL kit from Promega was used. For quantification of edema, organs were weighed immediately after collection and after being incubated overnight at 90°C. Edema was expressed as the wet weight/dry weight ratio.

Quantitative RT-PCR. Total RNA was extracted from pancreatic tissue by using TRIzol extraction and reverse-transcribed to cDNA. Quantitative RT-PCR was performed by using primers for murine pancreatitis-associated protein (Mnop0440616_g1) and regenerating islet-derived 1 (Mnop085651_m1) from Applied Biosystems. Data were normalized by expression of 18S RNA and expressed as the relative ratio to control WT mice.

Statistical Analysis. Data are expressed as mean ± SEM. Statistical significance was determined by factorial ANOVA by using the XLStat software (Addinsoft).

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