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IL-10 Is Not Protective in Intestinal Ischemia Reperfusion Injury

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Background. **Ischemia/reperfusion of the small intestine disrupts gut barrier function, increases bacterial translocation, and activates systemic pro-inflammatory responses. Pharmacological treatment with the anti-inflammatory cytokine interleukin-10 (IL-10) following ischemia to muscle reduces the severity of local and systemic inflammation. While endogenous IL-10 is protective in murine models of acute endotoxemia, its physiological role during direct gut injury is unknown.**

Patients and materials. **Mice genetically deficient in IL-10 (IL-10$^{-/-}$) and their normal littermates (IL-10$^{+/+}$) underwent 20 to 50 min of gut ischemia by occlusion of the superior mesenteric artery.**

Results. **Both short- and long-term (>16 h) survival after reperfusion of IL-10$^{-/-}$ mice was identical to that of the wild-type littermates, with 50% mortality observed at 35 min of occlusion. The small bowel demonstrated discrete gross areas of hemorrhage and ischemia localized to the jejunum. No significant difference in the extent or time for occurrence of macroscopic or microscopic intestinal damage to the small bowel was observed in IL-10$^{-/-}$ or IL-10$^{+/+}$ mice, despite the marked elevation in serum IL-6.**

Conclusions. **The absolute serum concentration of IL-6 in the presence or the absence of IL-10 does not affect local or systemic response to ischemic intestinal injury. These results also demonstrate that the anti-inflammatory cytokine IL-10 does not play a significant local or systemic protective role in this model of ischemia/reperfusion.**

Key Words: **shock; sepsis; ischemia; reperfusion; multisystem organ failure; SIRS; IL-6; IL-10; TNF-α; intestine.**

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INTRODUCTION

Uncontrolled systemic inflammation caused by the release of locally produced inflammatory mediators often results in multisystem organ failure (MSOF) with significant morbidity and mortality [1]. There are multiple initiators of the pro-inflammatory cytokine cascade that results in systemic inflammation [2]. These include multisystem trauma, pancreatitis, intra-abdominal sepsis, as well as direct and indirect injury to the intestine [3–6]. Common to most critical conditions is gut hypoperfusion with subsequent ischemic damage to enterocytes and their supporting structures [7]. This insult results in epithelial cell damage, loss of brush border enzymes, decreased absorptive function, and the loss of basement membrane integrity leading to translocation of bacteria [8]. Early enteral feedings in the critically ill patient blunts systemic inflammation by protecting mucosal integrity [9]. Thus, the gastrointestinal tract may directly or indirectly modulate systemic inflammation.

Current understanding of the systemic inflammatory cascade and the possible role of pro-inflammatory and anti-inflammatory cytokines in MSOF is derived from the endotoxin-induced (i.e., bacterial wall lipopolysaccharide, LPS) animal model of sepsis. Results from this model exhibit a reproducible pro-inflammatory cytokine cascade involving the sequential production of TNF-α, IL-1, and IL-6 [10]. The questionable relevance of this model to humans is partially due to the lack of efficacy of such anticytokine therapies as anti-TNF-α, IL-1 receptor antagonist, and soluble IL-6 receptor developed for patients with sepsis and/or MSOF, which contrasts with the results reported in the endotoxin model of sepsis [11]. Thus, the clinical correlation of the sequential production of cytokines in the endotoxin model of sepsis has not been established.

More recently, strategies for the treatment of septic

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shock have focused on the use of anti-inflammatory cytokines, such as IL-10, which regulates the production of their pro-inflammatory counterparts [12]. IL-10 is a homodimeric cytokine produced by monocytes, macrophages, T cells, and epithelial cells among others. *In vitro* IL-10 inhibits the synthesis of TNF-α, IL-1α, IL-1β, IL-6, and reactive oxidative intermediates [13, 14]. Experimentally, the administration of pharmacological doses of IL-10 has demonstrated protection in animal models of acute endotoxemia, cecal ligation and puncture, and intestinal ischemia [15–17]. However, when sepsis is induced by cecal ligation and puncture, the protective effect of endogenous IL-10 is controversial due to the conflicting results from two independent investigators [18, 19]. Thus, the physiologic role and clinical efficacy of this anti-inflammatory cytokine in the regulation of systemic inflammation and MSOF are unproven.

Intestinal damage due to hypoperfusion of the bowel in the absence of sepsis is often indirect and insidious and is manifested by loss of barrier function and translocation. Ischemic injury to the gut is believed to occur in most critically ill patients, resulting from such insults as adult respiratory distress syndrome (ARDS) or pancreatitis [3–6]. Therefore, gut injury may play a critical role in the initiation and /or propagation of the systemic inflammatory cascade. In contrast to the LPS-induced sepsis model, animal models of intestinal injury may better mimic the pathology of systemic inflammation and MSOF. We have previously developed a reproducible murine model of intestinal ischemic injury induced by reversible occlusion of the superior mesenteric artery (SMA) [20, 21]. Using this model we demonstrate in this report that endogenous IL-10, while still regulating pro-inflammatory cytokine production, is unable to protect against local intestinal ischemia reperfusion injury and systemic inflammation.

METHODS

Mice. C57BL/10 mice 8–10 weeks of age (20–22 g), genetically deficient in IL-10 (IL-$10^{-/-}$), and their wild-type littermate controls C57BL/10 (IL-$10^{+/+}$) were used for these experiments (Jackson Laboratory, Bar Harbor, ME). All animals were maintained in specific pathogen-free housing and were acclimated to the surgery lab for 24 h prior to experimentation. Animals had unlimited access to food and water except for the time of anesthesia.

Ischemia/reperfusion model. Mice were anesthetized with Avertin (tribromoethanol, 200 mg/kg) and resuscitated with a total of two subcutaneous administrations of normal saline (1 ml) at the start of the procedure and 30 min later. Anesthesia was maintained with supplemental doses of Avertin (2 mg in 0.1 ml of normal saline/10 g of body weight, given by intraperitoneal injection). Animals were subjected to ceiliotomy [20, 21], with subsequent isolation and occlusion of the SMA for variable periods (lethal and nonlethal) with a spring-loaded microvascular clamp. The abdomen was closed after reperfusion of the intestine and the animals were recovered with the assistance of a heating lamp and warming pad. For each study group shams underwent ceiliotomy alone. After the reperfusion period animals were evaluated for survival. Those alive at 16 h were considered long-term survivors based on studies showing their full recovery [20]. Preliminary studies in wild-type mice demonstrated that occlusion times greater than 35 min lead to 100% mortality. All protocols were approved by IACUC review.

TABLE 1

Histological Grading of Intestinal Ischemia (Small Intestine)

Grade[a]	Features
0	Normal; villous to crypt ratio 5 or 6:1 Minimal number of lymphocytes and plasma cells Tall columnar surface epithelial cells
1	Epithelial cell degenerative changes (cuboidal, vacuolated) but intact Mild increase of lymphocytes and plasma cells in lamina propria
2	Decreased villous height to V:C = 1:1 or less Epithelial cell necrosis, erosions More chronic inflammation in lamina propria; ± neutrophils Glandular dilatation
3	Villi effaced (flat surface) Epithelial cell necrosis, erosions May be pseudomembrane on surface Glandular destruction, inflammation extending deep to muscle layer
4	Transmural changes (all of the above plus change in muscle layer)

[a] Histological scoring of injury to the small bowel was developed specifically for the damage observed in this murine model.

Animal evaluation. After an extreme ischemic injury (>90 min) animals that succumb to the initial process of reperfusion never recover from the anesthetic and die within 60–90 min. Those animals that die due to the systemic inflammatory process recover from the anesthesia and subsequently demonstrate progressive disease hallmarked by lethargy, piloerection, periorbital discharge, and the loss of their righting ability.

Histology grading. The small bowel was collected from mice surviving 16 h or at the time of their premature demise. Tissue was divided into duodenum, jejunum, and proximal and distal ileum located 1, 6, 26, and 37 cm from the gastroduodenal junction, placed in Formalin, and subsequently embedded in paraffin for hematoxylin and eosin (H&E) staining. All specimens were evaluated microscopically in a blinded fashion for degree of damage based on a grading system of epithelial injury originally described by Park [22] and modified to reflect the variable nature of ischemic injury to the mouse intestine (Table 1).

Serum cytokine. Animals underwent cardiac puncture to obtain blood at the indicated times in the reperfusion period. Serum levels of the cytokines TNF-α and IL-6 were measured by ELISA (all from R&D Systems, Minneapolis, MN), according to the manufacturer's recommended protocols.

Reverse transcription–polymerase chain reaction (RT-PCR). Jejunal levels of TNF-α, IL-6, and IL-10 mRNA were measured via RT–PCR. Total RNA was isolated using TRIzol Reagent (Life Technologies, Basel, Switzerland) according to the manufacturer's protocol. The RT reaction contained 1 μg of RNA, 5 μL of 5× First Strand Buffer, 2.5 μL of 0.1 M DTT, 1.25 μL of 10 mM dNTP mix, 1 U of oligo(dT), and 1 U of Superscript RNase H RT (all from Gibco Life Technologies). PCR was performed in the following mixture: 2.5 mM each dNTP, 1 U of Taq polymerase (Boehringer Mannheim, Switzer-

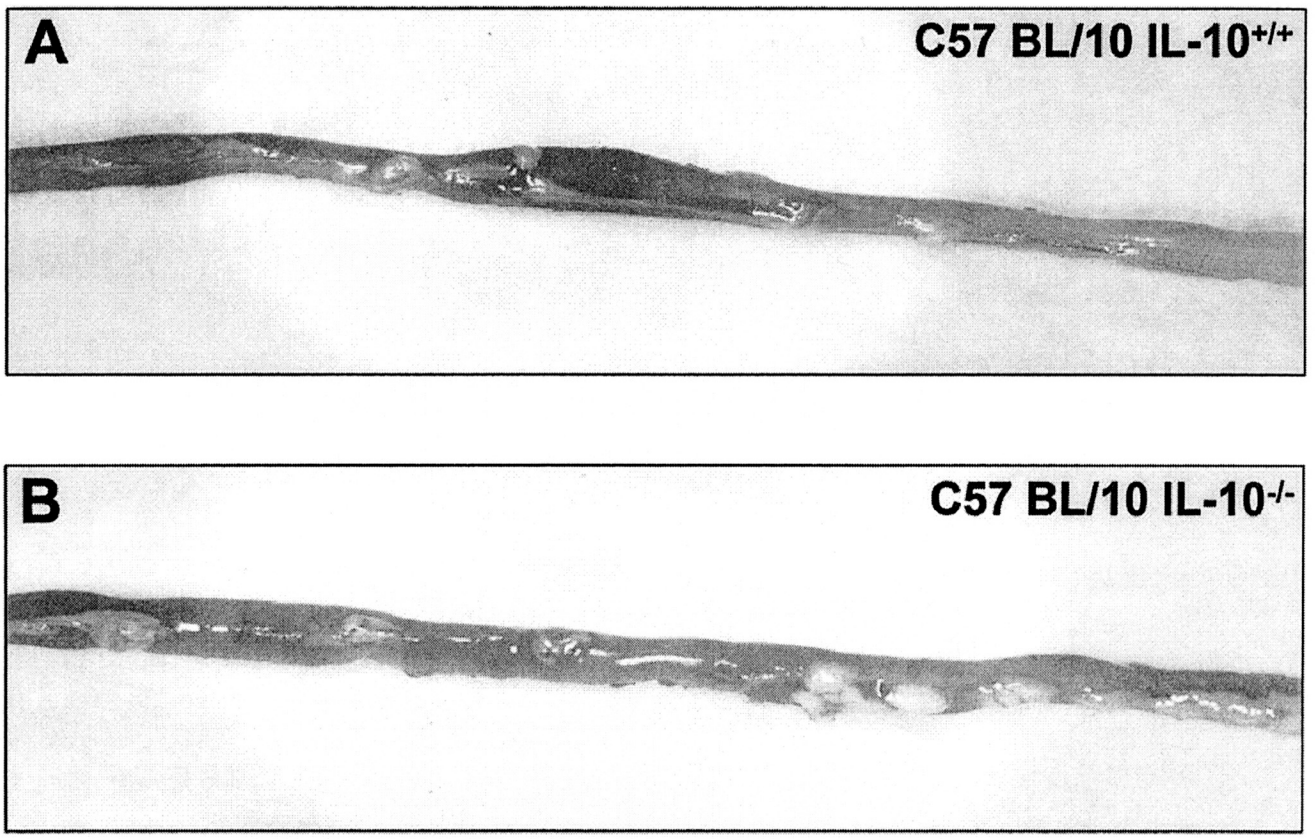

FIG. 1. Similar levels of necrosis and hemorrhagic damage to the small bowel are observed in the presence and the absence of IL-10. The SMA was occluded for 30 min, 2 h after reperfusion the mice were sacrificed, and a 10-cm section of the jejunum was photographed. Representative samples from more than six mice are shown. IL-$10^{+/+}$ (*A*); IL-$10^{-/-}$ (*B*).

land), 10X PCR buffer (Life Technologies), 5 μL of cDNA, and 2.5 μM PCR primer pairs. The following forward and reverse primers pairs were used: TNF-α, ACAAGCTTGTAGCCCACGTCGTAGC and TGACTCGAGAGTAGACCTGCCCGG (307); IL-6; ATGAAGTTCCTGTCTGCAACAGACT and CACTAGGTTTGCCGAGTAGATCTC (638 bp); IL-10, TCCTTAATGCAGGACTTTAAGGGTTACTTG and GACACCTTGGTCTTGGAGC TTATTAAAATC (373); GAPDH; ACCACAGTCCATGCCATCAC and TCCACCACCCTGTTG CTGTA (452 bp). Samples were fractionated on a 1.5% agarose gel in 0.5× TBE buffer and the bands stained with 0.5 μg/mL of ethidium bromide and destained with ddH_20 for 30 min each. Reaction conditions were optimized for each primer pair. Band intensity was recorded on a Bio-Rad Molecular Imager (Hercules, CA) and the results were normalized to the housekeeping gene GAPDH.

Statistics. Statistical analysis was performed using Student's *t* test for direct comparison between groups (Microsoft Excel, Richmond, WA). Significance was $P < 0.05$.

RESULTS

Macroscopic and Microscopic Damage to the Jejunum after Ischemia/Reperfusion Injury

Occlusion of the SMA and subsequent reperfusion in the C57BL/10 mouse resulted in a consistent dose-dependent intestinal injury determined by histology, directly correlating with the length of ischemia [20, 21]. At a sublethal dose, i.e., 30 min of occlusion, the greatest macroscopic damage was observed in the proximal ileum and entire jejunum at 2 h of reperfusion (Fig. 1A). These segments of the small bowel demonstrated gross intestinal dilatation and intramural hemorrhage. This correlated microscopically at both 2 and 16 h after reperfusion where histological evaluation of the jejunum showed neutrophilic infiltration of the lamina propria, blunted villi, and lifting of the epithelial from the underlying tissue (Figs. 2A and 2B). There was minimal, if any, injury to the duodenum and colon, as would be expected due to extensive collateral blood supply.

Genetic Ablation of IL-10 Does Not Alter the Rate or Severity of Intestinal Ischemia/Reperfusion (I/R) Injury

The anti-inflammatory and immunosuppressive functions of IL-10 have been demonstrated to play a protective role in a variety of immune and inflammatory models [23–27]. In these models the absence of IL-10 renders the animals more susceptible to inflammatory insults. In our study when IL-$10^{-/-}$ mice were subjected to intestinal I/R, the expected increased sensitivity to the I/R injury was not seen. There were no differences in the gross appearance of the small bowel from IL-$10^{-/-}$ mice, when compared to IL-$10^{+/+}$ mice, observed at any time point after reperfusion (Fig. 1B).

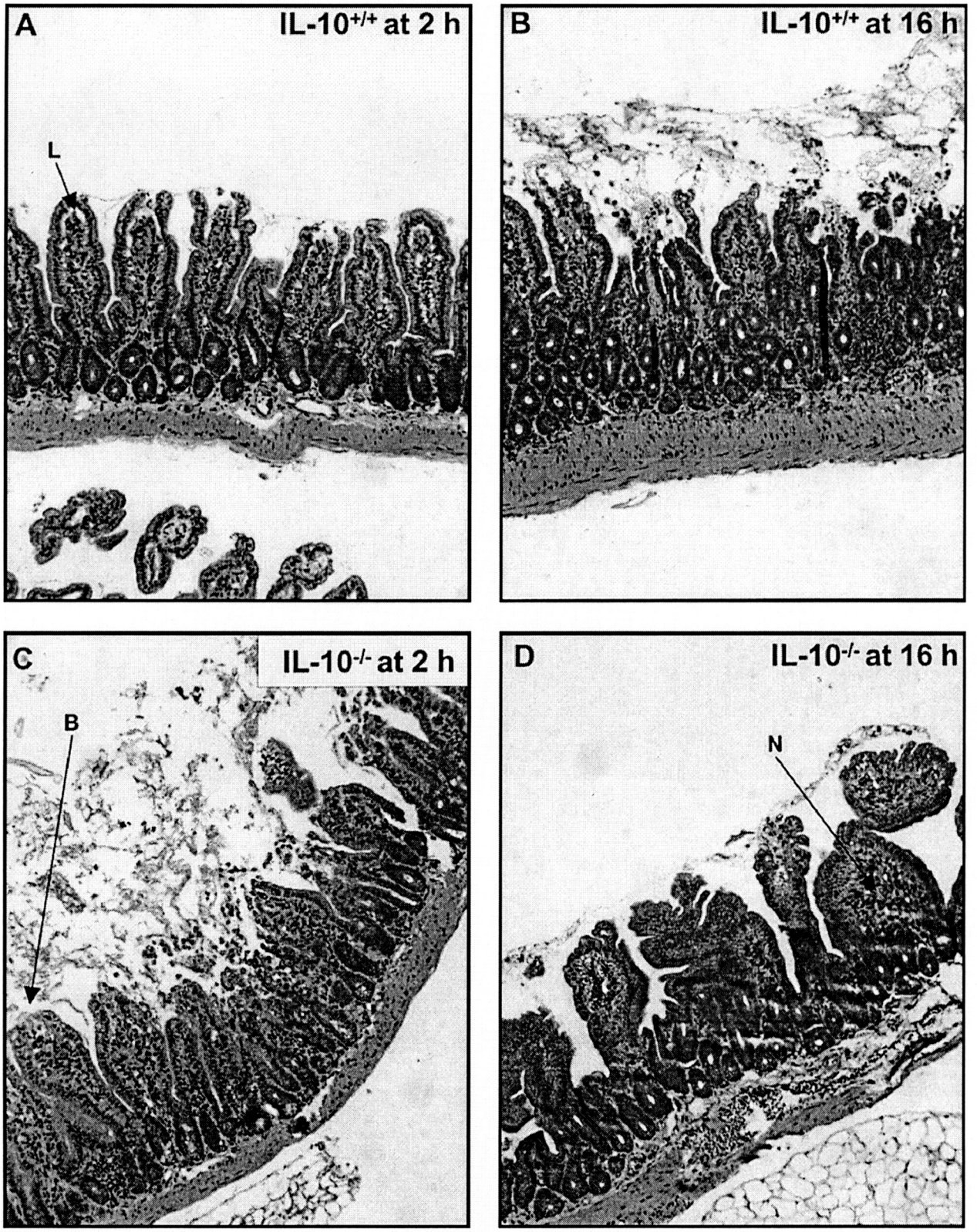

FIG. 2. Mucosal injury after SMA occlusion is similar in IL-$10^{+/+}$ and IL-$10^{-/-}$ mice at 2 and 16 h after reperfusion. Mice underwent 30 min of SMA occlusion, and sections from the jejunum of IL-$10^{+/+}$ (A and B) and IL-$10^{-/-}$ (C and D) after 2 h (A and C) and 16 h (B and D) were fixed and stained with H&E. Areas of epithelial lifting (L), neutrophil infiltration (N), and blunting of the villi (B) are noted.

Similarly, at 2 and 16 h postischemia there was no microscopic difference in intestinal damage between IL-$10^{+/+}$ and IL-$10^{-/-}$ mice (Figs. 2C and 2D) when quantitated by blinded histological scoring (Fig. 3).

TNF-α Minimally Produced in Serum and Tissue

The amount of TNF-α protein present in the serum (Fig. 4) and mRNA synthesized locally in the jejunum

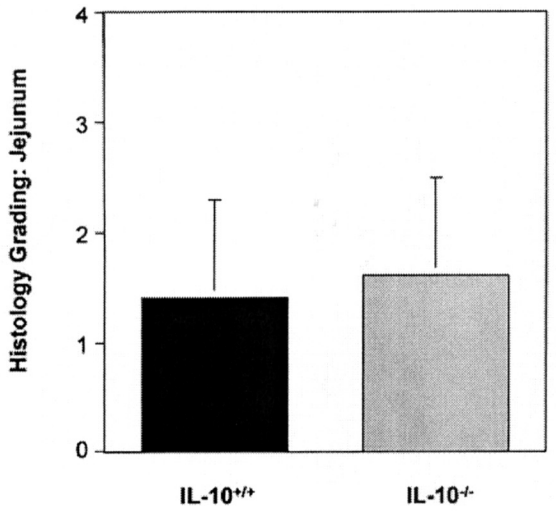

FIG. 3. Local intestinal damage is unaffected by genetic deletion of the anti-inflammatory cytokine IL-10. Groups of five IL-10-deficient mice (gray bars) and their wild-type littermates (black bars) underwent 30 min of SMA occlusion and were graded microscopically, as described in Table 1.

(Fig. 6) are below the minimal detectable level of ELISA and RT–PCR, respectively. Serum TNF-α levels were dramatically below those concentrations commonly observed in other models of sepsis [10].

Serum and Tissue IL-6 Concentrations Do Not Reflect Local Damage after I/R

As described in the cecal ligation and puncture model, early in the disease process IL-6 levels between survivors and nonsurvivors were similar. Yet, those animals that maintain persistent elevated serum IL-6 have greatly decreased survival [28]. We therefore investigated whether the profile of IL-6 synthesis, rather than the absolute concentration of serum IL-6, is predictive of outcome after ischemic injury to the gut. IL-$10^{+/+}$ and IL-$10^{-/-}$ mice were challenged with sublethal and lethal occlusions of the SMA, and serum IL-6 concentrations (Fig. 4) and tissue mRNA expression (Fig. 6) were measured. As expected, the absolute level of serum IL-6 was significantly increased in the absence of IL-10 ($P < 0.05$), yet local injury in IL-$10^{+/+}$ and IL-$10^{-/-}$ mice was independent of peak IL-6 concentrations.

In addition, IL-6 production at 2 h of reperfusion was not statistically different when wild-type mice were subjected to a sublethal or lethal intestinal injury. Similarly at 2 h, serum IL-6 levels in IL-$10^{-/-}$ mice were the same in surviving and nonsurviving animals. Together these results demonstrate that absolute serum IL-6 concentrations do affect local injury or mortality.

Systemic Inflammation Induced by I/R Injury Is Not Regulated by the Anti-inflammatory Cytokine IL-10

It is widely accepted that IL-10 is protective in the LPS/endotoxemia model of sepsis by regulating the inflammatory response through its inhibition of the production of pro-inflammatory cytokines (TNF-α, IL-1, IL-6). If IL-10 is a primary regulator of systemic inflammation and MSOF, one may expect that a genetic deficiency in IL-10 would render an animal more susceptible to insult. We compared the survival after I/R of genetically engineered IL-10-deficient mice on a C57BL/10 background with their wild-type littermates. There was no difference in survival between the two congenic strains when the mice were exposed to increasing times of SMA occlusion (Fig. 5), suggesting a direct, IL-10-independent link between local intestinal injury and systemic inflammation, despite the local production of IL-10 mRNA in the damaged jejunum (Fig. 6).

DISCUSSION

In this report, the role of intestinal injury in initiating the systemic inflammatory response syndrome (SIRS) and the production of inflammatory mediators is elucidated. The critically ill patient with eventual systemic manifestations has persistent inadequate perfusion resulting in intestinal injury from progressive ischemia. Using the dynamic model of intestinal I/R, which mimics what is observed clinically, we demonstrate the presence of gross as well as localized histological intestinal damage that initiates a previously undefined systemic cascade of cytokine synthesis. In particular, neither IL-10, TNF-α, nor IL-6 appears to significantly influence the progression of sepsis, suggesting that there are alternative pathways and actions of cytokines differing from those of previous reports. This is especially apparent with both the lack of TNF-α protein in the serum and mRNA in injured jejunal tissue. These results may explain why to date other animal models of sepsis have not lead to a clinically successful intervention for uncontrolled systemic inflammation and MSOF.

Targeted deletion of the gene encoding IL-10, or administering neutralizing antibodies to IL-10 to normal mice, dramatically increases their susceptibility to an endotoxin challenge [29, 30]. Similarly, the exogenous administration of pharmacological doses of IL-10 has a protective role in the same LPS model of sepsis [16, 17] and in an intestinal I/R model [26]. Although IL-10 is shown experimentally to have anti-inflammatory properties, the expected susceptibility of IL-10-deficient adult mice on a C57BL/10 background in the I/R model of local and systemic inflammation, as measured by intestinal damage and mortality respectively, was not observed. These results differ from those of another

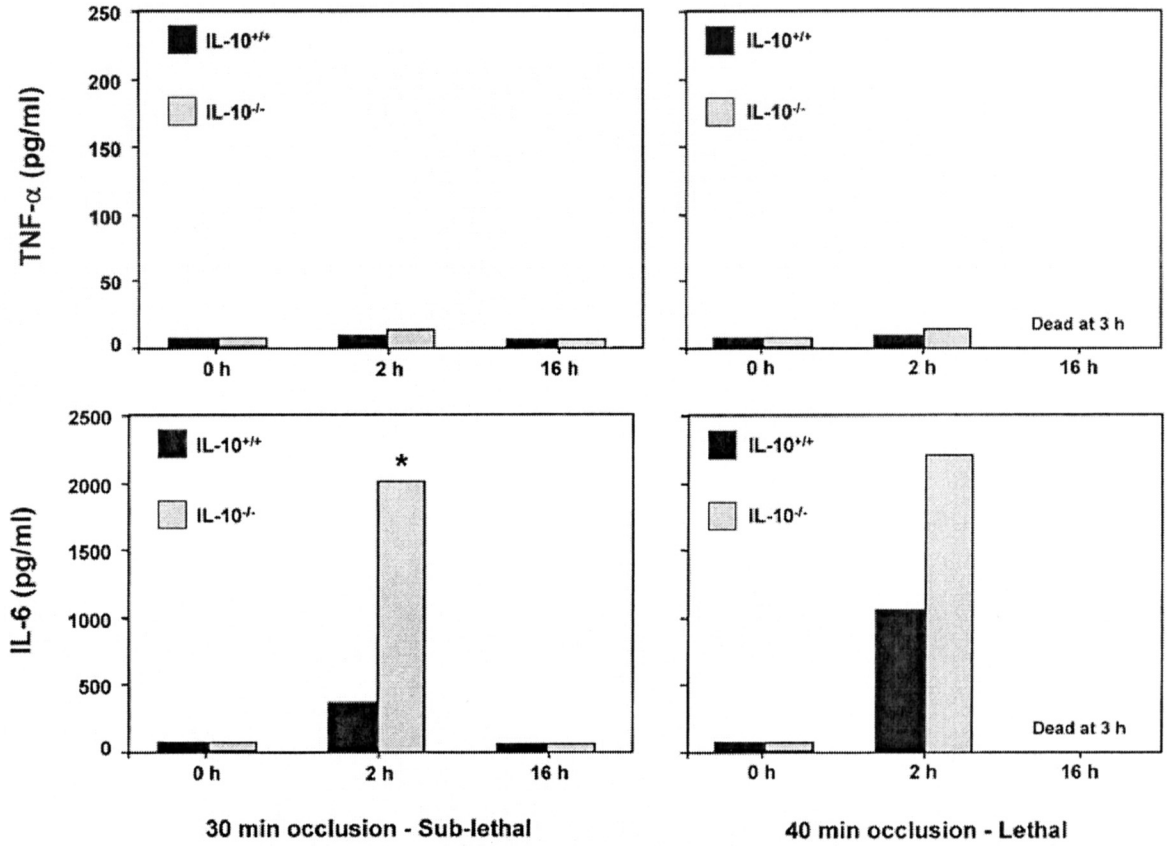

FIG. 4. Serum TNF-α and IL-6 concentrations do not reflect local damage after I/R. The SMA of IL-$10^{+/+}$ (black bars) and IL-$10^{-/-}$ (gray bars; $n = 5$) mice was occluded for sublethal (left) and lethal (right) times and serum TNF-α and IL-6 levels were measured by ELISA. Note that despite the increased expression of IL-6 in IL-$10^{-/-}$ mice the absolute levels of IL-6 did not predict the outcome. $^*P < 0.05$.

report, in which ischemia was introduced into the total foregut via occlusion of both the superior mesenteric and celiac arteries in a more immature animal [31]. It is currently unclear why these results differ; however, in the latter model younger animals were subjected to a more severe injury affecting the proximal and distal GI tract, while in our model damage was localized to the jejunum. In addition, our study evaluated histological and immunological effects for 16 h of reperfusion,

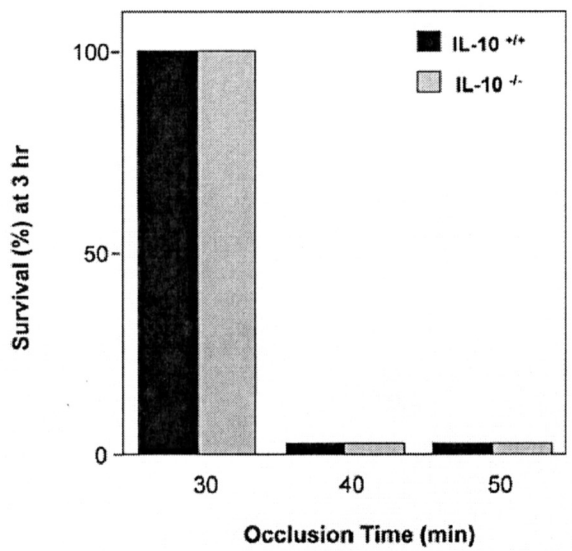

FIG. 5. Survival after SMA occlusion is unaffected by the absence or the presence of endogenous IL-10. Fifteen IL-$10^{-/-}$ mice (black bars) and their wild-type littermates (gray bars) underwent ischemic injury for the times indicated. The percentage of survival was evaluated at 3 and 16 h.

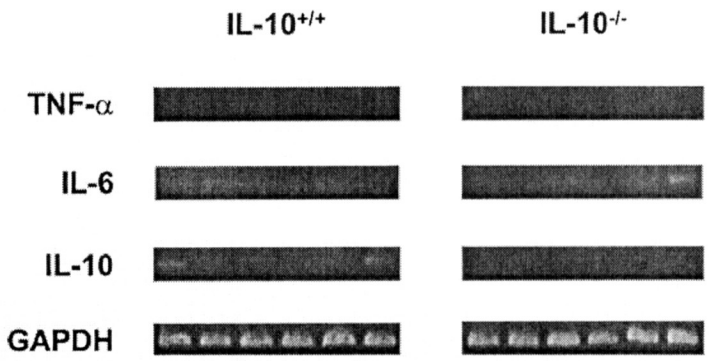

FIG. 6. Jejunum production of TNF-α, IL-6, and IL-10 was measured using RT–PCR. Two hours after reperfusion from a lethal (40-min occlusion) injury, jejunal RNA was isolated. RT–PCR analysis of two groups of individual IL-$10^{+/+}$ and IL-$10^{-/-}$ mice is shown ($n = 6$). TNF-α mRNA expression was minimal in both strains of mice. IL-6 mRNA expression in both IL-$10^{+/+}$ and IL-$10^{-/-}$ mice was elevated. IL-10 mRNA expression was detected only in IL-$10^{+/+}$ jejunum and absent, as expected, in IL-$10^{-/-}$ mice.

compared to the shorter 45 min previously reported. This discrepancy in findings may be better clarified by our planned studies using anti-IL 10 antibody in wild-type mice.

IL-6 has pro-inflammatory properties and is believed to play a role in systemic inflammation [10]. However, recent data with IL-6-deficient mice also suggest that IL-6 is protective after LPS administration [32]. Similarly, in the cecal ligation and puncture model of peritonitis, the relative serum concentration of IL-6 may be a marker for the onset and the continuation of systemic inflammation [28]. Yet, in the I/R model the absolute quantity of serum IL-6 appears to have no effect on outcome, in the absence or the presence of IL-10. After I/R the early elevation of IL-6 does not correlate with clinical outcome, even when unopposed by IL-10. The usefulness of IL-6 as a marker for the degree of inflammation, MSOF, and death is questionable.

The production of TNF-α is the initial event in the endotoxin-induced cytokine cascade. The administration of TNF-α alone will result in the same subsequent sequential production of cytokines [10]. However, in the intestinal I/R model there is no appreciable production of serum TNF-α when compared to the 500- to 1000-fold higher levels produced by an endotoxic challenge [29]. The lack of significant TNF-α expression in this dynamic model of SIRS may be one explanation for the ineffectiveness of the experimental therapies whose rationale was derived from the static endotoxin model of sepsis [11], raising the question of the importance of TNF-α in systemic inflammation and MSOF. The apparent lack of protection by endogenous IL-10 and the differences in the levels and kinetics of IL-6 and TNF-α production suggest that a possible alternative pathway of cytokine production results from intestinal I/R.

It is likely that the failure of anticytokine therapies is multifactorial. The majority of animal data are based on a single hit model of endotoxemia that is unlike the clinical setting, which most often involves an insidious and progressive insult. The interventions based on the LPS/endotoxemia model have the benefit of administration prior to or early after a known quantified insult, while the majority of clinical scenarios do not afford us the luxury of this knowledge or timing. The clinical effectiveness of these therapies may therefore have been impeded by the late administration of these anticytokine therapies after the initiation of a very complex systemic inflammatory process. Furthermore, these ineffective approaches target only one aspect of a multifaceted cascade of events. The failure of the LPS model to accurately predict the efficiency of potential therapies suggests that this simplified model does not reflect the intricate series of events and organ responses to localized or systemic insults.

This report suggests that damage to the gut is a pathway through which systemic inflammation is initiated and possibly amplified. This is consistent with studies demonstrating that intestinal mucosal protection, via the administration of enteral nutrition, reduces systemic catecolamine production, nosicomial infection, and length of stay in the intensive care unit [9, 33]. At what point protection of gut mucosal integrity is most beneficial demands further investigation, because of the role of intestinal injury as a propagator and initiator of systemic inflammation and MSOF.

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