Interleukin-1β is the primary initiator of pulmonary inflammation following liver injury in mice

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The technique of hepatic cryoablation involves the use of a supercooled probe to produce local destruction of liver tumors through the disruption of cell membranes (7). The use of this method of ablation has been largely abandoned due to a morbidity rate approaching 60%, with the lungs being particularly susceptible to injury (19, 32, 33, 36). In light of the clinical similarities in the lung injury patterns that occur in patients following hepatic cryoablation and prolonged cold ischemia during orthotopic liver transplantation, cryoinjury may represent a form of hepatic I/R injury. As such, hepatic cryoacllation is a simplified and reproducible model for investigating the pathophysiology of liver-mediated pulmonary inflammation, without such confounding factors as portal hypertension (as occurs during portal clamping techniques) or acute rejection (observed following transplantation). Previously, we demonstrated that members of the IL-1 cytokine family may mediate the ensuing lung inflammation following liver injury (17). The current study further explored this concept utilizing both short interfering RNA (siRNA) directed against IL-1β and homozygous IL-1 receptor 1 knockout (IL1R1KO) mice. We report the novel finding that IL-1β is an early initiator of liver injury-induced lung inflammation and that blocking IL-1β signaling using RNA interference can prevent the remote organ inflammatory response.

MATERIALS AND METHODS

In vitro-based siRNA sequence selection. An immortalized murine alveolar macrophage cell line (MH-S; American Type Culture Collection, Bethesda, MD) was grown under standard cell culture conditions using RPMI 1640 media supplemented with heat-inactivated 10% FBS and 0.05 mM 2-mercaptoethanol. MH-S cells constitutively express IL-1β, with greater expression observed following LPS-based stimulation (12, 24). Cells underwent transfection with each IL-1β siRNA or a nonsense siRNA as described below, with nontransfected or 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)-only exposure used as positive controls. Cultures were maintained for 24–72 h, following which the cells were pelleted and the supernatant removed for further analysis. IL-1β was measured in the culture supernatants using ELISA (R&D Systems, Minneapolis, MN). Following the selection of the siRNA sequence exhibiting the greatest degree of IL-1β knockdown, additional testing was performed to select the appropriate siRNA:DOTAP ratio and siRNA working concentration.

siRNA selection and preparation. Three IL-1β-specific siRNA sequences initially were selected from the Dharmaco (Lafayette, CO) siRNA library as potential candidates with high likelihoods of knockdown based on in silico screening techniques (28). The 21-nucleotide
sequences each included 3'-dUdU overhangs and underwent counter ion sodium exchange, dialysis, and endotoxin screening before administration. The mRNA targets were murine IL-1β sequence A (5'-GAA AGA AUG UAU ACC UCG-3'), murine IL-1β sequence B (5'-GCU CCG AGA UGA ACA ACA A-3'), and murine IL-1β sequence C (5'-UGG UUG AUC CCA AGU A A-3'). Transfection was accomplished using a cationic liposomal carrier molecule (DOTAP, Roche Applied Science, Mannheim, Germany) after incubation with 100 nM siRNA at 26°C for 15 min (35, 37). The siRNA:DOTAP mixture then was diluted in either OptiMEM media (for cell culture; Invitrogen, Carlsbad, CA) or phosphate-buffered physiological saline solution (for in vivo administration). Transfection vehicle without siRNA (DOTAP-only) was administered to control for potential toxicity, and nonsense siRNA (siCONTROL RISC-Free, Dharmaco) transfected under identical conditions to the IL-1β-specific siRNA was utilized as a control for non-specificity.

Animal care and surgical techniques. Transgenic 5'-HIV-LTR-Luciferase (HLL) mice were utilized for all siRNA-based experiments. Mice were housed in pathogen-free conditions with a 12-h diurnal light cycle and access to water and food ad libitum. Breeding pairs on a C57Bl/6 background were inbred, with subsequent generations used for this study (ages 8–13 wk). Additionally, IL-1 receptor 1 homozygous knockout (IL1R1KO) mice with C57Bl/6 backgrounds were purchased from Jackson Laboratories (Bar Harbor, ME) and housed under similar conditions. All experiments were approved by the Washington University Animal Studies Committee and conducted in accordance with criteria outlined by the National Institutes of Health in “The Guide for the Care and Use of Laboratory Animals.”

Experimental animals received an intraperitoneal dose of 7.5 nmol siRNA (either IL-1β-specific or nonsense siRNA) with DOTAP transfection agent and phosphate-buffered saline in a total volume of 1.5 ml 24 h before liver injury. The vehicle-only group received an identical volume containing only DOTAP. Sham animals were given 1.5 ml of PBS only. A separate group of animals received the siRNA:DOTAP mixture but did not undergo liver injury; they were anesthetized and then killed at the designated times as controls. All experimental groups contained four to six animals.

Our model of hepatic injury using cryoablation has been well characterized (7, 16, 17). In brief, mice are anesthetized with an intramuscular injection of a ketamine (87 mg/kg body wt) and lidocaine (13 mg/kg) cocktail. Active warming to maintain normothermia is accomplished using a heating pad. Following sterile preparation and using aseptic instruments, a midline laparotomy is performed, the abdomen is irrigated with 2 ml of warmed physiological saline and closed in two layers. Animals are subsequently killed at designated times using an overdose of anesthetic with rapid bilateral pneumothoraces. This hepatic injury reliably induces an acute, progressive, inflammatory lung injury with a mortality rate of ~45% at 24 h (1, 7, 38). Mice with evidence of intra-abdominal injury other than hepatic injury (e.g., hemoperitoneum or inadvertent gastrointestinal cryoinjury) were excluded from analysis. Blood was drawn from the inferior vena cava and centrifuged for plasma extraction. Tissue samples of the nonlabeled hepatic remnant and lungs were snap-frozen in liquid nitrogen and stored at −80°C in DNase/RNase-free containers.

Ex vivo determination of NF-kB activity. The 5'-HIV-LTR is a known NF-kB binding sequence, and its upstream location from the luciferase gene allows direct quantification of NF-kB-DNA interaction in HLL mice using luciferase activity measurements (2). Luciferase activity was determined in organ homogenates with slight modification from previously described techniques (23, 29). Briefly, 30-mg portions of frozen lung or liver tissue were homogenized in reporter lysis buffer 1× (Promega, Madison, WI), subjected to three freeze-thaw cycles, and centrifuged at 10,000 g to extract cytoplasmic protein. Protein extract (50 μl) was combined with 100 μl of reconstituted luciferase assay substrate (Promega), followed by measurement of relative light units (RLUs) using a standard luminometer (Berthold Detection Systems, Oak Ridge, TN). Luciferase activity was normalized to protein concentration, as determined by the Bradford method with spectrophotometric analysis (4).

Determination of pulmonary neutrophilic response. A myeloperoxidase (MPO) assay was used to quantify the degree of acute lung injury at 24 h after hepatic injury. The functional MPO assay is a reliable indicator of the degree of neutrophilic infiltration, particularly in the lungs (18, 30). Frozen pulmonary tissue (100 mg) was homogenized using a tissue rotator in a 1 M potassium phosphate buffer containing 10% hexadecyltrimethylammonium bromide (Sigma, St. Louis, MO), sonically disrupted, and centrifuged at 1,700 g for 30 min. Supernatant (5 μl) was added to a 0.1 M potassium phosphate buffer solution supplemented with 10% bovine serum albumin, 7.5% sodium bicarbonate, 1 M HEPES, and 10× HBSS. A solution of 0.05% H2O2 and α-diaminophenol initiated the functional assay and resulting color shift, and this reaction was terminated by the addition of 1% sodium azide after 15 min. Spectrophotometric shift at 460 nm was determined at 5 and 20 min in duplicate. Results were calculated as the change in optical density per minute per milligram of protein to yield a relative quantification of neutrophil content and subsequently normalized to noninjured control values.

ELISA for cytokines. Commercial ELISA kits for murine IL-1β, IL-6, macrophage inflammatory protein-2 (MIP-2), and keratinocyte-derived chemokine (KC) were used per the manufacturer’s instructions to quantify these proinflammatory cytokines/chemokines in both serum and tissue (R&D Systems). For tissue measurements, total protein was extracted from snap-frozen mouse lung tissue by homogenization in a 150 mM sodium chloride buffer containing HEPES (1 M), EDTA (0.2 M), PMSF in ethanol, and Nonidet P-40. Protein concentrations were measured using the Bradford assay. All samples were measured in duplicate.

Statistical analysis. Comparisons between groups were made using analysis of variance with the Newman-Keuls post-hoc analysis. Data are expressed as means ± SD. A P value less than 0.05 is considered significant.

RESULTS

Effective knockdown of IL-1β in murine alveolar macrophages using RNA interference. Variable IL-1β knockdown in the MH-S cell line was achieved using siRNA relative to vehicle-only control. Varying the concentration of the DOTAP transfection vehicle between 3.7 and 7.4 μL/μg siRNA did not affect the degree of IL-1β knockdown in the transfected MH-S cells in the absence of LPS stimulation (data not shown); subsequently, the lower concentration of DOTAP was used for the remaining in vitro and all in vivo experimentation to diminish possible off-target effects from the vehicle itself. Longer exposures to IL-1β siRNA resulted in greater knockdown as measured by ELISA (Fig. 1). Murine IL-1β siRNA sequence C exhibited the greatest cytokine knockdown, with almost undetectable IL-1β levels at 72 h posttransfection. Dose-response testing using siRNA sequence C following Escherichia coli LPS stimulation demonstrated a direct relationship between siRNA concentration and the degree of IL-1β suppression (at 72 h of continuous exposure: control, 118.1 ± 6.7; 1 nM, 111.8 ± 5.8; 10 nM, 96.8 ± 11.7; 100 nM, 92.2 ± 4.9 pg/ml). On the basis of its superior knockdown of IL-1β, siRNA sequence C was utilized for all subsequent in vivo experiments.
IL-1β siRNA suppresses systemic cytokine response after liver injury. Consistent with the acute increase in IL-1β transcription observed within the liver in prior research, hepatic cryoablation reliably induced a significant increase in serum IL-1β within 4 h of injury (17). The increase in IL-1β was similar in both the HLL and IL1R1KO mice (Fig. 2). Following liver injury, IL-1β levels increased significantly in the sham (462.7 ± 54.2 pg/ml; *P < 0.001), vehicle-only (559.5 ± 102.9 pg/ml; †P < 0.001), and nonsense (NS) siRNA (511.6 ± 109.9 pg/ml; ‡P < 0.001) groups relative to uninjured controls (Fig. 3). Notably, although levels of IL-1β were elevated following hepatic injury in mice pretreated with IL-1β-specific siRNA (202.5 ± 38.9 pg/ml), they did not statistically differ from the uninjured groups. However, IL-1β in the IL-1β-specific siRNA-pretreated group remained significantly lower than observed in both the sham and vehicle-only groups (‡P < 0.05). Not surprisingly, IL-1β also was significantly increased in the IL1R1KO mice after hepatic cryoablation (445.0 ± 22.7 pg/ml; ‡P < 0.005 relative to uninjured IL1R1KO mice).

IL-6 was markedly elevated in the control animals relative to the uninjured groups (sham: 944.2 ± 430.2 pg/ml; vehicle-only: 833.2 ± 488.1 pg/ml; NS siRNA: 1,099 ± 661.4 pg/ml; all *P < 0.05). Conversely, only a minimal elevation in IL-6 was observed in both the mice pretreated with IL-1β siRNA (102.5 ± 28.8 pg/ml, †P < 0.05 vs. uninjured controls) and the IL1R1KO mice (43.5 ± 13.3 pg/ml, ‡P < 0.05). Suppression of IL-1β production reduces hepatic and pulmonary NF-κB activation. Tissue luciferase assays were used to quantify NF-κB activation in both the liver and lungs of the transgenic mice. Baseline hepatic NF-κB activity was low in both uninjured controls (1,540 ± 208 RLU/mg protein) and in mice receiving IL-1β-specific siRNA but no liver injury (2,474 ± 40 RLU/mg protein). Mice pretreated with IL-1β-specific siRNA demonstrated significantly lower levels of NF-κB-dependent luciferase activity (63,754 ± 10.250 RLU/mg protein) compared to the control groups (*P < 0.001 vs. uninjured controls, †P < 0.05 vs. IL-1β siRNA group). NS, nonsense.
protein) within the liver at 4 h post-hepatic injury than did control groups (sham 194,097 ± 43,698; vehicle-only 152,464 ± 19,058; NS siRNA 181,768 ± 41,206 RLU/mg protein; for all, P < 0.05 vs. IL-1β-specific siRNA). Similarly, pulmonary NF-kB activation was significantly diminished in the IL-1β-specific siRNA-pretreated group relative to controls undergoing equivalent liver injuries (Fig. 5). This effect persisted through 24 h; pulmonary NF-kB activity remained statistically unchanged from baseline in mice that received siRNA specific for IL-1β before hepatic surgery.

Reduction in pulmonary NF-kB activation ameliorates the subsequent neutrophil influx. MIP-2 and KC are homologs to human IL-8 and are the primary murine C-X-C chemokines, mediating lung inflammation through neutrophil attraction (22, 26). Concentrations of these chemokines were measured in uninjured control mice and at 4 and 24 h after liver injury. As shown in Fig. 6, KC increased significantly from baseline in all untreated controls at 4 h, and it remained elevated at 24 h postcryoablation (sham 263.6 ± 16.7; vehicle-only 236.2 ± 22.3; NS siRNA 291.7 ± 65.9 pg/mg protein; for all, P < 0.01 vs. baseline and IL1R1KO at 4 h). Conversely, mice treated with IL-1β siRNA exhibited a less pronounced increase in pulmonary KC at 4 h (158 ± 10.5 pg/mg protein, P < 0.01 vs. baseline) and did not differ statistically from the IL1R1KO group (60.9 ± 10.9 pg/mg protein). In addition, at 24 h following liver injury, lung KC levels were not significantly different from the baseline in the mice with impaired IL-1 signaling, whereas KC remained elevated in control groups. Small elevations in MIP-2 levels were found in all groups at 4 and 24 h (data not shown).

MPO is a heme-containing compound found almost exclusively in the azurophilic granules of neutrophils (31). Figure 7 depicts pulmonary MPO levels at 24 h post-liver injury. Control mice undergoing hepatic cryoinjury reliably demonstrated increases in pulmonary MPO content of 30–35% over baseline (P < 0.05). However, pulmonary MPO in mice receiving IL-1β-specific siRNA did not differ significantly from baseline and remained much lower than in other injured animals. Similar to the IL-1β-specific siRNA-pretreated mice, the IL1R1KO mice did not exhibit significant increases in pulmonary MPO at 24 h following hepatic injury (1.00 ± 15.5% at baseline, 1.02 ± 10.6% at 24 h, P = NS).

**DISCUSSION**

Liver-mediated pulmonary inflammation occurs following hepatic cryoablation as well as coincident with other forms of liver injury such as during prolonged vascular inflow occlusion or I/R following liver transplantation (34, 39). These responses are of interest since they are induced under presumably sterile conditions, yet the ensuing systemic response, and in particular the acute lung injury, can resemble the response to bacterial components such as endotoxin. In fact, the potential role of liver-mediated systemic inflammation is just now being elucidated. We report the novel finding that IL-1β not only is a key early initiator of this inflammatory response but that the remote organ effects of hepatic injury can be abolished through the targeted knockdown of this cytokine. Specifically, circulating levels of IL-1β were significantly diminished in mice receiving systemic dosing of IL-1β-specific siRNA, and marked reduc-
tions in NF-κB activation were observed in both the liver and lungs. This inhibition of the proinflammatory state typically observed following hepatic cryoinjury led directly to a reduction in neutrophilic lung inflammation at 24 h. Findings similar to those obtained using siRNA directed against IL-1β were demonstrated in the IL1R1KO mice, which lack an intact IL-1 signaling pathway. Although the knockout mice had increased IL-1β following liver injury, they exhibited a blunted IL-6 response and no increase in pulmonary neutrophil infiltration. Together, these findings suggest that initiation of the proinflammatory response following hepatic cryoinjury is dependent on the activity of IL-1β and the IL-1 receptor pathway.

Moderate to large volume hepatic cryoablation induces a systemic inflammatory state affecting multiple organ systems, but the majority of research has focused on the heterogeneous acute lung injury that is characterized by a patchy, mixed neutrophil and macrophage infiltrate (1, 6, 7, 17). Since similar lung injury does not occur following resection or radiofrequency ablation of an equivalent liver volume, cryo-induced injury may represent a variant of I/R injury initiated by the nonablated liver remnant (7). Prior research has shown that IL-1β reaches pathological levels within hours of hepatic injury and that members of the IL-1 signaling pathway are upregulated within the lung in response to hepatic cryoablation (17). These findings were confirmed in the current study, where IL-1β was detectable in serum in both the HLL and IL1R1KO mice following liver injury. This cytokine acts through two cell membrane surface receptors; IL-1 receptor 1 transduces a signal, whereas IL-1 receptor 2 binds the cytokine but is nonfunctioning, presumably acting as a “decoy” receptor (3, 10, 14). Mice deficient in receptor 1 produce normal levels of IL-1β but lack the subsequent downstream effects (15, 20).

Accordingly, we observed equivalent IL-1β production following liver injury in the IL1R1KO mice as in the untreated controls. In comparison, pretreatment using IL-1β-specific siRNA resulted in a blunted (but non-zero) IL-1β response.

Upon stimulation with IL-1β, intracellular inhibitory-κB (IκB) is phosphorylated and rapidly degraded, leading to translocation of NF-κB to the nucleus and binding to the promoter regions of various proinflammatory genes. Increased hepatic NF-κB activation occurs in a variety of liver injury models, including following cryoablation, where the remnant liver exhibits markedly elevated NF-κB-mediated transcriptional activity (1, 38). In the HLL mice, IL-1β siRNA pretreatment resulted in NF-κB activity within the nonablated liver remnant approximating only 30% of that seen in the untreated control groups. Likewise, in the lungs, much lower NF-κB activity was observed in the siRNA group, with an even more pronounced difference observed at 24 h post-liver injury. It is unknown whether the large decrement of NF-κB activity in the lungs is due solely to the initial blunted hepatic response or whether intraperitoneal siRNA delivery produced systemic (and pulmonary) knockdown of IL-1β. Nonetheless, it is clear that treatment with IL-1β siRNA resulted in less NF-κB activation in both the liver and the lungs following hepatic injury.

IL-6 induction by IL-1β accounts for the hepatic acute phase protein response and further propagates the proinflammatory cytokine cascade (13). We observed marked increases in serum IL-6 following hepatic injury in control animals, whereas both the IL-1β siRNA-treated and the IL1R1KO mice demonstrated statistically insignificant increases in this cytokine, presumably secondary to disruption of the IL-1 signaling pathway. Likewise, although IL-1β is not a direct chemoattractant for neutrophils, it mediates neutrophil recruitment through IL-1-induced synthesis of C-X-C chemokines such as KC and MIP-2 (5, 14, 22). The lack of IL-1 signaling, either through the use of siRNA or receptor knockout, resulted in diminished KC accumulation in the lungs at both 4 and 24 h after liver injury. Interestingly, animals receiving IL-1β siRNA exhibited an intermediate KC response, suggesting that knockdown (but not complete elimination) via RNA interference still permitted some degree of chemokine generation. The reductions in NF-κB activity and KC generation within the lungs of non-control mice resulted in a less vigorous neutrophilic infiltration at 24 h post-liver injury, as evidenced by MPO quantification.

The potential utility of siRNA sequences as therapeutic agents has been previously described in the literature (11, 21, 25). The liver, in particular, seems an ideal target organ for RNA interference, due in part to its reticuloendothelial function and relative ease of transfection. To our knowledge, the current study is the first to demonstrate that RNA interference within the liver can prevent the ensuing pulmonary and systemic inflammatory response. Knockdown of IL-1β before hepatic injury resulted in reduced NF-κB activation within the liver and the lungs, reduced levels of systemic IL-1β and IL-6, diminished pulmonary chemokine generation, and a reduction in neutrophil accumulation in the lungs. Although our model of hepatic cryoablation shares many similarities to graft I/R injury during liver transplantation, it is possible the systemic inflammatory responses are mediated through different pathways. Nonetheless, IL-1β appears to play a central role in liver injury-mediated inflammation, and siRNA represents an attractive therapeutic option for the prevention of this response.

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REFERENCES
LIVER INJURY-INDUCED LUNG INFLAMMATION VIA IL-1β


