Little is known about interactions between endogenous anti-inflammatory paradigms and microvascular thrombosis in lung ischemia/reperfusion (I/R) injury. Interleukin (IL)-10 suppresses macrophage activation and down-regulates proinflammatory cytokine production, but there are no available data to suggest a link between IL-10, thrombosis, and fibrinolysis in the setting of I/R. We hypothesized that hypoxia/ischemia triggers IL-10 production, to dampen proinflammatory cytokine and adhesion receptor cascades and to restore vascular patency by fibrinolytic potentiation. Studies were performed in a mouse lung I/R model. IL-10 mRNA levels in lung were increased 43-fold over base line by 1 h of ischemia/2 h of reperfusion, with a corresponding increase in plasma IL-10. Expression was prominently localized in bronchial epithelial cells and mononuclear phagocytes. To study the link between IL-10 and fibrinolysis in vivo, the induction of plasminogen activator inhibitor-1 (PAI-1) was evaluated. Northern analysis demonstrated exaggerated pulmonary PAI-1 expression in IL-10 (−/−) mice after I/R, with a corresponding increase in plasma PAI-1/tissue-type plasminogen activator activity. In vivo, IL-10 (−/−) mice showed poor postischemic lung function and survival after I/R compared with IL-10 (+/+). mice. Despite a decrease in infiltration of mononuclear phagocytes in I/R lungs of IL-10 (−/−) mice, an increased intravascular pulmonary fibrin deposition was observed by immunohistochemistry and Western blotting, along with increased IL-1 expression. Recombinant IL-10 given to IL-10 (−/−) mice normalized the PAI-1/tissue-type plasminogen activator ratio, reduced pulmonary vascular fibrin deposition, and rescued mice from lung injury. Since recombinant hirudin (direct thrombin inhibitor) also sufficed to rescue IL-10 (−/−) mice, these data suggest a preeminent role for microvascular thrombosis in I/R lung injury. Ischemia-driven IL-10 expression confers postischemic pulmonary protection by augmenting endogenous fibrinolytic mechanisms.
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study as the critical inhibitor of fibrinolysis (17–22). Some studies have suggested a relation between the increased synthesis of PAI-1 and persistence or recurrence of thrombosis (17, 18) even after thrombolytic therapy. We have shown the physiologic relevance of hypoxia-induced modulation of the fibrinolytic response in the pathogenesis of fibrin accumulation in lungs using PAI-1, -tPA-, and uPA-deficient mice (23). Since hypoxia is an important component of the ischemic vascular milieu, these data suggest that I/R injury might involve not only induction of the inflammatory response but also abnormalities in the fibrinolytic system that lead to clot formation.

In most biological systems, when one set of pathways is triggered, countervailing forces are activated to modulate the effects of uncontrolled activation of the primary pathway. The current studies were driven by an endogenous IL-10 plays a pivotal role in regulating the fibrinolytic system in human endotoxemia (31), no data are available supporting an anti-inflammatory role of IL-10 in lung inflammatory injury by suppression of cytokine induction and thrombosis in lung I/R injury. IL-10 is one of the Th2 type cytokines that is believed to exert negative regulatory effects of IL-10 on critically relevant issues including apoptosis and nitric oxide (25–27). With regard to the lung, several studies have shown that IL-10 reduces the intensity of cellular recruitment in pulmonary inflammation and is an inhibitor of the induced release of several proinflammatory cytokines such as TNF-α and macrophage inflammatory proteins 1 and 2, supporting an anti-inflammatory role of IL-10 in the lung (28). Furthermore, some studies have shown that IL-10 has significant protective effects in lung inflammatory injury by suppressing the expression ICAM-1 (29, 30). Although a recent report shows that IL-10 may inhibit coagulation and potentiate the fibrinolytic system in human endotoxemia (31), no data are available with respect to its effects on the coagulant/fibrinolytic mechanism in I/R. Therefore, the current studies were driven by a 2-fold hypothesis: 1) that microvascular thrombosis represents a significant component of lung I/R injury and 2) that endogenous IL-10 plays a pivotal role in regulating the fibrinolytic system in lung I/R injury.

EXPERIMENTAL PROCEDURES

Animals—IL-10-deficient mice (IL-10−/−, C57/6/IL-10tm1cgn, male, 10 weeks old) (32) and their wild-type controls (IL-10+/+, C57/6J, male, 10 weeks old), which were purchased from Jackson Laboratories (Bar Harbor, ME), were used in these experiments according to a protocol approved by the Institutional Animal Care and Use Committee at Columbia University, in accordance with guidelines of the American Association for the Accreditation of Laboratory Animal Care.

Murine Ischemia/Reperfusion Model—Animals were anesthetized intraperitoneally with 0.1 mg/g of muscle weight of ketamine and 0.01 mg/g of muscle weight of xylazine, followed by intraperitoneal continuous infusion of one-third of the initial dose per hour using a syringe pump (model 100 series, KD Scientific Inc.). After ensuring appropriate depth of anesthesia, mice were intubated via tracheostomy and placed on a Harvard ventilator (tidal volume = 0.75 ml, respiratory rate = 120/min) with room air, followed by bilateral thoracotomy. The left hilum was clamped for a period of 1 h, after which the cross-clamp was released. Reperfusion proceeded from 1 to 3 h according to the following groups: untreated lung in sham operation; group I, 1-h ischemia without reperfusion; and R-1, R-2, or R-3 groups, consisting of 1-h ischemia followed by 1-, 2-, and 3-h reperfusion, respectively. After observation, blood samples were obtained for ELISA (IL-10, IL-1α, and sICAM-1) and PAI-1 activity assays, and lung specimens were taken for the measurement of wet/dry ratios or Northern blot analyses.

In a separate series of survival experiments, lung function was ascertained by arterial blood gas analysis (sampled from the left ventricle) in mice that survived for 30 min after right hilar ligation. Immediately after determination of lung function, mice were heparinized, and lung specimens were taken for Western blot or immunohistochemical analysis for fibrin. These experiments were performed as a separate group so that obtaining the left ventricular sample of blood did not impact on mouse survival.

Wet/Dry Ratio—When mice were sacrificed after the survival experiments, the left hilum was ligated, and then the left lung (including residual blood) was taken and weighed as a wet weight. The lung specimen was desiccated at 80 °C for 24 h and weighed again as dry weight. Wet weight was divided by dry weight for the calculation of wet/dry ratio.

ELISA for IL-10, IL-1α, and sICAM-1—I-10−/−mice were divided into untreated, I, R-1, R-2, and R-3 groups. In each group, blood was drawn from the heart, kept at 4 °C overnight, and centrifuged at 13,000 rpm for 20 min to obtain serum, which was then divided into aliquots and frozen at −80 °C until the time of use. The serum IL-10 level was assayed by ELISA kits (R & D Systems), and IL-1α and sICAM-1 levels were assayed by an ELISA kit (Endogen). The lower limits of detection for IL-10, IL-1α, and sICAM-1 assays are 4 pg/ml, 6 pg/ml, and 5 μg/ml, respectively. Values are expressed as the mean ± S.E. of duplicate determinations.

RNA Extraction from Lung Tissues and Northern Blot Analysis—In dedicated experiments, the left lung was rapidly exposed and snap-frozen in liquid nitrogen until the time of mRNA extraction. After tissue homogenization using a Brinkmann Polytron homogenizer, total RNA from the lung tissues was isolated by the Trizol method (Life Technologies, Inc.), and then poly(A) mRNA were purified using Poly(A)Ttract® RNA Isolation Systems (Promega, Madison, WI).

To detect IL-1α, IL-10, PAI-1, and tPA transcripts, equal amounts of poly(A) mRNA (2.5 μg/lane) or total RNA (25 μg/lane) were loaded onto an 0.8% agarose gel containing 2.2× formaldehyde for size fractionation and then transferred overnight to nylon membranes (Duralon-UY™ membranes; Stratagene) with 20× SSC buffer. A murine IL-10 (1.5 kb), American Type Culture Collection, IL-1α (789 bp), PAI-1 (900 bp), the plasmid, containing a PBS vector and a 3014 bp insert, was generously provided by M. Cole, and PAI-800 bp; composed of a 2.5-kb insert from a PKS−/−/plasmid vector (33) CDNs were purified using a Qiagen II gel extraction kit (QIAGEN Inc.). These fragments were used as cDNA probes after 2P-Random primer labeling (Prime-It RmT®; Stratagene) with [α-32P]dCTP. After prehybridization and hybridization using QuikHyb hybridization solution (Strategene) at 68 °C for 1 h, the blots were washed twice for 15 min with 2× SSC, 0.1% SDS at room temperature, followed by one wash for 30 min with 0.1× SSC, 0.1% SDS at 60 °C. Blots were developed with X-Omat AR film exposed with an intensifying light screen at −80 °C for 3 days. Normalized absorption values were obtained by densitometry scanning (Molecular Imager® System; Bio-Rad) of CDNs including β-actin bands.

In Situ Hybridization—In order to make RNA probes for in situ hybridization, the polymerase chain reaction was first performed using poly(A) mRNA from the lung tissues by the method of Rehman et al. (17–22) reverse transcription. Reverse transcription was performed on total RNA with oligo(dT) primers, and amplification was carried out for 35 cycles by polymerase chain reaction with specific primers for IL-10 (CLON-TECH): 5′ primer, 5′-ATGCGAGACTTGAAGGTTACTGCTTT-3′; 3′ primer, 5′-ATTTCGGAGAAGGAGAACTCAAAGGAGTTT-3′. An aliquot of the polymerase chain reaction product mixture was run in a 1% agarose
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gel stained with ethidium bromide. The polymerase chain reaction products (455 bp) were recovered using a Qiagen II gel extraction kit (QIAGEN) and inserted to pGEM-T<sup>®</sup> Easy Vector using the T4 ligation method (Promega). The RNA expression plasmid was linearized with NcoI and Sall enzymes to allow in vitro run-off synthesis of both sense- and antisense RNA probes. Sense and antisense RNA probes were labeled by transcription with a digoxigenin RNA labeling kit (Roche Molecular Biochemicals), and the labeled probes were then purified.

Both untreated lungs and left lungs after 1 h ischemia/2 h reperfusion were snap-frozen embedded in OCT compound (Miles Scientific) in a cryostat in liquid nitrogen. The sections were cut 5 μm thick and placed on glass slides precoated with opaque (VWR Scientific Products). Briefly, slides were prefixed in 4% parafomaldehyde for 20 min and then digested with 14 μg/ml proteinase K in Tris-EDTA (pH 8.0) for 15 min at 37 °C, fixed in 4% parafomaldehyde for 10 min. Sections were acetylated with 0.25% (v/v) acetic anhydride. Sections were then equilibrated for 60 min in hybridization buffer consisting of 4× SSC, 50% formamide, 5% dextran sulfate, 0.1 mg/ml yeast tRNA, and 0.05 mg/ml salmon sperm RNA. Hybridization was carried out overnight at 45 °C with either IL-10 sense or antisense probe (1:25 dilution in prehybridization buffer). Sections were subjected to stringent washes consisting of a single wash with 2× SSC, two 30-min washes with 1× SSC at room temperature. Sections were then washed with 1× SSC, two 30-min washes with 0.25% (v/v) acetic anhydride. Sections were then incubated for 4 h at room temperature with a 1:100 dilution of anti-digoxigenin antibody (Roche Molecular Biochemicals) for 2 h at room temperature. After four washes, color was allowed to develop for 4 h, and development was stopped by dipping the slides briefly in Tris-EDTA buffer (pH 8.0) and then rinsing. Sections were covered with coverslips with water-soluble mounting medium.

**PAI and tPA Activity Assay**—PAI/tPA activity was determined by a functional rate assay described by Ranby et al. (34) and its adaptation to plasma samples, as described by Wiman et al. (35). Blood samples (F, n = 9; IL-10 (+/+), n = 9; IL-10 (−/−), n = 9; and IL-10 (−/−) plus rmIL-10, n = 9) were drawn at the end of survival experiments and acidified by acetate buffer immediately. The samples were centrifuged at 2000 × g for 5 min. Equal volumes of acetate buffer and Tris buffer were added to acidified plasma and incubated at 37 °C for 20 min. The activity was assayed by Spectrolyse<sup>®</sup> PAI/tPA activity assay kits (American Diagnostica). In brief, each sample was added to reaction mixture containing a known quantity of tPA, soluble fibrin (Desatin; American Diagnostica), and a plasmin substrate (Spectrozyme PL; American Diagnostica). Plasmin generated by the reaction of tPA and fibrin cleaves the Spectrozyme substrate to generate a yellow color, which can be measured at an OD of 405 nm. PAI activity is expressed as the amount of PAI that inhibits 1 IU of tPA.

**Western Blotting for Fibrin Accumulation**—Lung tissues were harvested at the time of fibrin extraction. These tissues were 4 mm thick and placed on glass slides precoated with opaque (VWR Scientific Products). Briefly, slides were prefixed in 4% paraformaldehyde for 20 min, washed with 2× SSC, two 30-min washes with 1× SSC at room temperature. More slides were washed with 1× SSC, two 30-min washes with 0.25% (v/v) acetic anhydride. Sections were then equilibrated for 4 h at room temperature with a 1:100 dilution of anti-digoxigenin antibody (Roche Molecular Biochemicals) for 2 h at room temperature. After four washes, color was allowed to develop for 4 h, and development was stopped by dipping the slides briefly in Tris-EDTA buffer (pH 8.0) and then rinsing. Sections were covered with coverslips with water-soluble mounting medium.

**Localization of IL-10 mRNA Expression in I/R Lungs**—To investigate the role of IL-10 in lung I/R injury, we first examined the serum levels of IL-10, IL-1α, and sICAM-1 in both IL-10 (+/+), IL-1α (+/+), and IL-10 (−/−) mice during ischemia and reperfusion. Serum levels of IL-10 in IL-10 (+/+), IL-1α (+/+), and IL-10 (−/−) mice were significantly higher than those in IL-10 (−/−) mice. Administration of rmIL-10 to IL-10 (−/−) mice reduced levels of IL-1α significantly (Fig. 1B). No significant differences in sICAM-1 levels were noted among these four groups (Fig. 1C).

**Time Course of IL-10 and IL-1α mRNA Expression in Mouse Lung I/R**—To investigate the time course of proinflammatory cytokine IL-1α and anti-inflammatory cytokine IL-10 expression in our model, 2.5 μg of poly(A) RNA was derived from the untreated, IL-10 (−/−), and IL-1α (−/−) groups (five lungs were homogenized for each group to isolate 2.5 μg of poly(A) RNA for each group). mRNA was loaded into each lane of an agarose gel, and Northern blotting procedures were performed as described above. IL-1α mRNA expression was up-regulated noticeably as early as 1 h after ischemia and reperfusion. Samples were hybridized using a 5.0 Abacus Concepts). Analysis of variance was used to compare different conditions among the groups of mice. The product limit (Kaplan-Meier) estimate of the cumulative survival was assessed with the log-rank test to evaluate significance differences. Differences were considered significant at the level of p < 0.05.

**RESULTS**

**ELISA for IL-10, IL-1α, and sICAM-1**—To investigate the role of IL-10 in lung I/R injury, we first examined the serum levels of IL-10, IL-1α, and sICAM-1 in both IL-10 (+/+), IL-1α (+/+), and IL-10 (−/−) mice during ischemia and reperfusion. Serum levels of IL-10 in IL-10 (+/+), IL-1α (+/+), and IL-10 (−/−) mice were significantly higher than those in IL-10 (−/−) mice. Administration of rmIL-10 to IL-10 (−/−) mice reduced levels of IL-1α significantly (Fig. 1B). No significant differences in sICAM-1 levels were noted among these four groups (Fig. 1C).

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Fig. 1. Serum levels of IL-10, IL-1α, and sICAM-1 were measured by ELISA. IL-10 in the I/R model showed a time-dependent increase after reperfusion (A). For IL-1α and sICAM-1, the values were compared among untreated, IL-10 (+/+/), (−/−), and (−/+) plus rmIL-10 mice. IL-1α levels in the IL-10 (−/−) group showed elevated values compared with other groups, which were reduced by exogenous rmIL-10 administration (B). There was no significant difference between sICAM-1 levels among these groups (C). Means ± S.E. are shown. *, p < 0.05.

Produced by I/R, in situ hybridization was performed using murine sense and antisense probes. Lung tissue from the R-2 group demonstrated increased IL-10 mRNA levels in bronchial epithelial cells (Fig. 3A) and mononuclear cells (Fig. 3C), but not in the endothelial cells. As negative controls, this staining was not observed in antisense-stained adjacent sections (Fig. 3, B and D) or in untreated lungs (Fig. 3, E–H). Quantitative analysis of these data indicates a 17-fold increase in IL-10 mRNA under ischemic conditions compared with untreated control conditions for epithelium and an 11-fold increase for mononuclear phagocytes.

PAI-1 mRNA Expression in IL-10 (+/+/), (−/−), and (−/+). Plus rmIL-10 Mice Lungs—To investigate the contribution of IL-10 to the fibrinolytic balance in I/R, PAI-1 mRNA expression was studied by Northern blot analysis in IL-10 (+/+/), IL-10 (−/−), and IL-10 (−/+) plus rmIL-10 mice. Blots were performed four separate times using four mice in each group, and normalized absorption values (by densitometry scanning) were analyzed statistically. One-h ischemia/2 h reperfusion up-regulated PAI-1 mRNA levels compared with untreated lungs in IL-10 (+/+/) mouse (2.6-fold increase) (Fig. 4, A and B). PAI-1 expression was significantly up-regulated in IL-10 (−/−) mice (4.7-fold increase); this up-regulation was suppressed by administration of exogenous rmIL-10 (Fig. 4, A and B). In contrast to increased PAI-1 mRNA expression, although tPA mRNA appeared to increase in IL-10 (−/−) mice, this difference did not achieve the level of a significant difference on multiple blots (data not shown). These data suggested that in wild-type mice, IL-10 induction might contribute to up-regulation of fibrinolytic activity in lung I/R injury.

PAI and tPA Activity Assay—Because PAI can circulate in both active and latent forms (40), plasma PAI activity was measured using a microtiter system that monitors PAI-mediated inhibition of plasminogen activator activity (34, 35) as well as tPA activity. IL-10 (−/−) mice showed significantly higher PAI activity compared with that of IL-10 (+/+) mice. PAI activity was significantly reduced by administration of exogenous rmIL-10 (Fig. 5A). Although lack of the IL-10 gene did not appear to alter tPA activity, reconstitution of the IL-10 null mice with rmIL-10 appeared to augment tPA activity (Fig. 5B).

Although tPA mRNA is reportedly unchanged after endothelial exposure to anoxia (41) or hypoxia (42), the PAI/tPA activity ratio appears to be increased (42), which may contribute to the apparent hypofibrinolytic state of endothelial cells exposed to hypoxia in vitro (42). In a whole animal hypoxia model, it appears that tPA mRNA levels are actually reduced in the lungs, which, along with induction of PAI-1 mRNA, may provide a potent stimulus for thrombus accrual (23). In the current lung I/R model, the PAI/tPA ratio was therefore calculated to provide insights into the relative fibrinolytic “balance” in this model. The PAI/tPA ratio in IL-10 (−/−) mice was significantly greater than that observed in IL-10 (+/+) mice, and this ratio was normalized by reconstitution of IL-10 null mice with rmIL-10 (Fig. 5C).

Detection of Fibrin—The data shown so far regarding the role of IL-10 in modulating the fibrinolytic state suggest that in vitro, changes in the fibrinolytic balance in IL-10 (−/−) mice incited by I/R are likely to be of pathologic significance with respect to the accrual fibrin. Immunohistochemical analysis revealed that I/R-driven fibrin accumulation occurred predom-
 redundantly at intravascular sites (Fig. 6A); controls showed a relative absence of fibrin accumulation in untreated lung sections stained with identical procedures or in I/R lung tissue subjected to similar staining procedures in the absence of the primary anti-fibrin antibody (Fig. 6B). To confirm that IL-10 (−/−) mice actually exhibit I/R-induced accumulation of fibrin, fibrin accumulation was quantified using two different methods in tissue from mice heparinized immediately prior to sacrifice to reduce nonspecific/postmortem thrombosis. In the first method, vessels staining for fibrin were counted by an observer blinded to experimental conditions (Fig. 6C). Although I/R increased the number of fibrin-positive vessels significantly, there was an even more marked increase in fibrin-positive vessels in the IL-10 (−/−) mice. Recombinant murine IL-10 reduced the number of fibrin-positive vessels, suggesting a direct role of IL-10 in fibrin accumulation following I/R. In the second method for quantifying fibrin accumulation, immunoblotting for fibrin was performed on lung tissue. IL-10 (1/1) mice showed that the I/R stimulus does indeed cause fibrin accumulation, compared with the absence of detectable fibrin in untreated lung (Fig. 6D). IL-10 (−/−) mice showed a marked increase in fibrin accumulation compared with that seen under identical I/R conditions in IL-10 (+/+) mice. Note that IL-10 (−/−) mice given hirudin (1.0 mg/kg) also had a marked diminution in I/R-induced fibrin accumulation. Provision of exogenous rmIL-10 to reconstitute the IL-10 null mice resulted in marked suppression of fibrin accumulation in lung tissue (Fig. 6C and D). These data demonstrate that endogenous IL-10 plays a pivotal role in potentiating fibrinolysis and reducing fibrin accumulation after I/R injury.

Quantification of Leukocyte Infiltration—In order to deter-
mine whether IL-10 modulates the recruitment of leukocytes (mononuclear phagocytes or polymorphonuclear leukocytes) in the setting of lung I/R injury, specific immunostaining and myeloperoxidase assays were performed. These data show that IL-10 (+/−) mice demonstrated increased recruitment of both leukocyte types following lung I/R injury (Fig. 7, A and B). Mice in which the IL-10 gene was absent exhibited reduced accumulation of both leukocyte types, but particularly of mononuclear phagocytes. Reconstitution of IL-10 null mice with rmIL-10 resulted in an intermediate level of accumulation.

Arterial Blood Gas Analysis—Because these data show pathological accumulation of fibrin in I/R and especially in IL-10 (−/−) mice exposed to I/R, additional experiments were performed to show that the pathological accumulation of fibrin is likely to be pathologically relevant. Arterial blood samples were taken 30 min after 1-h ischemia/reperfusion from IL-10 (+/+), IL-10 (−/−), rmIL-10-reconstituted IL-10 (−/−), and IL-10 (−/−) plus hirudin mice. For these experiments, the contralateral (nonischemic right) lung was excluded from the circulation so that both animal survival and gas exchange were completely dependent upon the function of the postischemic left lung. Arterial oxygenation and PaO2 deteriorated in IL-10 (−/−) mice compared with IL-10 (+/+) mice, while exogenous rmIL-10 significantly ameliorated these hallmarks of lung function. IL-10 (−/−) mice given hirudin also showed significant improvement in PaO2 compared with IL-10 (−/−) mice (Fig. 8 A). PaCO2 tracked the arterial oxygenation data in inverse relationship, as one would expect (Fig. 8 B).

Wet/Dry Ratio—To further assess lung tissue damage after I/R, we measured wet/dry ratio after the completion of the survival experiments. The data showed that IL-10 (−/−) mice contained significantly more water than did IL-10 (+/+) mice, while edema formation was reduced by the administration of rmIL-10 or hirudin (Fig. 8 C).

Survival—Because in vivo, there are many different mechanisms contributing to lung injury and demise of an animal after an ischemic insult, survival experiments were performed to “summate” the multitude of competing forces and to establish the role of endogenous IL-10 and thrombosis in lung I/R injury. Again for these experiments, following ischemia and reperfusion, the contralateral (nonischemic right) lung was excluded from the circulation so that survival depended entirely on the postischemic left lung. IL-10 (+/+) mice subjected to 1 h of ischemia followed by 2 h of reperfusion showed 67% survival.
values expressed are the means among five groups, including untreated lung (weight ratio of the excised lung tissue. Wet/dry ratio was calculated each group).

C. Hirudin mice that survived for 30 min after right hilar ligation (+/−), IL-10 (−/−), IL-10 (−/−) plus rmIL-10, and IL-10 (−/−) plus recombinant hirudin (n = 9 in each group) were used. The product limit (Kaplan-Meier) estimate of the cumulative survival was assessed with the log-rank test to evaluate for significant differences in survival. *, p < 0.05.

During 60 min of observation after ligation of the right hilum. Survival was significantly less in IL-10 (−/−) (11%) mice during the same observation period. Reconstitution of IL-10 null mice with exogenous rmIL-10 improved not only lung function but also the survival (44%) significantly. To demonstrate that thrombus accumulation is a critical mechanism responsible for the poor survival of IL-10 null mice after lung I/R, a direct and specific thrombin inhibitor, recombinant hirudin was administered to IL-10 (−/−) mice prior to ischemia. One mg/kg of recombinant hirudin markedly improved survival of IL-10 (−/−) mice (78%). These data suggest that thrombus accumulation is a significant cause of high mortality in IL-10 (−/−) mice (Fig. 9).

**DISCUSSION**

The major findings in these experiments are as follows: 1) endogenous IL-10 expression increases following lung ischemia and reperfusion in parallel with IL-1α expression; 2) the absence of the IL-10 gene increases PAI-1 expression and results in augmented fibrin accumulation in postischemic lungs; 3) thrombus accumulation is a significant adverse event responsible for poor postischemic lung function and survival; and 4) provision of recombinant IL-10 (or an anti-thrombin agent) can rescue IL-10 null mice from thrombus accumulation and lung failure following ischemia. Since IL-10 null mice exhibited reduced recruitment of mononuclear phagocytes but the highest levels of fibrin, the potentiation of fibrinolysis by IL-10 cannot be explained on the basis of IL-10 suppressing mononuclear phagocyte infiltration into the lungs following I/R. More likely, IL-10 has a direct effect on macrophages to reduce PAI-1 expression, a claim that is indirectly supported by in vitro data (not shown) in which mononuclear phagocytes exhibited IL-10-mediated suppression of hypoxic induction of PAI-1. In vivo, IL-10 not only potentiates fibrinolysis but suppresses the expression of a potent proinflammatory cytokine (IL-1α), whose expression in ischemia also contributes to leukocyte recruitment and tissue damage (43).

Like many other cytokines, IL-10 is produced by many cell types and mediates diverse cellular functions. In addition to T cells, IL-10 is also expressed by stimulated B lymphocytes, monocytes-macrophages (44), keratinocytes (45, 46), mast cells (46), and epithelial cells (47). With regard to the lung, it has been demonstrated that alveolar macrophages can produce significant amounts of IL-10 (48). In our study, in situ hybridization identified mononuclear phagocytes and bronchial epithelial cells as major cellular sources of IL-10 in lung. Bonfield et al. (49) have demonstrated that bronchial epithelial cells from healthy control subjects constitutively produce IL-10, which appears to be down-regulated in cystic fibrosis patients. However, in our acute lung I/R model, Northern blot analysis and serum levels showed that IL-10 was expressed little constitutively but strongly up-regulated in a time-dependent fashion after reperfusion; this may serve as compensatory regulation against the inflammatory response after ischemic injury.

The prevailing belief is that the mechanism by which IL-10 exerts cytoprotective effects against I/R injury is due to 1) the ability of IL-10 to inhibit macrophage function and to inhibit the synthesis of several proinflammatory cytokines (25–28) and 2) its ability to suppress leukocyte-endothelial cell interactions (29, 30). In order to elucidate the role of endogenous IL-10 in lung I/R injury, we used IL-10-deficient mice, which could be reconstituted with exogenous rmIL-10, as a critical way to dissect the mechanism by which IL-10 works in vivo. According to our data, IL-10 (−/−) mice showed the greatest expression of IL-1α, prominent edema formation, the worst postischemic lung function, and the lowest survival compared with IL-10 (+/−) mice. Whereas exogenous rmIL-10 administration reversed these adverse effects (including the high mortality),
sICAM-1 levels were not significantly affected by the IL-10 deficiency (nor was ICAM-1 on Northern blots; data not shown). These data suggest that although endogenous IL-10 suppresses IL-1 expression, its protective role in lung ischemia is not likely to be mediated by inhibiting ICAM-1 expression.

In contrast to our initial expectations that macrophage infiltration might be suppressed by IL-10, our data showed exactly the opposite effect, that the presence of IL-10 was associated with increased accumulation of mononuclear phagocytes. Similar albeit much less pronounced effects were seen with regard to neutrophil infiltration when tissue was analyzed for the relatively neutrophil-specific enzyme myeloperoxidase. Although the I/R procedure caused a dramatic increase in the number of infiltrating MPs, lack of the IL-10 gene was associated with a significant reduction in MP recruitment. “Rescue” of the IL-10 null mice with IL-10 caused a significant increase in MP recruitment following I/R, albeit absolute levels were highest in mice capable of expressing the IL-10 gene. Although IL-10 generally has anti-inflammatory properties, there is support in the literature for an effect of IL-10 to increase levels of monocyte chemoattractant protein-1 under certain conditions (dependent upon cell type and activation state) (50).

Recent evidence is emerging that, in concert with the shift toward a procoagulant phenotype, endothelial cells exhibit a diminished fibrinolytic response under conditions of oxygen deprivation, especially when followed by reoxygenation and attendant production of reactive oxygen intermediates (41, 51). We have shown the physiologic relevance of hypoxia-induced modulation of the fibrinolytic response in the pathogenesis of fibrin accumulation using PAI-1-, tPA-, and uPA-deficient mice (52, 53) and microvascular thrombosis. However, our reflow generally consists of multiple effector mechanisms such going tissue damage and edema formation. Postischemic no-reflow generally consists of multiple effector mechanisms such as neutrophil infiltration following I/R, albeit absolute levels were highest in mice capable of expressing the IL-10 gene. Although IL-10 generally has anti-inflammatory properties, there is support in the literature for an effect of IL-10 to increase levels of monocyte chemoattractant protein-1 under certain conditions (dependent upon cell type and activation state) (50).

IL-10 Potentiates Endogenous Thrombolysis in Ischemic Lungs

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