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Neutrophil-Derived IL-6 Limits Alveolar Barrier Disruption in Experimental Ventilator-Induced Lung Injury

Paul J. Wolters,* Charlie Wray,† Rachel E. Sutherland,* Sophia S. Kim,* Jon Koff,* Ying Mao,† and James A. Frank2*†‡

IL-6 is a biological marker of ventilator-associated lung injury that may contribute to alveolar barrier dysfunction in acute respiratory distress syndrome (ARDS), 3 is a common cause of respiratory failure characterized by alveolar barrier dysfunction (1). Although treatment options for patients with acute lung injury are limited, one intervention associated with decreased mortality in patients requiring mechanical ventilation is tidal volume reduction (2). The mechanisms for the beneficial effects of lower tidal volume breathing in this patient population are incompletely understood, but previous clinical trials have identified associations between plasma IL-6 levels and ventilator-associated lung injury (3).

Several clinical studies have reported an association between plasma IL-6 levels and adverse outcomes, including mortality and organ failure, in patients with acute lung injury (2–4). In the ARDS clinical trials network study of lower tidal volume compared with higher tidal volume ventilation in patients with acute lung injury, plasma IL-6 levels positively correlated with mortality (2). Lower tidal volume ventilation was associated with a decrease in IL-6 consistent with a causal role for IL-6 in ventilator-associated lung injury (3). In contrast, another clinical study of trauma patients with acute lung injury found that higher plasma IL-6 levels were associated with lower mortality (5). It remains uncertain whether IL-6 levels are merely a marker of lung injury or whether IL-6 contributes to the pathogenesis of ventilator-associated lung injury.

Experimental studies suggest a complex role for IL-6 in lung injury. In some experimental models, IL-6 prevents lung injury. For example, in an aerosolized endotoxin model of lung injury, IL-6 decreased levels of TNF-α, MIP-2, and airspace neutrophils (6). Similarly, IL-6-blocking Abs increased lung protein permeability in a model of IgG-mediated lung injury (7). In another study, transgenic mice overexpressing IL-6 in the lung were protected from hyperoxia-mediated lung injury (8). However, the effect of IL-6 on alveolar barrier function may be dependent on the mechanism of lung injury. For example, in a mouse model of lipoteichoic acid-induced lung injury, IL-6 attenuated lung inflammation, but in the same study IL-6 null mice were protected from peptidoglycan-mediated lung injury (9). Other experimental studies support a deleterious role for IL-6 in acute lung injury. In a mouse sepsis model, IL-6 signaling was associated with increased mortality and increased lung complement 5a receptor expression (10), an effect expected to increase lung neutrophil recruitment. A possible explanation for these conflicting results is that the cellular source of IL-6 may influence measures of injury. For example, IL-6 knockout (IL6KO) mice are highly susceptible to Listeria monocytogenes infection and infusion of rIL-6 reverses this susceptibility (11, 12). Because neutrophil-depleted wild-type (WT) and IL6KO mice have similar susceptibility to L. monocytogenes, IL-6 signaling in neutrophils may be critical to infection prevention (11).
This study was undertaken to test the hypothesis that IL-6 is a mediator of alveolar barrier dysfunction in a mouse model of ventilator-induced lung injury (VILI). When initial studies revealed that mice treated with IL-6-blocking Ab were more susceptible to VILI, we investigated the effect of IL-6 on lung neutrophil recruitment and tested the hypothesis that neutrophil-derived IL-6 limits alveolar barrier dysfunction in this neutrophil-dependent model of lung injury using WT and IL6KO chimera mice.

Materials and Methods
Lung injury model and study groups
These protocols were approved by the University of California, San Francisco and San Francisco Veterans Affairs Medical Center Institutional Animal Care and Use committee and are consistent with National Institutes of Health guidelines on the use of research animals. C57BL/6 mice (20 – 25 g) were anesthetized with ketamine/xylazine followed induction anesthesia with 4% isoflurane. Lung injury was induced using a previously described protocol (13). Briefly, the trachea was cannulated and mechanical ventilation was initiated with a peak airway pressure of 20 cm of H2O and an end expiratory pressure of 0 cm of H2O. Ventilation was continued for 3 h. Anesthesia was maintained with ketamine/xylazine, muscle relaxation was maintained with pancuronium, and mice received 0.25 ml of saline i.p. every hour. For these studies, mice were given IL-6-blocking Ab (2.5 mg/kg, i.v.) or a control Ab of the same isotype immediately before mechanical ventilation, or recombinant mouse IL-6 (rmIL-6; 4 μg/kg, i.v.) 2 h and immediately before mechanical ventilation. Additional groups of ventilated and spontaneously breathing control WT mice and IL-6-deficient mice (IL6KO) were also compared.

Alveolar barrier protein flux
Alveolar endothelial and epithelial permeability to protein were measured as the flux of radiolabeled albumin from the circulation to the interstitium and airspaces as previously described (13, 14). Briefly, mice were given 0.5 μCi of 125I-labeled albumin i.p. 2 h before the ventilation protocol. At the end of the experiments, a plasma sample was collected and the ratio of radioactivity in the blood-free lung to the plasma normalized to plasma volume is reported as extravascular plasma equivalents (EVP%).

Plasma IL-6 levels, cell counts, myeloperoxidase (MPO) assay, and histology
IL-6 levels were measured using a species-specific ELISA (Pierce Biotechnology) in plasma collected at the conclusion of select experiments. At the conclusion of select experiments, bronchoalveolar lavage (BAL) was done using 0.5 ml of PBS and recovered fluid used to determine differential cell counts. In separate studies, whole lung MPO activity was measured as the flux of radiolabeled albumin from the circulation to the interstitium and airspaces as previously described (13, 14). Briefly, mice were given 0.5 μCi of 125I-labeled albumin i.p. 2 h before the ventilation protocol. When initial studies revealed that mice treated with IL-6-blocking Ab were more susceptible to VILI, we investigated the effect of IL-6 on lung neutrophil recruitment and tested the hypothesis that neutrophil-derived IL-6 limits alveolar barrier dysfunction in this neutrophil-dependent model of lung injury using WT and IL6KO chimera mice.

Neutrophil adhesion and migration assays
Bone marrow-derived neutrophils from WT or IL6KO mice were isolated by Percoll gradient centrifugation (17) and suspended at 3.5 × 10⁶ cells/ml in RPMI 1640 and plated on low-adhesion plastic dishes as previously described (18). After 2 h at 37°C and 5% CO₂, plates were washed with PBS and the average number of cells in five ×40 fields per well was determined. Some cells were incubated for 30 min with rmIL-6 (10 ng/ml) before plating.

The movement of neutrophils through cultured human pulmonary microvascular endothelial cells (ScienCell) was measured as previously described, with a slight modification (19). Briefly, 10⁶ neutrophils isolated from bone marrow as above were labeled with calcine-AM (Invitrogen) and plated in the apical chamber of Transwells with confluent monolayers of human pulmonary microvascular endothelial cells in serum-free conditions. Endothelial cells were plated at 10⁵ cells per well and grown to confluence in complete growth medium for 3 days before migration studies. FMLP (10⁻⁵ M) (Sigma-Aldrich) was added to the basolateral compartment and the number of neutrophils in the basolateral compartment was determined as 480 nm fluorescence compared with a standard curve 1 h later. Data are expressed as an index of the ratio to unstimulated control wells. In some wells, rmIL6 (10 ng/ml) was added to both the apical and basolateral chambers just before the migration experiment. In separate wells, polymorphonuclear leukocytes of each genotype were incubated with rmIL6 (10 ng/ml) for 35 min and then washed twice in PBS-calcium and magnesium free before the migration experiments.

Effect of IL-6 on epithelial albumin flux and transepithelial electrical resistance
The effect of IL-6 on albumin permeability (flux) in primary rat alveolar type II cells was measured following 3 h of IL-6 exposure (1–100 ng/ml). Primary rat cells were isolated as previously reported and grown to confluence in Transwells. Following exposure to IL-6 in serum-containing medium, transepithelial electrical resistance was measured with a volthometer and the flux of FITC-labeled albumin was determined by adding 100 μg/ml albumin to the basolateral compartment of the Transwell and measuring fluorescence in the apical chamber 1 h later.

Statistics
Data are reported as mean and SD or SE where indicated. Differences between groups were compared using ANOVA and post hoc the Student-Newman-Keuls test for multiple comparisons. Within-group differences were compared using paired t tests. Comparisons of nonparametric data were made using the Mann-Whitney U test. Values of p < 0.05 were considered significant.

Results
Effects of blocking IL-6 and IL-6 pretreatment on alveolar barrier disruption in VILI
To study the role of IL-6 in VILI, we measured alveolar barrier protein flux in C57BL/6 (WT) mice treated with an IL-6-blocking Ab. We found that mice treated with an IL-6-blocking Ab had higher alveolar barrier protein flux (Fig. 1). Pretreatment of mice with rmIL-6 decreased the severity of alveolar barrier disruption (Fig. 1). These findings suggest IL-6 limits alveolar barrier disruption in VILI.

Effects of genetic deletion of IL-6 on alveolar barrier disruption in VILI
To confirm a role for IL-6 in VILI, we next compared alveolar barrier protein flux in WT and IL6-deficient (IL6KO) mice. Baseline albumin flux without mechanical ventilation was higher in WT mice than in IL6KO mice (3.8 ± 0.9 vs 2.8 ± 1.2% (EVP%)), p < 0.05, n = 9–11/group). Surprisingly, lung albumin flux was similarly increased in IL6KO and WT mice subjected to the VILI model (Fig. 2). Pretreatment with rmIL-6 decreased alveolar barrier albumin flux to a similar level in both WT and IL6KO mice. Histology showed similar changes in cellularity and edema in WT and IL6KO mice, but decreased cellularity and edema following rmIL-6 pretreatment (Fig. 3). Lung wet/dry weights were lower in

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mice treated with rmIL6 compared with IL6-deficient mice ($p < 0.05$; Fig. 2B). Lung compliance decreased more in IL-6-deficient mice compared with both WT mice and rmIL6-treated mice, which showed a significantly smaller decrease in compliance compared with the other two groups (compliance decreased 4.4 (1.4) μl/cm H$_2$O in WT mice, 6.3 (2.5) μl/cm H$_2$O in IL6KO mice, and 2.8 (1.6) μl/cm H$_2$O in rmIL6-treated mice ($p < 0.05$ for WT and rmIL6 compared with IL6KO).

Airspace neutrophil counts and lung MPO activity in WT and IL6KO mice

BAL fluid neutrophil counts were lower in IL6KO mice compared with WT mice; however, lung MPO activity was similar in both groups (Fig. 4). These data suggest that although the total number of neutrophils in the airspace and lung parenchyma was similar in the two groups, more neutrophils remained sequestered or adherent in the IL6KO mice. IL-6 pretreatment decreased BAL cell counts and MPO activity. IL6AB resulted in increased lung neutrophil accumulation, but similar to IL6KO mice, IL6AB decreased BAL neutrophil counts. These findings are consistent with IL-6 acting to limit neutrophil adhesion or promote neutrophil migration.

Alveolar barrier protein flux in WT/IL6KO chimera mice in VILI

To further explore whether the cellular source of IL-6 influences the effect on alveolar barrier disruption in VILI, WT mice were irradiated and reconstituted with bone marrow from IL6KO mice (WT/KO) or bone marrow from WT mice (WT/WT). WT/KO mice showed significantly greater alveolar barrier protein leak than WT/WT mice, which were comparable to nonirradiated WT mice,
KO/WT, and KO/KO. (Fig. 5 B). Baseline EVP% in WT/WT mice was 3.8 ± 0.8 and 3.0 ± 0.6 in WT/KO mice (p < 0.05, n = 6 in each group). Therefore, the absence of IL-6 exclusively in hematopoietic cells resulted in increased barrier leak.

Reconstitution of WT/KO chimera mice with WT neutrophils
Infusion of 10^6 bone marrow-derived neutrophils into WT/WT mice (WT/WT/WT) had no effect on alveolar barrier albumin flux. However, the infusion of WT neutrophils to WT/KO mice (WT/KO/WT) significantly decreased alveolar barrier albumin flux to a level comparable to that of WT/WT mice (Fig. 5 B). Infusion of WT neutrophils into nonirradiated WT mice did not affect alveolar barrier permeability (data not shown). Therefore, infusion of neutrophils expressing IL-6 is sufficient to restore the WT phenotype in WT/KO chimeric mice.

Reconstitution of WT/WT and WT/KO chimeras
Total lung neutrophil accumulation as measured by lung MPO activity was significantly higher in WT/KO than in WT/WT mice (Fig. 6). Similar to IL6KO mice, WT/KO mice had fewer airspace neutrophils than WT/WT mice. This finding supports the hypothesis that hematopoietic cell IL-6 limits neutrophil adhesion or promotes migration in the lung. Airspace neutrophil counts in KO/KO KO/WT, and KO/KO. (Fig. 5A). Baseline EVP% in WT/WT mice was 3.8 ± 0.8 and 3.0 ± 0.6 in WT/KO mice (p > 0.05, n = 6 in each group). Therefore, the absence of IL-6 exclusively in hematopoietic cells resulted in increased barrier leak.

Effect of neutrophil depletion on lung injury in WT and WT/KO chimera
Anti Gr-1 Ab decreased peripheral neutrophils >95%. WT mice given Gr-1 Ab showed decreased lung albumin flux with VILI compared with isotype control Ab-treated mice (n = 4 in each group; Fig. 5C). WT/KO mice given the Gr-1 Ab showed a proportional decrease in alveolar barrier albumin flux compared with control Ab-treated chimeras (n = 3 in each group; Fig. 5C). These data show that neutrophils are the major mediators of injury in this VILI model.

Airspace cell counts and lung MPO activity in WT/WT and WT/KO chimeras
Total lung neutrophil accumulation as measured by lung MPO activity was significantly higher in WT/KO than in WT/WT mice (Fig. 6). Similar to IL6KO mice, WT/KO mice had fewer airspace neutrophils than WT/WT mice. This finding supports the hypothesis that hematopoietic cell IL-6 limits neutrophil adhesion or promotes migration in the lung. Airspace neutrophil counts in KO/KO KO/WT, and KO/KO. (Fig. 5A). Baseline EVP% in WT/WT mice was 3.8 ± 0.8 and 3.0 ± 0.6 in WT/KO mice (p > 0.05, n = 6 in each group). Therefore, the absence of IL-6 exclusively in hematopoietic cells resulted in increased barrier leak.

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mice were comparable to those of IL6KO mice (2.6 ± 0.8 vs 2.4 ± 0.5, n = 5 in each group). Airspace neutrophil counts in KO/WT mice were comparable to those of WT mice (4.8 ± 2.2 vs 5.3 ± 1.3, p < 0.05, n = 5 in each group). In nonventilated WT mice reconstituted with GFP-positive bone marrow, 85 ± 5% of BAL cells were GFP positive, indicating that airspace macrophages were not entirely replaced with the protocol used in this study.

Neutrophil adhesion and migration

To determine whether there are differences in neutrophil adhesion between WT and IL6KO neutrophils, adhesion to plastic was compared in a widely used assay (18). IL6KO neutrophils were more adherent to plastic than WT neutrophils (Fig. 7A). Coincubation with rmIL-6 decreased adhesion of neutrophils isolated from IL6KO mice. Incubation of WT neutrophils with rmIL-6 had no significant effect on adhesion to plastic in this assay. In a migration assay, fewer IL6KO than WT neutrophils moved through the lung microvascular endothelial cell monolayer in response to FMLP (Fig. 7B) and incubation of neutrophils with rmIL-6 restored IL6KO neutrophil migration to a level comparable to that of WT neutrophils. The addition of rmIL6 to the apical and basolateral chambers just before the migration experiment resulted in decreased neutrophil migration in both WT and KO neutrophils.

Effect of IL-6 on epithelial permeability

The effect of IL-6 on albumin permeability (flux) in primary rat alveolar type II cells was measured following 3 h of IL-6 exposure. IL-6 had no significant effect on alveolar epithelial albumin flux. Albumin flux normalized to control (no IL-6) was 1.0 ± 0.1, 1.2 ± 0.25, and 1.4 ± 0.1 for a dose range of 1, 10, and 100 ng/ml, respectively (mean ± SEM, p > 0.05, n = three replicates of three wells each). Similarly, IL-6 had no effect on transepithelial electrical resistance in primary rat alveolar type II epithelial cells over 3 h. Transepithelial electrical resistance was 1434 ± 309 Ohm cm² in control cells and 1363 ± 245 Ohm cm² in IL-6-treated cells (p > 0.05). These data indicate that IL-6 does not directly influence permeability of the epithelial cell barrier.

FIGURE 6. Lung neutrophil accumulation in chimera mice. Whole lung MPO activity, a measure of neutrophil sequestration in the lung, was significantly higher in WT/KO mice than in WT/WT mice (++; p < 0.05, n = 4 –5 in each group). BAL fluid neutrophil counts following VILI were significantly lower in WT/KO mice than in WT/WT mice (++; **, p < 0.05, n = 6 –7 in each group). Accordingly, the ratio of total lung neutrophils to airspace lavage neutrophils was higher in WT/KO mice.

FIGURE 7. Adhesion and transendothelial migration in IL6KO bone marrow-derived neutrophils in vitro. A, Neutrophils from IL6KO mice were significantly more adherent to low-adhesion plastic than WT neutrophils (*, p < 0.05 compared with all other groups; nine replicates of eight wells each per condition. Data are mean ± SEM of the mean number of cells per 40 field). Note that incubation with rmIL-6 before plating resulted in a significant decrease in adhesion in IL6KO neutrophils, but not WT neutrophils. B, FMLP increased neutrophil migration through primary human pulmonary microvascular endothelial cells (+, p < 0.05 compared with baseline). IL-6 did not affect neutrophil migration in the absence of FMLP. However, fewer IL6KO neutrophils migrated through human primary pulmonary microvascular endothelial cell monolayers in response to FMLP stimulation (+, p < 0.05 compared with FMLP-stimulated WT neutrophils; three to six replicates of three wells each). Incubation of IL6KO neutrophils with IL6 before migration (10 ng/ml) increased the number of migrating neutrophils to a level comparable to that of WT neutrophils (far right). The addition of IL-6 to endothelial cells just before and during neutrophil migration decreased migration in response to FMLP in neutrophils of both genotypes (+, p < 0.05 compared with baseline and #, p < 0.05 compared with FMLP only with IL6KO neutrophils).

IL-6 plasma levels

Circulating IL-6 levels were not different in WT, WT/WT, WT/KO, or rmIL-6-pretreated IL6KO mice (Table I). IL-6 levels were
lower in KO/WT mice compared with WT/WT and WT/KO mice ($p < 0.05$). WT/WT/WT mice and WT/KO/WT mice had similar IL-6 plasma levels (Table I). IL-6 levels were undetectable in IL-6 KO mice (data not shown). Baseline IL-6 levels (mean 95% confidence interval) in unventilated WT mice (156 (112) pg/ml) were not significantly different compared with unventilated WT/WT mice or WT/KO mice (17 (34) and 194 (173) pg/ml, respectively, $n = 3–6$ in each group).

**Discussion**

The primary objective of this study was to determine the role of IL-6 in the pathogenesis of VILI with a focus on alveolar barrier dysfunction, a hallmark of ARDS and an important determinant of outcome in ARDS patients (1, 20). Our initial hypothesis was that IL-6 blockade would prevent VILI. Contrary to this hypothesis, we found that mice treated with IL-6-blocking Ab were more susceptible to alveolar barrier disruption during high tidal volume ventilation and that pretreatment with IL-6 reduced lung protein permeability. These data support a protective role for IL-6 in VILI.

To confirm a role for IL-6 in VILI, we subjected WT and IL6KO mice to the VILI model and found that lung protein permeability was similar. To explore the mechanisms for this unexpected finding, we examined lung MPO activity as a measure of lung neutrophil accumulation and that pretreatment with IL-6 reduced lung protein permeability. These data support a protective role for IL-6 in VILI.

We then examined lung and airspace neutrophil accumulation in the chimeric mice. Similar to IL-6-blocking Ab-treated mice, WT/KO mice also had greater lung neutrophil accumulation compared with WT/WT mice. However, as was observed in IL6KO mice, WT/KO mice had fewer airspace neutrophils than WT/WT mice, suggesting increased neutrophil sequestration, impaired adhesion, or decreased migration in the WT/KO mice. Therefore, an infusion of WT neutrophils was sufficient to restore the WT phenotype to WT/KO mice, implicating neutrophils as the source of IL-6 important to limiting alveolar barrier disruption in VILI.

To determine whether there were differences in WT and IL6KO neutrophil adhesion, we compared neutrophil adhesion in an in vitro assay. Consistent with the in vivo studies, IL6KO neutrophils were more adherent than WT neutrophils and adding IL-6 decreased adhesion in IL6KO neutrophils. In addition, we assessed the effect of IL-6 expression on neutrophil migration through endothelial layers in response to FMLP. IL-6KO neutrophils showed a small but significant decrease in migration across endothelial cells compared with WT cells and the addition of IL-6 to neutrophils before the migration assay increased the number of migrating IL6KO neutrophils to a level comparable to WT cells. Coincubation of endothelial cells and neutrophils with rmIL6 prevented migration in neutrophils of both genotypes. These observations suggest that neutrophil IL-6 can regulate neutrophil adherence and migration through the endothelium. However, the effect of neutrophil IL-6 on migration in this assay was small and of uncertain biological importance. The net effect of IL-6 from all sources on neutrophil migration in vivo may not be modeled by this assay.
neutrophils in the airspaces was not addressed in this study. However, the absence of neutrophil IL-6 potentially results in prolonged neutrophil-endothelial contact and greater lung injury. This possibility is consistent with a recent study reporting that prolonged neutrophil-endothelial contact promotes lung injury in neutrophil elastase-deficient mice following mechanical ventilation (21). Further supporting an effect of IL-6 on neutrophils in this model is the finding that IL-6 alone did not affect alveolar epithelial albumin flux of transepithelial electrical resistance in vitro, suggesting that the effect of IL-6 on alveolar barrier protein permeability is not primarily due to a direct effect on epithelial cells.

Although these data indicate that IL-6 from hematopoietic cells limits lung neutrophil accumulation, they are also consistent with the possibility that IL-6 from nonhematopoietic cell sources plays a detrimental role in VILI. Specifically, lung neutrophil accumulation was lower in IL6KO mice compared with WT/KO mice. IL-6-blocking Ab as used in this study resulted in conditions more comparable to WT/KO mice than IL6KO mice. A potential explanation for these findings is that IL-6 may up-regulate proteins important to endothelial cell activation and neutrophil-endothelial cell adhesion in this model and that IL-6 from nonhematopoietic cells is sufficient for this effect (22). It is noteworthy that the specific proteins involved in transmigration of neutrophils across the alveolar barrier are somewhat specific to the mode of lung injury (23) and neutrophil transmigration was not specifically examined in vivo in this study.

Differences among the groups in alveolar barrier disruption were not explained by differences in circulating IL-6 levels as plasma IL-6 levels were not different in WT, WT/WT, WT/KO, or mIl6-treated mice. IL-6 levels were lower in KO/WT mice compared with these other groups, but their lung albumin flux was similar to that of WT/WT mice. IL-6 was not measured in the plasma of KO/KO chimeras after ventilation, but because these are essentially identical to IL-6 KO mice, the levels are almost certainly undetectable. Airspace IL-6 levels were not measured in this study; therefore, it remains unresolved whether there were differences in airspace IL-6 levels among the groups. These data suggest that plasma IL-6 levels may be a poor measure of the IL-6 that plays a critical role in VILI. In addition, they suggest that the majority of circulating IL-6 during VILI is released from nonhematopoietic cells and not the hematopoietic cell source (likely neutrophils) that is responsible for the differences in alveolar barrier permeability measured in this study. Therefore, locally released IL-6 may modulate barrier properties or neutrophil-mediated injury in VILI and it is possible that neutrophil-derived IL-6 signals a down-regulation of alveolar barrier disruption in a spatially restricted fashion.

Clinical data have suggested a role for IL-6 in the pathogenesis of ventilator-associated lung injury (2–4), but a causal role for IL-6 in alveolar barrier disruption has not been reported. Data from the present study contradict the hypothesis that IL-6 mediates alveolar barrier disruption and support a protective role for hematopoietic cell-derived IL-6, which acts to decrease neutrophil adhesion, sequestration, and possibly migration in this lung injury model. These data raise the possibility that modulation of IL-6 production in a cell-specific fashion may be a therapeutic target for patients at risk for VILI.

Disclosures
The authors have no financial conflict of interest.

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