Generation and functional significance of CXC chemokines for neutrophil-induced liver injury during endotoxemia

Robert B. Dorman,1 Jaspreet S. Gujral,1,2 Mary Lynn Bajt,1,2 Anwar Farhood,3 and Hartmut Jaeschke1,2

1Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, Little Rock, Arkansas; 2Liver Research Institute, University of Arizona, Tucson, Arizona; and 3Department of Pathology, University of Texas Health Science Center, Houston, Texas

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Neutrophils can accumulate in portal venules, sinusoids, and adherent cells of the liver, extravasation into the parenchyma, and the adherence-dependent cytotoxicity against hepatocytes (31). Neutrophils can accumulate in portal venules, sinusoids, and post-sinusoidal venules (4, 12, 14, 18, 34, 53, 54, 56) and extravasate from all these locations (15, 18, 34, 54). However, extravasation from sinusoids is most critical for liver injury (6, 18). Blocking strategies directed against β2-integrins (15, 26, 29) and intercellular adhesion molecule-1 (11, 18), which eliminated neutrophil extravasation, proved to be highly effective in preventing neutrophil cytotoxicity in the liver. However, the chemotactic signal responsible for neutrophil extravasation has not been conclusively identified in any model.

Chemokines are a class of chemotactic agents with a relatively high specificity for individual leukocytes (40). α-Chemokines are characterized by a structural feature where the first two cysteine residues are separated by one amino acid (CXC). The members of a subgroup of this family, which contains the amino acid sequence glutamic acid-leucine-arginine (ELR), are chemotactants for neutrophils (40). In the mouse, the most prominent ELR+ CXC chemokines expressed in the liver are the chemokine KC and macrophage inflammatory protein-2 (MIP-2) (47), which bind to the CXC receptor 2 (CXCR2) (3, 36). Although CXC chemokines are less potent activators of neutrophils than are cytokines and complement factors (1), excessive CXC chemokine formation in parenchymal cells can recruit neutrophils into the liver vasculature (13, 41, 50, 58), induce transmigration, and cause injury (41). The most convincing evidence for the importance of CXC chemokines under pathophysiological conditions was provided in hepatic ischemia-reperfusion injury (23, 45). Neutralizing CXC chemokines attenuated hepatic neutrophil recruitment and the neutrophil-mediated injury phase in rat and mouse models of warm ischemia-reperfusion (7, 21, 33, 38). On the other hand, in a model of α-naphthylisothiocyanate (ANIT)-induced liver injury, which involves injury by neutrophils (8), neutralizing CXC chemokines had no effect on neutrophil recruitment or injury (57). Thus the importance of CXC chemokines as a neutrophil chemotactant and the exact mechanism may vary between different pathophysiological situations. We previously demonstrated that CXC chemokines are unlikely to be involved in neutrophil activation and recruitment into the hepatic vasculature during endotoxemia (1). However, we showed that parenchymal apoptosis is a critical event triggering neutrophil extravasation (30, 35). Because hepatocytes undergoing apoptosis can generate CXC chemokines (13), we tested the hypothesis that CXC chemokines formed by healthy or apoptotic hepatocytes might represent the signal for neutrophil extravasation and attack on hepatocytes in the galactosamine/endotoxin (Gal/ET) model of liver injury.

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MATERIALS AND METHODS

Animals. Male C3HeB/FeJ and C3H/HeJ mice (20–25 g body wt) or wild-type (BALB/c) and CXCRR2−/− mice (BALB/c background) were obtained from Jackson Laboratories (Bar Harbor, ME). The animals had free access to food (certified rodent diet 5002C, PMI Feeds, Richmond, IN) and water. The experimental protocols were approved by the University of Arkansas and the University of Arizona. Animals were treated with Salmonella abortus equi endotoxin (ET, 100 μg/kg), galactosamine (Gal, 700 mg/kg), or Gal + ET (Gal/ET; Sigma, St. Louis, MO). Some of the animals were treated intravenously with anti-KC (5 mg/kg) and anti-MIP-2 (5 mg/kg) antibodies (R & D Systems, Minneapolis, MN) or nonimmune murine IgG (10 mg/kg; PharMingen, San Diego, CA) as described elsewhere (13). Other animals were treated intravenously with murine recombinant TNF-α, IL-1α, or IL-1β (2 μg/kg each; all from Endogen, Woburn, MA). Groups of animals were killed 1 or 3 h after injection of an inflammatory mediator or 0.5–7 h after Gal/ET or ET. Blood was collected from the vena cava into a heparinized syringe for analysis of alanine transaminase (ALT) activities and chemokine levels. Sections of the liver were fixed in phosphate-buffered formalin for histological analysis or frozen in liquid nitrogen.

Methods. To assess liver cell injury, ALT activities were measured in plasma by means of a kinetic test kit (catalog no. 68-B, Biotron Diagnostics, Herent, CA). Plasma levels of MIP-2 and KC were measured with the respective ELISA kits (R & D Systems) according to the manufacturer’s instructions. Caspase-3 activities were determined as described in detail elsewhere (30) with some modifications (16, 17). Briefly, liver samples were homogenized in 25 mM HEPES buffer (pH 7.5) containing 5 mM EDTA, 2 mM DTT, and 0.1% CHAPS. The homogenized samples were centrifuged at 14,000 g, and the diluted supernatant was assayed for the Z-Val-Ala-Asp-fluoro-methylketone (Z-VAD-fmk)-inhibitable portion of the caspase activity with use of the synthetic fluorogenic caspase-3 (CPP32) substrate acetyl-Asp-Glu-Val-Asp-4-methylcoumaryl-7-amide (Peptide Institute, Osaka, Japan) at 50 μM. The samples were assayed in duplicate with or without 10 μM Z-VAD-fmk, and the kinetics of the proteolytic cleavage of the substrate were monitored in a fluorescence microplate reader (Spectromax Gemini EM, Molecular Devices, Sunnyvale, CA) with 360-nm excitation and 460-nm emission wavelengths. Caspase-3 activity was calculated from the slope of the recorder trace and expressed as change in fluorescence per minute per milligram of protein. Protein concentrations in the supernatant were assayed using the bicinchoninic acid kit (Sigma).

Histology. Formalin-fixed portions of the liver were embedded in paraffin, and 5-μm sections were cut. Sections of the livers were stained for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay as described in the manufacturer’s instructions (Roche Molecular Biochemicals, Indianapolis, IN). The number of apoptotic hepatocytes was quantified in these sections on the basis of positive TUNEL staining and morphological criteria, as described previously (16, 17). The AS-D chloroacetate esterase technique was used to stain neutrophils in liver sections, as described in detail previously (27). Neutrophils were identified by positive staining and morphology and were counted in 20 high-power fields (×400) with a Nikon Labophot microscope. Only neutrophils within sinusoids or extravasated into the tissue were counted; the small numbers of neutrophils marginated within large vessels, e.g., hepatic venules, were not included, because they are not involved in the injury (6). Cell damage was evaluated in parallel sections stained with hematoxylin and eosin. The percentage of necrosis was estimated by evaluating the number of microscopic fields with necrosis compared with the entire histological section. The pathologist (A. Farhood) performing the histological evaluation was blinded with regard to the treatment of animals.

Ribonuclease protection assay. All protocols followed the instructions for the RiboQuant Multi-Probe RNase Protection Assay System (PharMingen). The In Vitro Transcription Kit and a customized template set (containing mouse MIP-2, mouse KC, and L32) were used to synthesize a radiolabeled probe set using [α-32P]UTP. These probes were hybridized with total RNA isolated from liver tissue for 16 h. After digestion of nonhybridized RNA with RNase, the protected probes were separated on a denaturing acrylamide gel. The gel was dried and then exposed to X-ray film (Kodak X-OMat, Fisher Scientific, Pittsburgh, PA) for 12 h at −80°C. The developed X-ray films were scanned using a calibrated imaging densitometer (model GS-710, Bio-Rad Laboratories, Hercules, CA) and Quantity-One software (Bio-Rad).

RESULTS

CXC chemokine formation during endotoxemia. To assess CXC chemokine formation during endotoxemia, plasma levels of KC and MIP-2 were measured by ELISA. ET or Gal/ET resulted in a rapid increase in plasma levels of KC and MIP-2 (Fig. 1). Peak levels of 2,000–2,500% (KC) and 3,600–4,000% (MIP-2) of baseline were reached 1.5 h after ET injection and then gradually declined over the next 4–5 h. Although drastically reduced compared with peak levels, plasma concentrations of KC and MIP-2 were still significantly above baseline at 6 and 7 h (Fig. 1). There was no significant difference in chemokine plasma levels between ET and Gal/ET injection. In contrast, plasma KC levels reached a peak of 3,600–4,000% (MIP-2) of baseline were reached 1.5 h after ET injection and then gradually declined over the next 4–5 h. Although drastically reduced compared with peak levels, plasma concentrations of KC and MIP-2 were still significantly above baseline at 6 and 7 h (Fig. 1).
at any time. On the other hand, Gal alone had no effect on CXC formation (Fig. 1). To verify that the CXC chemokines were formed in the liver, KC and MIP-2 mRNA levels were measured in the tissue with an RNase protection assay (Fig. 2). Similar to the time course of plasma protein levels, KC and MIP-2 mRNA levels were highest 1.5 h after Gal/ET (Fig. 2) and ET (data not shown) and then gradually declined. Again, the 6-h values were still significantly elevated compared with controls (Fig. 2).

Cytokines as inducers of CXC chemokine formation in vivo. To evaluate whether cytokines are actually responsible for KC and MIP-2 formation during endotoxemia, ET-resistant (C3H/HeJ) mice were treated with Gal/ET. C3H/HeJ mice have a Toll-like receptor-4 deficiency (46) and, therefore, are unable to produce any cytokines in response to ET or Gal/ET in vivo (11). Gal/ET did not cause a significant increase of plasma KC or MIP-2 levels (data not shown), suggesting that CXC chemokine formation during endotoxemia in vivo is largely dependent on the production of cytokines. To confirm the role of cytokines as inducer of CXC chemokine formation in vivo, C3H/HeJ mice were injected intravenously with TNF-α, IL-1α, or IL-1β (2 μg/kg each). In general, all three cytokines proved to be potent inducers of KC and MIP-2 (Fig. 3). However, some clear differences emerged between these cytokines. Substantially higher and more sustained plasma levels of KC or MIP-2 were induced by IL-1α than by IL-1β or TNF-α (Fig. 3).

Pathophysiological relevance of CXC chemokine formation during endotoxemia. To investigate a potential role of KC and MIP-2 in the pathophysiology of Gal/ET-induced liver injury, animals were treated with combined antibodies of KC and MIP-2. Gal/ET caused apoptosis, as indicated by the 20-fold increase in caspase-3 activities in the liver (Fig. 4). In addition, elevated plasma ALT activities were observed together with histological evidence of hepatocellular necrosis at 7 h (Fig. 5). The overall injury in this model is initiated by TNF-mediated apoptosis, which triggers neutrophil extravasation and neutrophil-mediated injury (30, 35). Consistent with this hypothesis, large numbers of neutrophils were present in the liver 7 h after Gal/ET; ~35% of the total hepatic neutrophils were located in the parenchyma (Fig. 6). Treatment with control IgG or the combined anti-KC and anti-MIP-2 antibodies did not attenuate any injury parameter, including caspase-3 activities (apoptosis; Fig. 4), plasma ALT, and histological assessment of necrosis (Fig. 5). Furthermore, the antibodies had no effect on Gal/ET-induced hepatic neutrophil accumulation or extravasation into the parenchyma (Fig. 6). Because previous reports suggested that hepatocytes undergoing apoptosis can produce CXC chemokines (9, 13), we treated animals with the pancaspase inhibitor Z-VAD-fmk (10 mg/kg) 3 and 4.5 h after Gal/ET and measured CXC chemokine formation at 5.5 h. These doses of Z-VAD-fmk and other pancaspase inhibitors are known to eliminate apoptosis in the Gal/ET model and prevent extravasation of neutrophils (17, 25, 30). Consistent with previous observations, Z-VAD-fmk attenuated the increase of caspase-3 activities by 92% and the number of TUNEL-positive hepatocytes by 87% (Fig. 7). However, Z-VAD-fmk treatment had no significant effect on KC or MIP-2 formation, despite its profound effect on apoptosis.

Gal/ET-induced liver injury in CXCR2−/− mice. Although the doses of anti-KC and anti-MIP-2 antibodies used in this study were effective in reducing hepatic neutrophil accumulation in the Fas receptor-induced apoptosis model (13), we wanted to confirm our findings with a different approach. Therefore, we tested the susceptibility of CXCR2−/− mice to Gal/ET-induced injury. CXCR2 is the only receptor for CXC proteins.
chemokines in mice and is used by KC and MIP-2 (3, 36, 44). Wild-type BALB/c and CXCR2−/− control animals had normal levels of plasma ALT activities, no necrosis, and baseline levels of hepatic caspase-3 activities (Table 1). Untreated CXCR2−/− mice showed significantly elevated numbers of neutrophils in the liver, all of which were located in sinusoids (Table 1). Similar to C3Heb/FeJ mice, Gal/ET-induced hepatic neutrophil sequestration and extravasation resulted in severe liver injury in wild-type BALB/c mice at 6.5 h (Table 1). However, all parameters of cell injury were similar in CXCR2−/− and wild-type animals (Table 1). The overall numbers of neutrophils in the liver as well as the numbers of extravasated neutrophils were higher in CXCR2−/− mice. However, the percentage of extravasated cells was similar in wild-type (42 ± 4%) and CXCR2−/− mice (39 ± 6%).

DISCUSSION

ET-induced CXC chemokine formation. The objective of this investigation was to determine CXC chemokine formation and assess its pathophysiological relevance in the Gal/ET model of apoptosis and inflammatory liver injury. Measurement of plasma chemokine protein levels demonstrated a massive formation of KC and MIP-2 in response to Gal/ET and ET but not Gal alone. These chemokines were at least in part generated within the liver, as indicated by the parallel rapid increase and gradual decline of hepatic mRNA levels and the plasma KC and MIP-2 protein levels. However, it cannot be excluded that other, extrahepatic sources also contributed to CXC chemokine concentrations in plasma. The increase in KC and MIP-2 mRNA within the liver developed parallel to enhanced TNF-α formation during endotoxemia in C3Heb/FeJ mice (55). On the other hand, Toll-like receptor-4-deficient C3H/HeJ mice, which are unable to generate cytokines in response to Gal/ET in vivo (11), did not produce chemokines during endotoxemia. However, injection of murine recombinant TNF-α resulted in a rapid CXC chemokine formation in the liver. On the basis of these data, it can be concluded that CXC chemokine formation after ET is almost entirely cytokine dependent. This does not involve only TNF-α but most likely also IL-1. The delayed decline of CXC chemokine mRNA and plasma protein levels compared with TNF-α suggests that IL-1 might be involved in maintaining CXC chemokine levels after TNF-α formation has stopped. Indeed, IL-1α and IL-1β are also potent inducers of KC and MIP-2 formation.

Pathophysiological relevance of CXC chemokine formation. CXC chemokines are potent chemotactic factors for neutrophils (40, 44); KC and MIP-2 can effectively trigger neutrophil

Fig. 4. Hepatic caspase-3 activities [change in fluorescence (ΔF)·min⁻¹·mg protein⁻¹] were measured in controls (C) and 7 h after injection of Gal (700 mg/kg) + ET (100 μg/kg; GE). Some animals were treated with anti-KC (5 mg/kg) and anti-MIP-2 (5 mg/kg) antibodies or nonimmune murine IgG. Values are means ± SE; n = 6. *P < 0.05 vs. C.

Fig. 5. Effect of anti-CXC chemokine antibodies on Gal/ET-induced liver injury. Plasma alanine transaminase (ALT) activities (A) and area of necrosis (B) were determined in controls and 7 h after Gal/ET. Some animals were treated with anti-KC (5 mg/kg) and anti-MIP-2 (5 mg/kg) antibodies or nonimmune murine IgG. Values are means ± SE; n = 6. *P < 0.05 vs. C.

Fig. 6. Effect of anti-CXC chemokine antibodies on hepatic neutrophil (PMN) accumulation. Neutrophils were stained in liver sections, and total number of neutrophils accumulating in the liver and number of neutrophils extravasated into the parenchyma were counted in 20 high-power fields (HPF). Hepatic neutrophils were assessed in controls and 7 h after Gal/ET. Some animals were treated with anti-KC (5 mg/kg) and anti-MIP-2 (5 mg/kg) antibodies or nonimmune murine IgG. Values are means ± SE; n = 6. *P < 0.05 vs. C.
extravasation in a peritonitis model in vivo (1). In addition, transfection of the CXC chemokine CINC-1 gene into rat hepatocytes resulted in hepatic neutrophil recruitment and extravasation (41). Our previous data indicate that neutrophil extravasation is a prerequisite for neutrophil-mediated liver injury in the Gal/ET model (6). Inhibition of extravasation by blocking adhesion molecules attenuated liver injury by preventing neutrophil-mediated oxidant stress after Gal/ET (10, 11, 29) as well as in other liver disease models (15, 18). Thus, if CXC chemokines play a relevant role in the pathophysiology, it was expected that neutralizing KC and MIP-2 would result in less neutrophil extravasation and reduced neutrophil-induced injury without an effect on apoptosis. Indeed, the anti-KC and anti-MIP-2 antibodies did not affect apoptosis or the overall neutrophil recruitment into the liver. Because both effects are entirely TNF-α dependent in this model (30, 37, 48), these results are consistent with the hypothesis and previous observations that CXC chemokines may be of limited relevance for hepatic neutrophil recruitment if more potent activators of neutrophils are present (1). However, neutralizing antibodies did not prevent neutrophil extravasation and liver injury after Gal/ET. These data suggest that CXC chemokine formation is not the critical signal for migration of neutrophils into the parenchyma. Moreover, CXC chemokine formation was not significantly affected when apoptosis was almost completely prevented by the pancaspase inhibitor Z-VAD-fmk. Although these results appear to contradict those of a previous study where hepatocytes undergoing Fas receptor-mediated apoptosis generated CXC chemokines (13), the situation in the endotoxemia model is clearly different. Even the low doses of ET used in the Gal/ET model induce substantial formation of TNF-α and IL-1, both of which are potent inducers of KC and MIP-2. Under these circumstances, CXC chemokine formation due to apoptosis is most likely negligible compared with the direct cytokine effect. The results with neutralizing antibodies were completely confirmed in CXCR2<sup>−/−</sup> mice, which showed neutrophil localization and neutrophil-mediated injury similar to that in wild-type animals.

Although our experiments strongly suggest that the CXC chemokines MIP-2 and KC are not involved in the pathophysiology, we cannot exclude the possibility that other chemokines could play a role. Proinflammatory conditions can increase the expression of CC chemokine receptors 1 and 2 on neutrophils (5, 32). The neutrophils then respond to CC chemokines such as MIP-1α (32, 59), monocyte chemoattractant protein-1 (32), and leukotactin (59). Treatment with anti-MIP-1α antibodies resulted in reduced neutrophil infiltration and transmigration in models of Ig- (49) or ET-induced lung injury (51). The role of CC chemokines in the pathogenesis of Gal/ET-induced liver injury is unknown and needs to be investigated.

Interestingly, Li et al. (39) recently suggested that CXC chemokines may be the critical mediators of neutrophil extravasation in the Gal/ET model. However, there are serious concerns about the interpretation of the results. 1) The anti chemokine antibodies used by Li et al. reduced parenchymal cell apoptosis (39). Because apoptosis in this model is entirely mediated by TNF-α (30, 37), these findings suggest that Li et al. may have reduced TNF levels with these antibodies. Although it remains unclear how this occurred, IgG has the capacity to bind ET and attenuate cytokine formation (42). Less TNF-α formation will result in reduced apoptosis, which is a critical event in neutrophil extravasation and injury (17, 30, 35). Thus at least some of the data of Li et al. can be explained without invoking any involvement of CXC chemokines in the pathophysiology of Gal/ET-induced liver injury. Another concern is that Li et al. found protection with each antibody alone. Given the fact that MIP-2 and KC are potent chemotactic factors for neutrophils and each can trigger neutrophil extrav-

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**Table 1. Gal/ET-induced liver injury in CXCR2<sup>−/−</sup> mice**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Gal/ET</th>
<th>CXCR2&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
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<tr>
<td>ALT, U/l</td>
<td>27±14</td>
<td>96±18</td>
<td>2.985±711*</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>15±8</td>
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<tr>
<td>Necrosis, %</td>
<td>0±0</td>
<td>8±2</td>
<td>54±6*</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0±0</td>
</tr>
<tr>
<td>Caspase-3, ΔF·min&lt;sup&gt;−1&lt;/sup&gt;·mg·protein&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>41±23</td>
<td>976±85*</td>
<td>1.240±171*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>49±28</td>
</tr>
<tr>
<td>Total PMNs, 20 HPF&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>11±1</td>
<td>175±41*</td>
<td>171±18*</td>
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<td></td>
<td></td>
<td>141±5*</td>
</tr>
<tr>
<td>Extravasated PMNs, 20 HPF&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>0.5±0.5</td>
<td>33±9</td>
<td>72±7*</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>0±0</td>
</tr>
<tr>
<td>Extravasated PMNs, %</td>
<td>5±5</td>
<td>19±5</td>
<td>42±4*</td>
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Values are means±SE; n = 3-6. Liver injury [alanine transaminase (ALT), necrosis, caspase-3 activity] and hepatic neutrophil (PMN) accumulation [in 20 high-power fields (HPF)] and extravasation were determined in BALB/c wild-type (WT) or CXCR receptor 2 gene knockout (CXCR2<sup>−/−</sup>) control mice and after treatment with galactoaminase (700 mg/kg) + endotoxin (100 μg/kg). ΔF, change in fluoroescence. *P < 0.05 vs. WT or CXCR2<sup>−/−</sup> controls. †P < 0.05 vs. WT Gal/ET (6.5 h).

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asation (1), one would expect a relevant effect on neutrophil extravasation only if both chemokines are eliminated. These concerns and other issues are discussed in more detail elsewhere (24).

In summary, our data indicate that cytokines produced during endotoxemia are responsible for the massive formation of KC and MIP-2 in the liver and possibly other extrahepatic sites. However, a pathophysiological role of these CXC chemokines could be excluded on the basis of five strong arguments: 1) The time course of CXC chemokine formation did not correlate with neutrophil extravasation. 2) ET and Gal/ET induced a similar CXC chemokine response, but only Gal/ET triggered apoptosis and neutrophil extravasation. 3) Neutralizing antibodies against KC and MIP-2 had no effect on neutrophil extravasation and injury. 4) Wild-type and CXCR2−/− mice showed similar neutrophil extravasation and liver injury. 5) Eliminating apoptosis with a pancaspase inhibitor prevented neutrophil transmigration without affecting CXC chemokine formation. Thus, on the basis of these different experimental approaches, which yielded identical results, we can conclude that CXC chemokines are not responsible for neutrophil recruitment into the liver or for neutrophil extravasation and, therefore, are not involved as a chemotactic signal in the injury process after Gal/ET administration.

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GRANTS

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