Abstract: The NKT cell ligand α-galactosylceramide and its synthetic homologue KRN7000 stimulate rapid and copious secretion of IFN-γ and TNF-α release, both of which are key mediators of LPS-induced shock. We showed that KRN7000, injected before or within 2 h after LPS challenge, was able to prevent endotoxic shock. KRN7000 induced survival when the mice were injected 6, 9, or 12 days before the first injection of LPS, and this protective effect was associated with reduction upon subsequent challenge in the levels of IFN-γ, TNF-α, MCP-1, and an increase of IL-10. Further analysis showed that the animals treated with KRN7000 prior to LPS challenge had lower numbers of F4/80+, NKT, and NK cells and lower percentages of NKT cells that stained for intracytoplasmic IFN-γ when compared with mice that were not treated with KRN7000. When MCP-1 was injected in KRN7000-treated mice, the lethal effect of LPS challenge was restored, and the numbers of F4/80+, NKT, and NK cells increased to levels similar to those in untreated mice following LPS challenge. Taken together, our data demonstrated that KRN7000, injected from 6 to 12 days before the first administration of LPS, prevented endotoxin shock by inhibiting IFN-γ, TNF-α, and MCP-1 release. J. Leukoc. Biol. 84: 550–560; 2008.

Key Words: rodent · endotoxic · MCP-1 · NKT

INTRODUCTION

Endotoxic shock is mainly caused by an exuberant systemic cytokine response to Gram-negative bacteria and their characteristic cell wall component LPS. The uncontrolled production of Th1 cytokines causes a variety of pathologic effects, which ultimately form the endotoxic shock syndrome [1]. Consistent with an essential role for TNF-α and IFN-γ in the lethal effects of LPS-induced shock, neutralization of these cytokines in experimental models of endotoxic shock substantially decreases mortality [1–6]. In addition, the critical role of these cytokines in the pathogenesis of LPS-induced shock was confirmed using mice deficient for their receptors [7–9]. Thus, proinflammatory cytokines, in particular, TNF-α and IFN-γ, produced by macrophages [1] and lymphocytes expressing NK cell-associated markers such as NK1 in mice [10], play a key role in the pathogenesis of endotoxic shock.

NK1+ cells segregate into two major populations, namely, NKT and NK cells. Many NKT cells express an invariant TCR-α chain rearrangement (Vα14-Jα18 in mice and the highly homologous Vα24-Jα18 in humans) [1] and represent a T cell subset that expresses NK cell markers such as NKR-P1 (NK1.1). NKT cell development depends on TCR-mediated selection by the β2-microglobulin (β2m)-associated CD1d protein, and hence, β2m−/− mice lack most T cells belonging to this subset [11–16]. Upon stimulation, Vα14+ NKT cells promptly secrete large amounts of the types 1 and 2 cytokines, including IFN-γ and IL-4, respectively [1]. In contrast, NK cells develop independently of β2m and typically produce proinflammatory cytokines such as IFN-γ and TNF-α. Both of these cell types are found in all secondary lymphoid tissues and are particularly abundant in the liver [2].

Accumulating evidences suggest that NK and NKT cells participate in the pathogenesis of LPS-induced shock [17]. Vα14+ NKT cells have been demonstrated by our group to play an important role in the pathogenesis of the generalized Shwartzman reaction [18]. Whereas NKT cells are mainly involved in the Shwartzman reaction elicited by low doses of LPS, NK cells play a prominent role at higher doses of LPS [18]. The relative contribution of NKT and NK cells may be dependent on the amount of IL-12 produced in response to the dose of LPS administered. It has been demonstrated that NK and NKT cells constitutively express IL-12R [19], although its expression is higher on NKT cells than on NK cells [19]. Therefore, the large amount of IL-12 produced by the injection of high doses of LPS into NKT-deficient mice might be needed for NK cells to overcome the impairment of NKT cells, which would be preferentially responsive to lower doses of IL-12 (and by extension, of LPS).
The glycolypid \(\alpha\)-galactosylceramide (\(\alpha\)-GalCer), originally isolated as a natural component of a marine sponge [20], and its synthetic homologue KRN7000 are specifically recognized by V\(\alpha\)14\(^+\) NKT cells. In vivo treatment with KRN7000 induces a Th2 or a Th0 cytokine response characterized by variable amounts of IFN-\(\gamma\) and/or IL-4 depending on the time of in vivo exposure to KRN7000 [21, 22]. In particular, Singh et al. [21] reported that long-term exposure to KRN7000 (9 days) coadministered with OVA induced an antigen-specific immune response. Sireci et al. [21] reported that long-term exposure to KRN7000 [21, 22]. In particular, Singh et al. [21] reported that long-term exposure to KRN7000 (9 days) coadministered with OVA induced an antigen-specific immune response.

Comparison of plasma levels of these cytokines with their mRNA expression in liver lymphocytes after exposure to \(\alpha\)-GalCer reveals two substantial differences: IFN-\(\gamma\) mRNA precedes the plasma peak reached at 10 h, and in contrast to the marginal plasma IL-10 concentration, its mRNA expression is increased significantly 1 h after \(\alpha\)-GalCer treatment [24].

As KRN7000 administration before and after LPS challenge significantly modifies the outcome of endotoxic shock by a switch of cytokines produced by NKT cells and transactivated NK cells [25], we hypothesized that \(\alpha\)-GalCer could prevent endotoxic shock when administered before the first administration of LPS. In this report, we show that KRN7000, when administered 6, 9, or 12 days before the first injection of LPS, decreased lethality of the experimental, systemic Shwartzman reaction. These effects could be correlated with the inhibition of IFN-\(\gamma\) and TNF-\(\alpha\) by MCP-1 (Table 4), mice were injected i.p. 16 h before LPS challenge with 10 \(\mu\)g/mouse rmMCP-1 (R&D Systems, Minneapolis, MN, USA) 16 h before challenge. We chose the dose and time of administration of rmMCP-1 based on experiments using different doses and time-points of rmMCP-1 administration in mice receiving KRN7000 treatment and LPS challenge. The time-point and dose of rmMCP-1 chosen inhibited the protective effects of KRN7000 on LPS-induced shock. For in vivo neutralization of MCP-1 (Table 4), mice were injected i.p. 16 h before LPS challenge with 10 \(\mu\)g/mouse anti-MCP-1 mAb (clone 4E2, hamster IgG, eBioscience) dissolved in PBS. Hamster IgG (20 \(\mu\)g/mouse; clone eBio Arm 229) was injected i.p. in control mice at the same time as anti-MCP-1 to check the specificity of MCP-1 neutralization.

**Materials and Methods**

**Animals**

C57BL/6j mice were purchased from Harlan-Nossan (Correzzana, MO, Italy) and were used at 8–12 weeks of age. Each experimental group consisted of 35 male mice. Fifteen mice from each group were used to test survival in LPS-induced shock and for measurement of aspartate aminotransferase/alanine aminotransferase (AST/ALT) in the sera. Blood samples were obtained from 10 animals for serum cytokine assays. The remaining 10 mice were used to assess percentages of body weight loss.

**Induction of the experimental, systemic Shwartzman reaction**

Mice were injected i.p. with 40 \(\mu\)g phenol-extracted *Escherichia coli*, *Salmonella typhimurium*, or *Salmonella abortus equi* derived LPS (first administration of LPS), purchased from Sigma-Aldrich (Milan, Italy). Twenty-four hours later, mice were challenged by i.v. injection of 400 \(\mu\)L LPS (second injection of LPS) dissolved in 0.2 ml PBS [18]. Negative controls were injected with the LPS priming dose and 0.2 ml PBS i.v. 24 h later or with 0.2 ml PBS i.p. and 24 h later, with the LPS challenge dose. The occurrence of a systemic Shwartzman reaction was evaluated by monitoring mortality of mice for 7 days after challenge. Each batch was used to induce the reaction three times, and the results reported in Tables 1–4 are the mean of three different experiments performed using in each reaction one of the three phenol-extracted preparations of LPS to avoid contaminating lipoprotein effects as reported previously [27]. Body weights were recorded before the first administration of LPS and monitored over a period of 24 h after challenge. Data were represented as mean percent of control body weight (100%). For in vivo neutralization of IFN-\(\gamma\) (Table 2), mice were injected i.p. 2 h before LPS challenge with 0.5 mg purified anti-IFN-\(\gamma\) mAb (clone XMG1.2, rat IgG1, BD Biosciences, San Jose, CA, USA) or isotype control mAb (rat IgG1, BD Biosciences). In the experiments reported in Table 3, the KRN7000-protected mice (mice pretreated with KRN7000 6, 9, or 12 days before the first injection of LPS) were injected i.p. with 10 \(\mu\)g/mouse rmMCP-1 (R&D Systems, Minneapolis, MN, USA) 16 h before challenge. We chose the dose and time of administration of rmMCP-1 based on experiments using different doses and time-points of rmMCP-1 administration in mice receiving KRN7000 treatment and LPS challenge. The time-point and dose of rmMCP-1 chosen inhibited the protective effects of KRN7000 on LPS-induced shock. For in vivo neutralization of MCP-1 (Table 4), mice were injected i.p. 16 h before LPS challenge with 10 \(\mu\)g/mouse anti-MCP-1 mAb (clone 4E2, hamster IgG, eBioscience) dissolved in PBS. Hamster IgG (20 \(\mu\)g/mouse; clone eBio Arm 229) was injected i.p. in control mice at the same time as anti-MCP-1 to check the specificity of MCP-1 neutralization.

**Serum transaminase**

In mice treated with KRN7000 before the first administration of LPS, sera were collected 2 h after LPS challenge and pooled (Tables 1–4). Serum ALT (EC 2.6.1.2) and AST (EC 2.6.1.1) were measured by the standard photometric method using an Hitachi-type 7350 automatic analyzer (Hitachi Chemical Diagnostic, Freiburg, Germany) and a commercial kit (Sigma-Aldrich) adapted to small sample volumes [24].

**KRN7000 treatments**

\(\alpha\)-GalCer (KRN7000) was kindly provided by R&D Collaboration Pharmaceutical Division, Kirin Brewery Co. (Tokyo, Japan). It was injected at indicated times i.p. at a dose of 4 \(\mu\)g/mouse in 400 \(\mu\)l vehicle (0.025% polysorbate-20), as suggested by the supplier; control mice received injections of the vehicle only.

**Cytokine detection**

For measurement of cytokine levels in pooled mouse sera, we used IFN-\(\gamma\), IL-10, and TNF-\(\alpha\) quantikine mouse ELISA kits (R&D Systems). Detection limits were 5 and 4 pg/ml, respectively. Serum samples were collected after 1 h for TNF-\(\alpha\), 6 h for IFN-\(\gamma\), or 2 h from LPS challenge for IL-10 [28]. Data obtained from sera collected at different time-points after challenge consistently confirmed the differences between LPS-KRN7000 and LPS-vehicle-treated animals (see Fig. 2). To measure MCP-1 expression in the liver, mice were sacrificed 2 h after challenge, and organs were homogenized in a lysis buffer containing 1% Nonidet P-40, 500 mM NaCl, 50 mM Hepes, 1 mg/ml leupeptin, and 100 mg/ml PMSF using a tissue homogenizer (Biospec Products, Inc., Bartlesville, OK, USA). The crude lobe of the liver was removed and homogenized in 2 ml homogenization buffer. Homogenates were incubated on ice for 30 min and were then centrifuged at 2500 rpm for 10 min. Supernatants were collected and stored at –20°C. For measurement of MCP-1, flat-bottom, 96-well microtiter plates (Immu-Plate I 96-F, Nunc A/S, Roskilde, Denmark) were coated with 50 \(\mu\)l/well purified rabbit anti-mMCP-1 antibodies (1 \(\mu\)g/ml in 0.6 M NaCl, 0.26 M H\(_3\)BO\(_4\), and 0.01 M NaOH, pH 9.6) for 16 h at 4°C and were then washed with PBS and incubated for 90 min at 37°C. Plates were rinsed four times with washing buffer, and diluted (neat and 1:10) cell-free supernatants (50 \(\mu\)l) in duplicate were added, followed by incubation for 1 h at 37°C. Plates were washed four times, and 50 \(\mu\)l well...
ELISAs did not cross-react with other members of the chemokine C-C family, this method did not cross-react with IL-1, IL-2, or IL-6. In addition, the ELISA method consistently detected MCP-1 concentrations.

**Preparation of liver mononuclear cells**

Liver mononuclear cells were prepared as described [18]. In brief, livers were minced, passed through a mesh, and suspended in complete RPMI 1640. Cells were resuspended in 30% Percoll containing 100 U/ml heparin and centrifuged at 2000 rpm for 20 min at room temperature.

**Intracellular cytokine detection**

Cells for intracellular cytokine staining were cultured for 6 h in 3 μM monensin (Sigma-Aldrich). Lymphocytes were incubated in PBS-0.1% Na3 with anti-mouse CD16/32 mAb (clone 2.4G2, BD Biosciences) at the concentration indicated by the manufacturer for 5 min at 4°C to block nonspecific binding to FcR γ. Spleen cells were harvested and fixed with 4% (w/v) paraformaldehyde (Sigma-Aldrich) in PBS for 10 min at room temperature. For intracytoplasmic staining, fixed cells were resuspended and washed twice with permeabilization buffer containing 0.1% saponin (Sigma-Aldrich), 1% heat-inactivated FCS, and 0.1% Na3 in PBS. To analyze cytokines in TCR down-regulated NKT, permeabilized cells were incubated with mCD1d-α-GalCer/PE-labeled tetramers or mCD1d-unloaded-PE-labeled tetramers as negative control after staining for intracellular cytokine. Permeabilized cells were incubated with FITC-conjugated anti-mouse IFN-γ mAb (clone XMG1.2, rat IgG1, BD Biosciences). As a control, FITC-conjugated isotype mAb (clone R3-34, rat IgG1, BD Biosciences) were used. Cells were analyzed with a FACScan™ flow cytometer (Becton Dickinson, San Jose, CA, USA). Viable cells were gated by forward- and side-scatter, and analysis was performed on 100,000 acquired events for each sample.

**Detection of F4/80⁺, NKT, and NK cells**

Following isolation from liver, cells were incubated in PBS, 0.1% Na3, with anti-mouse CD16/32 mAb (clone 2.4G2, BD Biosciences) at the concentration indicated by the manufacturer for 5 min at 4°C to avoid nonspecific binding to FeRγ. Cells were labeled with F4/80-PE-conjugated (CL3A-1, rat IgG2b, Serotec, Oxford, UK). For separation of F4/80-positive cells, anti-PE microbeads (Miltenyi Biotech, Copenhagen, Denmark) were added, and positive fractions were collected by immunomagnetic methods (Miltenyi Biotech) and counted to obtain the data (see Fig. 5). For NKT counting during KRN7000-LPS treatments, liver cells from treated mice were incubated with α-GalCer/mCD1d-PE-labeled tetramers and then incubated with anti-PE microbeads (Miltenyi Biotech). NKT cells were stained with α-GalCer/mCD1d-PE-tetra-

**TABLE 1. KRN7000 Effects in Systemic Shwartzman Reaction. Treatments before LPS Priming**

<table>
<thead>
<tr>
<th>Days before LPS priming</th>
<th>Deaths/tested</th>
<th>Mortality %</th>
<th>AST U/L</th>
<th>ALT U/L</th>
<th>% of Body weight loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive controls*</td>
<td>13/15</td>
<td>87</td>
<td>238 ± 10</td>
<td>134 ± 5</td>
<td>9</td>
</tr>
<tr>
<td>Negative controls</td>
<td>0/15</td>
<td>0</td>
<td>29 ± 3</td>
<td>18 ± 3</td>
<td>0</td>
</tr>
<tr>
<td>Negative controls*</td>
<td>7/15</td>
<td>47</td>
<td>186 ± 16</td>
<td>127 ± 12</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0/15</td>
<td>0</td>
<td>128 ± 35</td>
<td>53 ± 10d</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0/15</td>
<td>0</td>
<td>134 ± 15</td>
<td>49 ± 4d</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>0/15</td>
<td>0</td>
<td>123 ± 24</td>
<td>55 ± 8d</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>3/15</td>
<td>20</td>
<td>151 ± 21</td>
<td>113 ± 12</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>6/15</td>
<td>40</td>
<td>194 ± 23</td>
<td>125 ± 17</td>
<td>4</td>
</tr>
<tr>
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<td>15/15</td>
<td>100</td>
<td>230 ± 15</td>
<td>142 ± 6</td>
<td>12</td>
</tr>
<tr>
<td>1</td>
<td>15/15</td>
<td>100</td>
<td>285 ± 15</td>
<td>248 ± 36</td>
<td>8</td>
</tr>
</tbody>
</table>

Every experimental group was repeated treating animals with vehicle only at the same time. Each experiment was repeated with different phenol-extracted LPS preparations described in Materials and Methods, obtaining the same results reported above. * Positive controls correspond to LPS-primed and -challenged mice, as reported in Materials and Methods. These groups of mice were primed with LPS and after 24 h, injected with 0.2 ml PBS. * These groups of mice were injected with 0.2 ml PBS and challenged with LPS 24 h later. P < 0.0001 using the χ² test comparing data obtained from KRN7000-treated groups with positive controls.
mers and checked by FACS analysis. As more than 90% of cells were stained with tetramers, they were counted. For detection of NK cells, liver cells were incubated with anti-TCRα/β mAb-PE-labeled (clone H57-597, hamster IgG, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 15 min at 4°C. After two washes in PBS, cells were incubated with anti-PE microbeads, and the negative fractions were recovered (Miltenyi Biotech). Negative fractions were incubated with pan-NK microbeads (DX5-coated microbeads, Miltenyi Biotech). Purification of TCRα/β-DX5+ was tested by FACS analysis. More than 93% of cells were TCRα/β-DX5+. These cells, obtained at different time-points of KRN7000 pretreatment, were counted. The data (see Figs. 5 and 7) were obtained by counting separated cells in 0.05% Trypan blue to exclude dead cells.

Statistics

The χ² and the Student’s t-test were used to compare significance of differences between experimental and control groups.

RESULTS

KRN7000 pretreatment modifies transaminase levels

As the treatment with KRN7000 alone induces liver injury [24], we tested AST and ALT levels in sera of mice exposed to α-GalCer at the same time-points that we used for the induction of endotoxic shock. Sera were collected after 1, 3, 6, 9, and 12 days of α-GalCer or vehicle-alone injection (Fig. 1). AST and ALT levels in mice exposed to KRN7000 were increased significantly when compared with transaminase levels in sera of control mice. AST and ALT detected 1 or 3 days after exposure to KRN7000 were higher than those found at 6, 9, or 12 days after exposure. These data confirmed that the administration of KRN7000 alone caused transient and reversible hepatic damage.

Prevention of systemic Shwartzman reaction by KRN7000

We investigated whether KRN7000, injected at different time-points before the first administration of LPS, could protect mice against endotoxic shock. Percentages of mortality, transaminase levels, and body weight loss in LPS-induced shock in C57BL/6 mice were assessed. Mice were divided into positive controls (animals primed and challenged with LPS), negative controls (animals primed or challenged only), and experimental groups treated with KRN7000 1, 3, 4, 5, 6, 9, or 12 days before the first injection of LPS (priming). All C57BL/6 mice survived when treated with KRN7000 6, 9, or 12 days before the first administration of LPS (Table 1), and groups treated 1 or 3 days before the first injection of LPS did not survive endotoxic shock. When KRN7000 was injected 4 or 5 days before the first exposure to LPS, 20–40% of mice died. Additionally, liver injury assessed by transaminase levels revealed less AST and ALT in the sera of animals in vivo exposed to KRN7000 for 6, 9, or 12 days before the first administration of LPS compared with those of unprotected mice. Moreover, in nonprotected mice, we observed considerable loss of body weight (8–12%) as a result of severe dehydration and diarrhea, compared with animals protected against endotoxic shock by KRN7000 treatments (0–1%). Similar results were obtained with phenol-extracted S. abortus equi- or S. typhimurium-derived LPS (data not shown), confirming that the reaction was induced by LPS independently from the source of LPS. No protection against endotoxic shock was observed in control groups mock-injected without KRN7000 (data not shown). Thus, KRN7000 treat-
KRN7000-induced protection by endotoxic shock correlates with a decrease of systemic IFN-γ and TNF-α

Because of the possible contribution of endogenous TNF-α, IFN-γ, and IL-10 to LPS-induced shock [18, 28], the systemic release of these cytokines was analyzed in mice treated with KRN7000 before the LPS priming. Sera were collected 1, 2, and 6 h after LPS challenge to detect the peak of systemic TNF-α, IL-10, and IFN-γ production, respectively [28]. Figure 2 shows a statistically significant increase of serum TNF-α and IFN-γ levels detected in mice treated with KRN7000 3 days before the first injection of LPS, and in the other groups treated with KRN7000 6, 9, or 12 days before the LPS priming, TNF-α and IFN-γ were reduced significantly when compared with control levels. Serum IL-10 levels increased in protected mice when compared with nonprotected animals. Differences between LPS-KRN7000 and LPS-vehicle-treated animals were reproduced when sera tested were collected at different time-points following LPS challenge (data not shown). Thus, KRN7000 prevention of LPS-induced shock was related to inhibition of release of systemic IFN-γ and TNF-α.

In vivo neutralization of IFN-γ protects mice by endotoxin shock

To analyze the role of IFN-γ in α-GalCer prevention of LPS-induced shock, we injected a neutralizing anti-IFN-γ mAb in mice treated 1 or 3 days with KRN7000 before the LPS priming (Table 2). Our data indicate that the LPS-induced shock is inhibited after IFN-γ neutralization, as the majority of KRN7000-nonprotected mice survived when anti-IFN-γ mAb was administered (underlined data in Table 2). Data obtained injecting isotype control mAb (data in parentheses) confirm that IFN-γ mAb specifically modify the immunomodulation of endotox shock exerted by KRN7000. These data confirm in our model evidence published previously [5, 6, 9], showing that IFN-γ plays a pivotal role in the immunopathogenesis of endotoxin shock.

Production of MCP-1 in KRN7000-LPS-treated mice

Macrophage recruitment is relevant for development of acute inflammatory responses, as shown, for example, for delayed-type hypersensitivity (DTH) to dinitrofluorobenzene (DNFB) [26]. As it was reported that MCP-1 induced the influx of macrophages, memory T cells, and NK cells in the sites of immune inflammation [26], we hypothesized that MCP-1 could be relevant in the analysis of the effects of KRN7000 in LPS-induced shock. To assess the production of MCP-1 in mice treated with KRN7000 before LPS priming, we tested MCP-1 in livers of animals, sacrificed 2 h after LPS challenge. As shown in Figure 3, at the different time-points of KRN7000 treatment (days pre-LPS), a rapid increase of MCP-1 levels was detected. In fact, MCP-1 levels in livers of mice pre-exposed to KRN7000 before LPS priming were increased significantly when compared with MCP-1 detected in livers of vehicle-LPS-treated mice. A statistically significant decrease of MCP-1 was detected in mice injected with KRN7000 8–12 days before LPS priming when compared with MCP-1 levels found in mice pre-exposed to vehicle 8–12 days before the first LPS injection. Plasma MCP-1 levels increased from undetectable levels to 9.03 ± 1.9 ng/ml when the mice were pretreated with KRN7000 1 day before the first dose of LPS (data not shown). Minimal levels of MCP-1 were detected in livers of mice exposed to KRN7000 alone.

These data indicate that pre-exposure to KRN7000 induced an increase of MCP-1 levels during the first 2 days of treatment and a decrease from 8 to 12 days of KRN7000 treatment. These data suggest that a decrease of MCP-1 could correlate with KRN7000-induced prevention of the Shwartzman reaction.

IFN-γ-producing NKT cells increase in α-GalCer-treated, nonprotected mice

As a relevant role for NKT cells in the immunopathogenesis of the generalized Shwartzman reaction has been reported previously [18, 25], we next investigated whether NKT cells, activated by KRN7000 and LPS, could be involved in modification of IFN-γ production that could change the outcome of the endotoxin shock. It has been shown that in vivo exposure to KRN7000 causes a transient TCR down-regulation of NKT

<table>
<thead>
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<tr>
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<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>Negative controls&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>186 ± 16</td>
<td>127 ± 12</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>3/15 (15/15)</td>
<td>20 (100)</td>
<td>130 ± 15 (205 ± 21)</td>
<td>102 ± 36 (141 ± 23)</td>
<td>2 (12)</td>
</tr>
<tr>
<td>1</td>
<td>3/15 (15/15)</td>
<td>20 (100)</td>
<td>165 ± 15 (232 ± 25)</td>
<td>88 ± 26 (127 ± 21)</td>
<td>3 (8)</td>
</tr>
</tbody>
</table>

Every experimental group was repeated treating animals with vehicle only at the same time. Each experiment was repeated with different phenol-treated LPS preparations as described in Materials and Methods, obtaining the same results reported above. Two hours before challenge, mice, exposed to KRN7000 3 or 1 days before LPS priming, were injected with 0.5 mg anti-IFN-γ per mouse or isotype control mAb. Underlined data were obtained by neutralization of IFN-γ, and data in parentheses were obtained in mice treated with 0.5 mg isotype control mAb 2 h before challenge. <sup>a</sup> Positive controls correspond to LPS-primed and challenged mice as reported in Materials and Methods. <sup>b</sup>This group of mice was primed with 40 μg LPS and after 24 h, injected with 0.2 ml PBS. <sup>c</sup>This group of mice was injected with 0.2 ml PBS and challenged with 400 μg LPS 24 h later.
We investigated if TCR down-regulation occurs in NKT cells of mice treated with KRN7000 before the first injection of LPS. Liver cells from controls and KRN7000-treated mice were stained with surface TCR mAb versus KRN7000/CD1d tetramers. We found a transient down-regulation of surface TCR on NKT cells with a peak reached from 6 h to 96 h of treatment with α-GalCer (data not shown).

As IFN-γ was shown previously to be relevant for monitoring α-GalCer immunomodulation of endotoxic shock (Table 2 and Fig. 2), we stained NKT cells obtained from livers of KRN7000-protected (Day 3) or -nonprotected mice (Day 6) for detection of intracellular IFN-γ. Liver cells were stained intracellularly with FITC-conjugated anti-IFN-γ mAb and unloaded (Fig. 4A) or KRN000-loaded CD1d tetramers (Fig. 4B) to detect TCR-down-regulated NKT+ -IFN-γ+ cells during LPS-KRN7000 treatments. More IFN-γ+ cells were detected in nonprotected mice (14.7%) than those detected in mice protected by KRN7000 (8%; Fig. 4A). When the cells were stained with intracellular, PE-labeled, α-GalCer/CD1d tetramers and FITC-labeled anti-IFN-γ (B), we detected more IFN-γ+ NKT cells in mice treated with KRN7000 3 days before LPS priming (4%) than in livers of mice treated with KRN7000 6 days before LPS priming (1.4%). NKT cells could transactivate NK cells to have intracellular “stores” of the same cytokine. In fact, we found more IFN-γ+ NK cells in nonprotected than in protected mice (data not shown). In addition, we analyzed the state of cellular activation of NK and NKT cells by staining these subsets for CD69 expression. We found more CD69+ NK and NKT cells in nonprotected than in protected mice (data not shown). In fact, we found more IFN-γ+ NK cells in nonprotected than in protected mice (data not shown). In fact, we found more IFN-γ+ NK cells in nonprotected than in protected mice (data not shown). In fact, we found more IFN-γ+ NK cells in nonprotected than in protected mice (data not shown). In fact, we found more IFN-γ+ NK cells in nonprotected than in protected mice (data not shown).

Decreased numbers of F4/80+ NKT and NK cells in KRN7000-protected mice

As the recruitment of macrophages, NKT cells, and NK cells in the liver is relevant for the development of the endotoxic shock [25], we counted these cells in the livers of protected and nonprotected mice. Figure 5A shows the numbers of F4/80+ cells during KRN7000 treatment before the first injection of LPS.
LPS or their controls. We detected higher numbers of macrophages in nonprotected (4.9–5.5 x 10^5 per liver of mice exposed 1 or 3 days to KRN7000, respectively) than in protected animals (1.1–1.5 x 10^5 per liver of animals exposed 6, 9, or 12 days to KRN7000). The data presented in Figure 5, B and C, demonstrate that NKT and NK cell numbers decreased in protected groups (mice treated 6, 9, or 12 days before LPS priming). In Figure 5D, FACS analyses of F4/80^+ cells from untreated (T0), protected (Day 6), and nonprotected (Day 3) mice were shown. An increased percentage of F4/80^+ cells (50.8%) was detected in nonprotected mice by 3 days of KRN7000 exposure before LPS priming, as compared with percentages of cells of protected (Day 6) mice (33.5%) or untreated animals (29.2%). These data showed that a decreased recruitment of F4/80^+, NKT, and NK cells was observed in livers of mice protected from endotoxic shock by KRN7000 treatment.

**MCP-1 modifies KRN7000 effects in LPS-induced shock**

We further investigated whether MCP-1 injection in KRN7000-protected mice could reverse the protection against lethal LPS-induced shock. For this purpose, we performed experiments injecting rmMCP-1 into α-GalCer-protected mice. To test time and dose of rmMCP-1 necessary to inhibit KRN7000-induced protection, we first carried out experiments in which we injected 0.1, 1, 5, or 10 μg rmMCP-1 at 2, 4, 8, or 16 h before challenge in mice protected from LPS-induced shock.
shock by injection of KRN7000 6 days before LPS priming. These experiments showed that the maximal biological effect of rmMCP-1 in KRN7000-protected mice was obtained with an i.p. injection of 10 µg rmMCP-1 16 h before challenge (Fig. 6).

We next tested if MCP-1 was able to modify KRN7000-induced protection from endotoxic shock. Data reported in Table 3 showed that rmMCP-1 administration caused death in KRN7000-protected mice (animals treated 12, 9, or 6 days before the first administration of LPS). In vivo neutralization of MCP-1 was able to block endotoxic shock in KRN7000-non-protected mice (animals treated with α-GalCer 1 and 3 days before LPS priming; Table 4). The effects of KRN7000 injected 1 or 3 days before LPS priming were not modified by isotype control mAb injection (data not shown).

To test if the effects of MCP-1 in the KRN7000-induced immunomodulation of LPS-induced shock could be related to numbers of F4/80, NKT, and NK cells, we counted these cells in mice treated with MCP-1 during KRN7000-LPS treatments. In Figure 7, A–C, cell numbers of F4/80, NKT, and NK cells per liver were shown. In KRN7000-protected groups, in mice treated with MCP-1 during KRN7000-LPS treatments.

<table>
<thead>
<tr>
<th>Days before LPS priming</th>
<th>Deaths/tested</th>
<th>Mortality %</th>
<th>AST UI/L</th>
<th>ALT UI/L</th>
<th>% of Body weight loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive controls</td>
<td>13/15</td>
<td>87</td>
<td>245 ± 35</td>
<td>144 ± 15</td>
<td>9</td>
</tr>
<tr>
<td>Negative controls</td>
<td>0/15</td>
<td>0</td>
<td>32 ± 3</td>
<td>28 ± 3</td>
<td>0</td>
</tr>
<tr>
<td>Negative controls'</td>
<td>7/15</td>
<td>47</td>
<td>156 ± 26</td>
<td>107 ± 10</td>
<td>4</td>
</tr>
<tr>
<td>12</td>
<td>13/15</td>
<td>87</td>
<td>220 ± 10</td>
<td>151 ± 15</td>
<td>7</td>
</tr>
<tr>
<td>9</td>
<td>15/15</td>
<td>100</td>
<td>242 ± 15</td>
<td>140 ± 14</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>14/15</td>
<td>93</td>
<td>135 ± 15</td>
<td>45 ± 4</td>
<td>9</td>
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</tbody>
</table>

Each experiment was repeated using different phenol-treated LPS preparations, obtaining the same results reported above. Sixteen hours before challenge, mice were injected i.p. with 10 µg rmMCP-1, diluted in 0.2 ml PBS in mice treated with KRN7000 12, 9, or 6 days before LPS priming. Positive controls correspond to LPS-primed and challenged mice, as reported in Materials and Methods. These groups of mice were primed with LPS. These groups of mice were challenged with LPS.

DISCUSSION

In this study, we provide evidence that KRN7000, when injected before LPS priming, can prevent the experimental, systemic Shwartzman reaction. The novelty of our findings is that in vivo treatment with α-GalCer could prevent even a potent signaling pathway such as that induced by LPS when administered 6–12 days before the initial exposure to LPS. Moreover, our data showed that KRN7000 induced direct damage to the liver (Fig. 1). The prevention of the endotoxin shock was achieved when mice were treated with KRN7000 6, 9, or 12 days before the first injection of LPS. Interestingly, however, when KRN7000 was given 1 or 3 days before the first administration of LPS, we detected an increased production of IFN-γ, and most important, no protection from the lethal Shwartzman reaction was achieved. KRN7000 is known to induce a cytokine storm, particularly in the liver [24]. It seems reasonable to speculate that treatment of mice with KRN7000, depending on the timing of in vivo exposure, results in the activation of NKT and indirectly, of NK cells. This activation produced an increased ratio of IFN-γ/IL-4 from 6 h to 72 h of KRN7000 exposure as previously reported by Crowe et al. [23]. Our results confirm that temporal differences in production of ILs may potentially be a key event for the different types of responses with which NKT cells are associated.

The increase of systemic IFN-γ in nonprotected mice and the beneficial effects of in vivo IFN-γ neutralization in

<table>
<thead>
<tr>
<th>Days before LPS priming</th>
<th>Deaths/tested</th>
<th>Mortality %</th>
<th>AST UI/L</th>
<th>ALT UI/L</th>
<th>% of Body weight loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive controls</td>
<td>14/15</td>
<td>93</td>
<td>267 ± 43</td>
<td>162 ± 21</td>
<td>10</td>
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<td>Negative controls</td>
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<td>56 ± 10</td>
<td>38 ± 7</td>
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<td>Negative controls'</td>
<td>3/15</td>
<td>53</td>
<td>156 ± 26</td>
<td>107 ± 10</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>15/15 (2/15)</td>
<td>100 (13)</td>
<td>311 ± 15 (75 ± 14)</td>
<td>147 ± 13 (62 ± 13)</td>
<td>12 (2)</td>
</tr>
<tr>
<td>1</td>
<td>15/15 (3/15)</td>
<td>100 (20)</td>
<td>345 ± 15 (95 ± 16)</td>
<td>148 ± 13 (59 ± 8)</td>
<td>8 (2)</td>
</tr>
</tbody>
</table>

Each experiment was repeated with different phenol-treated LPS preparations, obtaining the same results reported above. Anti-MCP-1 mAb (10 µg; clone 4E2, hamster Ig, eBioscience, San Diego, CA, USA), diluted in 0.2 ml PBS, were injected i.p. 16 h before LPS challenge in KRN7000-unprotected mice. Results obtained with MCP-1 neutralization (underlined and in parentheses) were shown statistically significant when compared with positive controls (P < 0.0001). The same groups, treated with isotype control mAb (clone eBio 229 Arm) at the same time, had the same percentages of mortality, transaminase levels, and percent of body weight loss of mice treated with KRN7000 1 or 3 days before LPS priming (data not shown). Positive controls correspond to LPS-primed and challenged mice as reported in Materials and Methods. These groups of mice were primed with LPS. These groups of mice were challenged with LPS.
KRN7000-nonprotected mice indicated a pivotal role for IFN-γ in our model of prevention of endotoxic shock by KRN7000. Extensive evidence in the literature [4–6, 9] has previously described the central role of IFN-γ in lethal endotoxemia. In our attempt to find a prophylactic role of KRN7000 in endotoxic shock, we confirmed several findings reported previously. It should be emphasized that NKT cells are thought to act primarily as initiators of immune responses, involving downstream effector cells that also produce cytokines. IFN-γ, produced by NKT cells, activated by in vivo treatment with KRN7000, could sequentially activate NK and other subsets that amplify IFN-γ production. Smyth et al. [30], studying the antitumoral effects of α-GalCer, described a sequential production of IFN-γ by NKT and NK cells. KRN7000 could activate NKT and indirectly, NK cells previously activated by LPS treatments and/or by IFN-γ produced by NKT cells. The disappearance of NKT and NK cells at later times in our experiments could be explained by at least two effects. First, in vivo glycolipid activation induces a decrease of NKT cells at later times as a result of TCR down-regulation [23]. Decreased numbers of NKT cells could cause a decrease of IFN-γ that does not transactivate NK cells by the same mechanisms reported by other authors [29]. Another possible explanation for decreased numbers of NKT and NK cells in mice exposed for 6–12 days to α-GalCer could be a result of a decrease of F4/80+ cells. Lower numbers of macrophages cause low levels of IL-12 and IFN-γ that could inhibit proliferation of NKT and NK cells. Furthermore, we cannot exclude that NKT cell disappearance in mice treated with α-GalCer could be a result of a different localization in the body of mice. Vinay et al. [10] reported that in CD137-deficient mice, in which reduced numbers and impaired functions of NK and NKT cells were found, there is survival following LPS-induced shock. In particular, these authors observed that also in wild-type mice, induction of septic shock was completely reversed by depletion of NK cells or blockade of NKT cell–APC interactions. These data therefore support our studies and those of others suggesting that NK cells [28] and NKT cells [18, 25] could play an essential role in the Shwartzman reaction.

It was reported that α-GalCer induces liver injury in C57BL/6 mice [24]. This tissue damage is characterized by an early release of plasma IL-4, IL-2, IL-6, and TNF-α (within 4 h after glycolipid injection) and a subsequent peak of IFN-γ (after 12 h) together with an increase of ALT (after 18 h of treatment). In our study, the time course of LPS-KRN7000 treatment provoking different effects on endotoxic shock could be a result of opposing factors operating at different times. In the early phase of treatment (within 3 days before the first exposure to LPS), α-GalCer could induce secretion of Th1 and Th2 cytokines (IFN-γ, IL-4, and IL-10); this was a result of TCR down-regulation that promoted a cytokine burst. By inducing release of TNF-α from macrophages, IFN-γ plays a major role in the cause of endotoxic shock. From 6 to 12 days of exposure, the cytokine burst is lost, resulting in substantial decreases in IFN-γ and TNF-α, which leads to survival in the setting of LPS challenge.

As shock is LPS-mediated but KRN7000-prevented, we next studied macrophages, NKT, and NK cell numbers, as these subsets were found to be relevant in the immunopathogenesis of the Shwartzman reaction and its immunomodulation [25, 28].
F4/80\(^+\) cells increased in nonprotected animals, and F4/80\(^+\), NKT, and NK cells decreased significantly in protected mice. The increased number of F4/80\(^+\) cells suggested that secretion of chemokines and expression of their relative receptors, responding to inflammatory molecules released during shock, could attract more macrophages in the liver. When chemokine release was inhibited by long KRN7000 in vivo exposure, F4/80\(^+\) cells as well as NKT and NK cells could not migrate to the liver.

Administration of MCP-1-induced death in KRN7000-protected mice and increased the arrival of F4/80\(^+\), NKT, and NK cells in the liver. Thus, we conclude that MCP-1 down-regulation could be one of the targets of the immunomodulatory activities of KRN7000. In spite of the existence of many CC chemokines attracting monocytes in vivo, Lu et al. [26] suggest that the loss of MCP-1 alone is sufficient to impair monocyte trafficking. In fact, they report that infiltrates appearing in challenged tissues of MCP-1 knockout mice during DTH to DNFB have less F4/80\(^+\) cells when compared with wild-type mice. This low number is accompanied by unresponsiveness to the antigens, used as a result of reduced edema and cellular infiltration. These data indicate that also in a different experimental murine model, MCP-1 is relevant to tissue recruitment of macrophages. In our model, the arrival of F4/80\(^+\), NKT, and NK cells in the liver is related to an increase of MCP-1 levels. Most likely, MCP-1 activates F4/80\(^+\) cells that transactivate NKT and NK cells through the release of cytokines and/or chemokines. The increased lethality obtained in our experiments involving injection of MCP-1 in KRN7000-LPS-treated mice seems to be in contrast with another report [31]. Those authors found that MCP-1 is a protective cytokine expressed in murine endotoxemia, and it exerts this effect by shifting the balance in favor of anti-inflammatory cytokine expression in endotoxin-challenged animals. Many differences exist between our experimental conditions and those reported in this paper. They used pathogen-free CD1 mice, and we used C57BL/6J mice maintained without pathogen-free conditions. Zisman et al. [31] gave a range of 750 µg–1 mg LPS for each animal to induce endotoxemia. We pretreated mice at various time-points with α-GalCer, and then we primed with 40 µg and challenged the reaction after 24 h with 400 µg LPS per animal. In addition, we injected 10 µg rmMCP-1, and Zisman et al. [31] gave 500 ng rmMCP-1 per mouse.

In conclusion, our data suggest that a relatively brief α-GalCer exposure (1–3 days) before LPS priming induced Th1 cytokines and MCP-1 release and did not protect mice from lethal endotoxic shock, whereas a longer (6–12-day) exposure to LPS ligand that inhibited Th1 cytokine and MCP-1 release induced survival of mice following LPS-induced shock. A deeper analysis of chemokines and their receptors in our model could be necessary to clarify the impact of the chemotaxis in the mechanism of the tissue damage occurring in the systemic, experimental Shwartzman reaction. Therefore, our studies provide another mechanism of immunomodulation by KRN7000 treatment, even if the time necessary to get protective effects raises difficulties for direct therapeutic translation of our findings.

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15. Mendiratta, S. K., Martin, W. D., Hong, S., Boesteanu, A., Joyce, S., van Rynke, R. Blumberg for help and advice in writing the paper and Drs. Yamano and Tukamatsu (Kirin Brewery Co., Tokyo, Japan) for providing us with KRN7000.


