The Free Radical Scavenger NecroX-7 Attenuates Acute Graft-versus-Host Disease via Reciprocal Regulation of Th1/Regulatory T Cells and Inhibition of HMGB1 Release

Keon-Il Im, Nayoun Kim, Jung-Yeon Lim, Young-Sun Nam, Eun-Sol Lee, Eun-Jung Kim, Hyoung Jin Kim, Soon Ha Kim and Seok-Goo Cho

J Immunol 2015; 194:5223-5232; Prepublished online 24 April 2015;
doi: 10.4049/jimmunol.1402609
http://www.jimmunol.org/content/194/11/5223

Supplementary Material
http://www.jimmunol.org/content/suppl/2015/04/24/jimmunol.1402609.DCSupplemental

References
This article cites 45 articles, 16 of which you can access for free at:
http://www.jimmunol.org/content/194/11/5223.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Author Choice
Freely available online through The Journal of Immunology Author Choice option

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
The Free Radical Scavenger NecroX-7 Attenuates Acute Graft-versus-Host Disease via Reciprocal Regulation of Th1/Regulatory T Cells and Inhibition of HMGB1 Release

Keon-Il Im,*,† Nayoun Kim,*,† Jung-Yeon Lim,*,† Young-Sun Nam,*,† Eun-Sol Lee,*,† Eun-Jung Kim,† Hyo Jung Kim,‡ Soon Ha Kim,‡ and Seok-Goo Cho*,†,§

Graft-versus-host disease (GVHD) is a major complication associated with allogeneic hematopoietic stem cell transplantation. Despite the prominent role of the adaptive immune system, the importance of controlling the innate immune system in the pathogenesis of GVHD has recently been rediscovered. High-mobility group box 1 (HMGB1) is a crucial damage-associated molecular pattern signal that functions as a potent innate immune mediator in GVHD. In the present study, we investigated treatment of experimental GVHD through HMGB1 blockade using the compound cyclopentylamino carboxymethylthiazolylindole (NecroX)-7. Treated animals significantly attenuated GVHD-related mortality and inhibited severe tissue damage. These protective effects correlated with the decrease in HMGB1 expression and lower levels of reactive oxidative stress. Additionally, NecroX-7 inhibited the HMGB1-induced release of TNF and IL-6, as well as the expression of TLR-4 and receptor for advanced glycation end products. We also observed increased regulatory T cell numbers, which may be associated with regulation of differentiation signals independent of HMGB1. Taken together, these data indicate that NecroX-7 protects mice against lethal GVHD by reciprocal regulation of regulatory T/Th1 cells, attenuating systemic HMGB1 accumulation and inhibiting HMGB1-mediated inflammatory response. Our results indicate the possibility of a new use for a clinical drug that is effective for the treatment of GVHD. The Journal of Immunology, 2015, 194: 5223–5232.

Abbreviations used in this article: BMT, bone marrow transplantation; DAMP, damage-associated molecular pattern; DC, dendritic cell; GVHD, graft-versus-host disease; HMGB1, high-mobility group box 1; HSCT, hematopoietic stem cell transplantation; NecroX, cyclopentylamino carboxymethylthiazolylindole; PCK, protein kinase C; RAGE, receptor for advanced glycation end products; ROS, reactive oxygen species; Treg, regulatory T cell.

This article is distributed under The American Association of Immunologists, Inc., Reuse Terms and Conditions for Author Choice articles.

Copyright © 2015 by The American Association of Immunologists, Inc. 0022-1767/15/S25.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1402609
stimulatory molecules (22). Additionally, Th1 polarization of naive T cells is strongly increased by HMGB1 (23). Given its importance in both innate and adaptive immune responses, we postulated that HMGB1 may act as a potent innate immune mediator that may have affects on GVHD.

Cyclopentylamino carbboxymethylthiazoylindole (NecroX) is a class of indole-derived, cell-permeable, antioxidant molecules that exhibit cytoprotective effects in cells acting as a scavenger of reactive oxygen species (ROS). Recently, one member of this group of compounds, NecroX-7, was shown to inhibit formation of mitochondria-specific ROS/reactive nitrogen species in H9C2 cells and hepatocytes after induction by tert-buty1 hydroperoxide or doxorubicin (24, 25). NecroX-7 has also been used as an antidote to acetonaminophen toxicity (26) and exerts a protective effect by preventing the release of HMGB1 in ischemia/reperfusion injury (27). The ability of NecroX-7 to protect cells and cellular components against oxidative stress and HMGB1 provided a rationale for its use in preventing GVHD induction in the setting of allo-genic HSCT.

Here, we investigated the mechanism of action of NecroX-7 in the regulation of GVHD through immune regulation and disease control focusing on HMGB1 blockade. Our results demonstrated that blockade of extracellular HMGB1 by NecroX-7 significantly reduced GVHD-related mortality, with decreased proinflammatory cytokine production and increased numbers of regulatory T cells (Tregs). These results suggest that targeting the upstream molecular mechanisms of GVHD pathogenesis through HMGB1 blockade against ROS in proinflammatory events represents a novel approach to the treatment of GVHD.

Materials and Methods

Materials

NecroX-7 (C9H16N2O4S2; patent no. KR2008-0080519) was provided by LG Life Sciences (Daejeon, Korea). This small molecule has a molecular mass of 516.67 Da. Monochlorobimane was obtained from Molecular Probes (Eugene, OR); all other chemicals used were from Sigma-Aldrich (St. Louis, MO).

Mice

Eight-week-old female BALB/c (recipient, H-2k) and C57BL/6 (donor, H-2k) mice were purchased from OrientBio (Sungnam, Korea). The mice were maintained under specific pathogen-free conditions in an animal facility with a controlled humidity of 55% (6). Mice were housed in an animal facility with a controlled humidity of 55% (6) and were maintained under specific pathogen-free conditions in an animal facility with a controlled humidity of 55% (6). Mixed lymphocyte cultures

The MLCs were prepared in RPMI 1640 medium (Life Technologies) containing 20 mM HEPES, 2 mM L-glutamine, 5% heat-inactivated FBS, 100 mM sodium pyruvate, and penicillin (10 U/ml)-streptomycin (10 g/ml). CD4+ T cells (1 x 10^5) cells/well) were cultured together with 2 x 10^6 irradiated (2000 cGy) H-2k or H-2k splenocytes as stimulators (2 x 10^5 cells/well) in a total volume of 500 ml containing 20 mM HEPES, 2 mML-glutamine, 5% heat-inactivated FBS, 100 mM sodium pyruvate, and penicillin (10 U/ml)-streptomycin (10 g/ml). CD4+ T cells (1 x 10^5) from spleens of C57BL/6 mice were cocultured with irradiated (2000 cGy) H-2k or H-2k splenocytes for 3–4 d with irradiated (2000 cGy) H-2k or H-2k splenocytes as stimulators (2 x 10^5 cells/well) that were treated with or without NecroX-7 in 96-well plates. Cultures were pulsed during the final 12 h with 1 μCi/well thymidine (GE Healthcare, Piscataway, NJ) and harvested using the TopCount harvester. Cell proliferation was determined as the number of counts per minute.

Western blot analysis

Briefly, 500 μl culture media fluid was filtered (Centricon YM-100; Millipore, Billerica, MA) and then concentrated (Centricon YM-30) according to the manufacturer’s specifications. The levels of HMGB1 were determined on Western blots according to standard methods. Briefly, proteins were separated by gel electrophoresis using 12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with polyclonal rabbit antiserum against mouse HMGB1. Mouse HMGB1 has 100% homology to the rat HMGB1 protein. After washing the membranes, they were incubated with a biotinylated antirabbit secondary Ab. The membranes were sequentially incubated with alkaline phosphatase and then dianisobenzidine substrate to visualize the proteins of interest. Band density was estimated by image capture densitometry.

Induction and evaluation of GVHD

BALB/c (H-2k) mice were conditioned with 800 cGy total body irradiation and transplanted with 5 x 10^6 BMCs and 5 x 10^6 spleen cells from either C57BL/6 (H-2k) mice (allo-genic transplantation) or BALB/C (H-2k) mice (syngenic transplantation). The severity of GVHD was assessed using a clinical GVHD scoring system as described previously (28). Briefly, mice were individually scored every week using the following five clinical parameters on a scale from 0 to 2: weight loss, posture, activity, fur, and skin. A clinical GVHD index was generated by summation of the five criteria scores (0–10). Survival was monitored daily. Animals with scores >7 were considered moribund and were euthanized.

Clinicopathological evaluation

Mice were killed at day 14 after BMT for blinded histopathological analysis of GVHD targets (skin, liver, and intestine). Organs were harvested, cryo-embedded or paraffin-embedded, and subsequently sectioned. Tissue sections were fixed in 10% buffered formalin and stained with H&E for histological examination. The scoring system for each parameter denoted 0 as normal, 0.5 as focal and rare, 1 as focal and mild, 2 as diffuse and mild, 3 as diffuse and moderate, and 4 as diffuse and severe, in accordance with previously published GVHD histology.

ROS assay

A solution of 2,7’-dichlorofluorescein diacetate (Sigma-Aldrich) in methanol (Bio-Lab, Jerusalem, Israel) was added at a final concentration of 3 μM to PBS at (~1 x 10^6/ml) in PBS. After a 15-min incubation at 37°C in a humidified atmosphere of 5% CO2 in air, the cells were washed and resuspended in PBS to the original cell concentration. The cells were then either left unstimulated or were stimulated by 10 μM of ROS• for 10 min incubation with 1 mM H2O2, as indicated.

Flow cytometric analysis

Mononuclear cells were immunostained with various combinations of the following fluorescence-conjugated Abs: CD4, CD25, Foxp3, IFN-γ, IL-4, and IL-17. Intracellular markers were stained using the following Abs: IL-4, IFN-γ, IL-17, and Foxp3 (eBioscience, San Diego, CA). Before intracellular cytokine staining, cells were stimulated in culture medium containing PMA (25 ng/ml; Sigma-Aldrich), ionomycin (250 ng/ml; Sigma-Aldrich), and monensin (GolgIStop, 1 μl/ml; BD Biosciences) in an incubator with 5% CO2 at 37°C for 4 h. Intracellular staining was performed using an intracellular staining kit (eBioscience) according to the manufacturer’s protocol. Flow cytometry was performed using a FACSCalibur cytometer (BD Biosciences) and FlowJo software (Tree Star).

Real time RT-PCR

Isolated RNA was reverse transcribed into cDNA using the TLR10-LS reagent (Invitrogen, Carlsbad, CA) for real-time RT-PCR analysis. Quantitative PCR was performed using the FastStart DNA Master SYBR Green I kit and a LightCycler 480 detection system (both from Roche, Meylan, France). As specified by the manufacturer. For quantification, we represent the relative mRNA expression levels of specific genes obtained using the 2^-ΔΔCt method and used the β-actin housekeeping gene for normalization. The following gene-specific primers were used: HMGB1 (forward, 5’-GAT GGC GAA AGG AGA TCC TAA G-3’; reverse, 5’-TCA TT TTT TGT CTC TCC TTT GGG-3’), Foxp3 (forward, 5’-GGC CCT TCT C CA GGA CAG A-3’; reverse, 5’-GCT GAT CAT GCG TGG GTT GTT-3’), IL-10 (forward, 5’-AAC TGA TGC CCC CCG AGA CA-3’; reverse, 5’-TCT CAC CCA GGA AAT TCA AA-3’), and IL-17A (forward, 5’-CTT CAA AGC TCA GCG TGT CC-3’; reverse, 5’-GAG CT C ACT TTG GCG CCA AG-3’).
**Immunofluorescence**

RAW264.7 cells were cultured in monolayers on glass coverslips. Cells were fixed in 4% formaldehyde for 30 min at room temperature prior to detergent extraction with 0.4% Triton X-100 for 20 min at 37˚C. Non-specific binding sites were blocked for 30 min at 37˚C with 10% normal goat serum (Sigma-Aldrich) diluted in PBS and processed for immunofluorescence with rabbit anti-HMGB1 Ab (1:1000) followed by Alexa Fluor 488–conjugated anti-rabbit IgG. Between all incubation steps, the cells were washed three times for 5 min with PBS. The coverslips were mounted on the slides using 50% glycerin. The specimens were analyzed using a confocal microscopy system (LSM 510 Meta; Zeiss, Gottingen, Germany).

**ELISA kits**

Serum was collected and frozen at −80˚C until analysis. Triplicate samples were then subjected to ELISA with an HMGB1 ELISA kit according to the manufacturer’s protocol (IBL International, Hamburg, Germany). The assay was performed as recommended by the producer.

**Statistical analysis**

Data are presented as means ± SD. Data comparisons between more than two groups and between two groups were performed with a Kruskal–Wallis test and a Mann–Whitney U test or Student t test, respectively. To assess the Gaussian distribution and the equality of variance, the Shapiro–Wilk test and Leven test were used, respectively. Statistical analysis was performed using the SPSS statistical software package (standard version 16.0; SPSS, Chicago, IL). A p value of <0.05 was considered significant.

**Results**

NecroX-7 treatment reduces acute GVHD severity in a murine major mismatch model

Novel synthetic compounds based on indole backbone showed antioxidant effects on various toxic stimuli (24, 25). Boxes show the chemical structure of NecroX-7 (Fig. 1A). The efficacy of NecroX-7 in attenuating the course of acute GVHD was evaluated...
using a murine lethal GVHD model with complete MHC-mismatched BMT (C57BL/6 → BALB/c). In this model, lethally irradiated BALB/c (H-2b) recipients received bone marrow cells and spleen cells from C57BL/6 (H-2b) donor mice. Varying doses of NecroX-7 (0.03, 0.1, and 0.3 mg/kg) or PBS were administered as i.v. injections at 2-d intervals for 2 wk (Supplemental Fig. 1), and survival was followed during 50 d. Efficacy was evaluated in at least two experiments for each dose, and the combined results of two representative experiments are shown in Fig. 1. Typically, untreated mice died by days 30–40 in this model. Statistically significant prolonged survival was observed in mice that received NecroX-7 at doses ≥0.1 mg/kg: 30–60% of mice in these treatment groups survived for >50 d. NecroX-7 treatment significantly improved clinical signs (Fig. 1B) and prolonged survival (Fig. 1C), and the mice showed a reduction in clinical manifestations of acute GVHD, including weight loss, hunched posture, diarrhea, and ruffled fur (Fig. 1D, 1E). Survival curves at doses of 0.03, 0.1, and 0.3 mg/kg were comparable overall, and no trend toward dose-related differences was noted within the effective dose range. Thus, 0.3 mg/kg was subsequently used in this study, unless specified otherwise. Next, we examined GVHD pathology in the lung, liver, and intestine at 14 d after allogeneic BMT. GVHD pathological scores were significantly lower in the recipients transplanted with NecroX-7 than in the PBS group (Fig. 1F, 1G). The GVHD group showed severe blunting of villi architecture and inflammatory cell infiltration, whereas NecroX-7–treated recipients showed significant restoration of the intestinal villous architecture with little inflammatory infiltration. Liver histology in the GVHD mice showed lobular inflammation, with more lymphocytes infiltrating liver cell plates (Fig. 1F, arrowheads) compared with the NecroX-7–treated mice. Less lymphocyte infiltration was observed in the skin of NecroX-7–treated mice compared with that of allogeneic controls. In summary, the GVHD pathology scores in each organ were significantly lower in NecroX-7–treated mice than those in control, PBS-treated mice (Fig. 1G). These results indicate that systemic delivery of NecroX-7 after induction of GVHD onset can reduce the inflammatory responses in mouse models.

**NecroX-7 modulates alloreactive T cell responses in vitro**

The findings described above showed that NecroX-7 attenuates GVHD. The mechanism by which NecroX-7 regulates the immune response remains unclear. To clarify this issue, we evaluated the effects of NecroX-7 in allogeneic MLRs, which are relevant to the T cell alloresponse during acute GVHD. We cultured C57BL/6 CD4+ T cells in the presence of irradiated T cell–depleted BALB/c splenocytes and treated the cultures with NecroX-7. As expected, high [%H] thymidine incorporation was observed in the allogeneic setting; after treatment with NecroX-7 there was a marked reduction, in a dose-dependent manner, in splenocyte proliferation compared with no drug (Fig. 2A). Moreover, we performed a CCK-8 assay after the culture period to examine any significant apoptosis and found that T cells were not significantly affected by NecroX-7 at any concentration, including 40 μM (Supplemental Fig. 2). This suggests that NecroX-7 can suppress activated or proliferating T cells without causing apoptosis. We next determined whether the reduction in alloreactive T cells response is associated with decreased HMGB1 by NecroX-7. The concentrations of HMGB1 protein levels in culture supernatants
were measured using Western blot. Treatment with NecroX-7 markedly reduced HMGB1 levels in a dose-dependent manner (Fig. 2B).

**HMGB1 is associated with T cell proliferation during allogeneic responses in vitro**

To determine the impact of exogenous HMGB1 on the proliferative capacity of donor CD4+ T cells in response to alloantigens, T cell alloreactivity in the presence of rHMGB1 was assessed based on the incorporation of \[^{3}H\]thymidine. In vitro rHMGB1 stimulation significantly increased T cell proliferation in the allogeneic stimulation condition (Fig. 2C). The increase in T cell proliferation was associated with enhanced secretion of HMGB1. We next evaluated the effect of NecroX-7 (Fig. 2D). Treatment with NecroX-7 markedly reduced proliferation in a dose-dependent manner.

**FIGURE 3.** Analysis of HMGB1 temporal expressions on acute GVHD after allogeneic BMT. (A) HMGB1 mRNA expression in the splenocytes after BMT. Real-time PCR was performed to determine the mRNA levels of HMGB1. Quantitative analysis of HMGB1 mRNA was normalized with \(\beta\)-actin. (B) Serum levels of HMGB1 were measured by ELISA. HMGB1 levels were significantly lower in the NecroX-7 treatment groups than in the untreated groups. Data are shown as means \(\pm\) SD from at least three independent experiments. *\(p < 0.05\), **\(p < 0.01\).

**FIGURE 4.** Expression of extranuclear HMGB1 protein in the organ tissues. (A–C) HMGB1 translocation in organ tissues after BMT. HMGB1 cellular location was visualized by immunohistochemistry. Brown indicates HMGB1; blue indicates counterstaining of nucleus with hematoxylin; arrows indicate cytoplasmic HMGB1 staining. Original magnification \(\times200\) and \(\times800\). (D) The percentage of immune cells with only cytoplasmic HMGB1 staining out of the total number of tissues was calculated. Representative images from six mice per group were selected. (E) RAGE and TLR4 mRNA expression levels were measured by real-time PCR. Data are shown as means \(\pm\) SD from at least three independent experiments. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\).
manner. These data support the critical role of HMGB1 in the GVHD and that NecroX-7 inhibited the HMGB1–induced allo- geneic T cell proliferation.

Modulating immunostimulatory activities of extracellular HMGB1 for acute GVHD regulation

Next, we performed quantitative real-time PCR (Fig. 3A) and ELISA (Fig. 3B) of HMGB1 in acute GVHD. We observed a time-dependent increase in the HMGB1 mRNA and protein levels in GVHD from day 1 through days 7 and 14 after transplantation. The statistical analysis of HMGB1 concentration in acute allogeneic BMT mice group is almost doubled compared with the syngeneic BMT mice group. These experimental data indicate that the increased HMGB1 level is correlated with the severity of the GVHD. Additionally, the administration of NecroX-7 significantly reduced these levels.

NecroX-7 attenuates acute GVHD-induced systemic HMGB1 accumulation

Additionally, we assessed active HMGB1 secretion by infiltrating immune cells in GVHD-induced mice. The cellular expression of HMGB1 in immunohistochemically stained sections from syngeneic BMT group tissue showed a strictly nuclear pattern. This staining appearance changed dramatically in inflamed tissue during the course of allogeneic BMT. In the allogeneic BMT group, the

FIGURE 5. Effects of NecroX-7 on H2O2-induced HMGB1 translocation and release in RAW264.7 cells. RAW264.7 macrophages were pretreated for 30 min with NecroX-7 at the indicated concentrations before stimulation with H2O2 for 12 h and (A) monitored for HMGB1 cytoplasmic translocation by confocal laser immunofluorescence analysis. Blue, nuclei; green, HMGB1. Original magnification ×1000. (B) The levels of HMGB1 in the culture media were determined by ELISAs and are expressed as the mean of two experiments in duplicate. (C) The CCK-8 assay data are shown at the maximum treatment concentration of NecroX-7 using RAW264.7 cells. (D) Representative confocal laser immunofluorescence photomicrography of RAW264.7 cells showed the localization of mitochondrial ROS in the cells. Blue, nuclei; red, MitoTracker CM-H2ROS. Original magnification ×1000. (E) PKC phosphorylation was measured in whole-cell lysates by Western blot analysis using antiphosphorylated panPKC Ab. The cytoplasmic housekeeping protein actin was used as a loading control. Data are shown as the means ± SEM; results are representative of three independent experiments. *p < 0.05, **p < 0.01.
expression of HMGB1 was markedly higher and was primarily confined to the cytoplasm of the tissue and extracellular milieu. However, in the NecroX-7–treated group, cytoplasmic HMGB1 release was clearly reduced or absent (Fig. 4A–C). The percentage of immune cells with only cytoplasmic HMGB1 staining among the total number of immune cells was calculated (Fig. 4D). The relative proportion of immune cells with HMGB1 translocation in NecroX-7–treated mice was significantly lower than that in untreated mice. Additionally, NecroX-7 decreased the mRNA levels of TLR4 and receptor for advanced glycation end products (RAGE) (Fig. 4E), but it did not affect those of TLR2 (data not shown). These results demonstrate that NecroX-7 confers protection against lethal acute GVHD by attenuating systemic HMGB1 accumulation.

*NecroX-7 influenced the in vitro secretion of extracellular HMGB1 significantly*

HMGB1 secretion involves translocation from the nucleus to the cytoplasm as the first step (29). Accordingly, we evaluated the effects of NecroX-7 on oxidative stress-induced HMGB1 cytoplasmic translocation. To investigate whether treatment with NecroX-7 affected the translocation of nuclear HMGB1 from macrophages, RAW264.7 cells were pretreated for 30 min with NecroX-7 and then stimulated with H2O2 at a nontoxic dose (125 μM) for 12 h. Confocal microscopy showed that the cytoplasmic HMGB1 levels were much higher in H2O2-stimulated cells than in unstimulated cells. However, treatment with NecroX-7 significantly reduced the cytoplasmic levels of HMGB1 (Fig. 5A). Furthermore, we observed a significant increase in secreted HMGB1 in the H2O2-stimulated culture medium; however, this amount was significantly reduced following treatment with NecroX-7 for 20 h in RAW264.7 cells (Fig. 5B). These results suggest that NecroX-7 can significantly inhibit the in vitro secretion of extracellular HMGB1. Our CCK-8 assay data for NecroX-7 and the background level of HMGB1 secretion indicate that these results were not due to the NecroX-7–induced necrosis of RAW264.7 cells. RAW264.7 cell viability was not significantly affected by treatment with NecroX-7 at any concentration, including 80 μM (Fig. 5C).

*NecroX-7 inhibits HMGB1 secretion by suppressing the mitochondrial ROS and protein kinase C pathways*

ROS are signaling molecules most important in several pathways to accelerate HMGB1 release (30, 31). Based on these associations, we next investigated a possible connection between mitochondrial ROS production and HMGB1 translocation by measuring the mitochondrial ROS level in H2O2-stimulated RAW264.7 cells. We assessed mitochondrial ROS levels using CM-H2ROS staining and found a significant increase in mitochondrial ROS after H2O2 stimulation. However, treatment with NecroX-7 significantly reduced the levels of mitochondrial ROS (Fig. 5D). The posttranslational modification of HMGB1 by phospho–protein kinase C (PKC) is a significant mechanism leading to HMGB1 secretion (32). We next evaluated the effect of NecroX-7 on activation of the mitochondrial PKC pathway.

**FIGURE 6.** NecroX-7 treatment decreases inflammatory mediators in GVHD mice. (A) Two weeks after BMT, blood cells were stained with 2',7'-dichlorofluorescein diacetate (DCF) and analyzed for ROS by flow cytometry. Gray histogram lines represent the syngeneic transplantation group, black histogram lines the untreated treated GVHD group, and gray filled histogram lines represent the NecroX-7–treated group. (B) The data are mean fluorescence intensities (MFI) measured using flow cytometry at days 1, 7, and 14 after BMT. The experiment was performed once with six mice per group. (C and D) Splenic T cells from each recipient mouse were collected at each indicated time point. The expression levels of TNF-α and IL-6 in spleen cells obtained from each mouse were determined by flow cytometry. *p < 0.05, **p < 0.01, ***p < 0.0001.
PKC signaling pathway by Western blot analysis. The level of phospho-PKC in NecroX-7–pretreated cells was significantly lower than that in H2O2-stimulated cells at the same time points (Fig. 5E). These results suggest that NecroX-7 can inhibit HMGB1 secretion by suppressing the mitochondrial ROS and PKC pathways.

Effects of NecroX-7 on ROS production and proinflammatory responses in acute GVHD

Next, we evaluated ROS production in PBMCs after allogeneic BMT. The levels of ROS production cells were decreased in the NecroX-7–treated recipients compared with untreated recipients on day 1 through days 7 and 14 after BMT (Fig. 6A, 6B). Moreover, we compared splenic TNF-α (Fig. 6C) and IL-6 (Fig. 6D) levels of recipients after BMT. The levels of these proinflammatory cytokines in NecroX-7–treated recipients were significantly lower than those in untreated recipients after BMT. These finding indicate that the protective effect of NecroX-7 is mediated by suppression of ROS production and inflammatory cytokine production.

Effects of NecroX-7 on T cell polarization in acute GVHD after allogeneic BMT

We investigated the effect of NecroX-7 treatment on allogeneic T cell polarization. The proportions of CD4+IFN-γ+, CD4+IL-4+ (Fig.7A), CD4+IL-17+ (Fig. 7B), and CD4+CD25+Foxp3+ T cells (Fig. 7C) in spleens from each group were determined by FACS. The proportions of CD4+IFN-γ+ and CD4+IL-17+ T cells in spleens were decreased in NecroX-7–treated GVHD animals compared with the untreated group. Conversely, the proportions of CD4+Foxp3+ splenocytes were increased, and CD4+IL-4+ cells had no significant difference in the NecroX-7–treated group. Additionally, the mRNA expression of Foxp3 and IL-10 increased, whereas IL-17 expression was reduced in the NecroX-7–treated mice compared with the untreated GVHD mice (Fig. 7D). The serum levels of cytokine IFN-r and IL-17 were also decreased in the NecroX-7–treated GVHD mice (Fig. 7E).

Discussion

The immune reaction in GVHD has traditionally been thought to largely involve adaptive immunity. However, according to recent studies, innate immunity also exerts an important effect on the pathogenesis of GVHD (9, 10). Excessive conditioning regimens such as total body irradiation and chemotherapy damage host tissue, and the damaged cells secrete danger signals, including cytokines and DAMPs. These trigger or intensify GVHD and influence adaptive immunity by affecting DC activation and immune cells, such as alloreactive T cells (33). Thus, it is necessary to understand the role played by innate immunity in GVHD and develop new treatment methods that target these factors.
NecroX-7, a mitochondria targeting necrosis inhibitor (27), has recently been approved by the Ministry of Food and Drug Safety (formerly the Korean Food and Drug Administration) to demonstrate the safety and pharmacokinetics of i.v. single injection immediately before percutaneous coronary intervention in patients with ST-segment elevation myocardial infarction (https://clinicaltrials.gov, identifier NCT02070471). We expect the effect of NecroX-7 to be blockage of upstream events in early stages of GVHD and designed this study to evaluate these effects. To our knowledge, this is the first study to investigate the effects of NecroX-7 on the severity of GVHD using in vivo and in vitro assays.

NecroX-7 diminished GVHD severity scores, which was associated with inhibition of HMGB1 release in vivo. After demonstrating these effects we investigated the potential mechanisms of action of NecroX-7. HMGB1 is a DNA-binding nuclear protein that acts as a transcription factor (11). However, intranuclear HMGB1 released by immune cells and necrotic cells damaged by TNF-α, IL-6, IFN-γ, and ROS (30, 34, 35) acts as a DAMP that accelerates immune and inflammatory responses, activates innate immunity, and acts as a mediator of various pathological conditions such as allograft acute rejection (36), sepsis syndromes (37), autoimmune diseases (14), and type 1 diabetes (38). Additionally, evidence from studies in allogeneic hematopoietic cell transplantation patients indicates that HMGB1 is also associated with successful transplant outcomes (39). Thus, HMGB1 is a potential molecular target for GVHD therapies. However, the exact role of HMGB1 in GVHD, particularly its relationship with alloimmune reactions, has not been reported. To evaluate the role of HMGB1 in GVHD, we investigated its release by host tissue after syngeneic and allogeneic transplantation, in the presence of other differences between the syngeneic and allogeneic transplantation cohorts (Fig. 3). We found that after allogeneic stimulation, nuclear HMGB1 in host tissue is translocated to the cytoplasm and secreted in a time-dependent manner. In this study, we show that HMGB1 is secreted by host tissues of GVHD models. Hence, we suggest that HMGB1 can be considered a novel marker of host tissue inflammation with important implications for the pathogenesis of GVHD.

Interestingly, NecroX-7 attenuated allogeneic BMT-induced systemic HMGB1 secretion (Fig. 4) and dose-dependently inhibited alloresponse-induced HMGB1 release in host APCs and donor T cell cocultures (Fig. 2D). We also performed CCK-8 assays to determine whether NecroX-7 treatment affected cell viability. NecroX-7 treatment at concentrations up to 40 μM did not induce apoptosis or affect the viability of murine splenocytes. Additionally, when released into the extracellular environment, HMGB1 can bind to cell-surface receptors, including RAGE and TLR4 (31). HMGB1 interaction with these receptors promotes intracellular signaling and mediates cellular responses, including activation of immature DCs and enhancement of the production of proinflammatory cytokines such as TNF-α and IL-6 (40). In mice with GVHD, NecroX-7 treatment restored the upregulated TLR4 and RAGE expression (Fig. 4E) and normalized the levels of TNF-α and IL-6 in splenocytes (Fig. 6C, 6D). We clearly showed that NecroX-7 protected mice with GVHD from death through the reduction of HMGB1 and the subsequent HMGB1-mediated inflammatory response.

Recent studies have clearly shown that it is important to control the balance between Th1 cells and Tregs during regulation of GVHD (41–43). Various drugs have shown Th1 suppression and Treg increase as an indicator of efficacy. Furthermore, there are studies where Tregs are directly injected to supply additional Tregs and induce endogenous Tregs (44, 45). NecroX-7 suppressed GVHD and inhibited the secretion of IFN-γ and IL-17 by Th1 and Th17 cells, as well as increased the number of Foxp3+ Tregs. These results confirm the direct effects of NecroX-7 in the GVHD model and in vitro. After administering NecroX-7 under the differentiation conditions of T cells that react to alloreactive cells, Treg expression was increased in a dose-dependent manner and IFN-γ expression was markedly suppressed. These immunoregulatory effects of NecroX-7 are a mechanism of GVHD suppression; this is a novel finding.

In summary, our results demonstrate that NecroX-7 has a therapeutic effect in an animal model of GVHD, and that this effect is mediated by reducing HMGB1 release. Additionally, NecroX-7 inhibited HMGB1-induced release of TNF and IL-6, as well as the expression of TLR4 and RAGE. In vivo, NecroX-7 improved animal survival in a mouse model of GVHD. More specifically, NecroX-7 increased HMGB1 in Tregs independently through direct regulation of differentiation signals. Taken together, these experimental data indicate that NecroX-7 protects mice against lethal GVHD by reciprocal regulation of Tregs/Th1 cells and attenuation of systemic HMGB1 accumulation, which are mediated in part by inhibition of the HMGB1-mediated inflammatory response. Our results indicate the possibility of using NecroX-7 for effective treatment of GVHD.

Acknowledgments
We thank Prof. Christopher Contag at Stanford University for helpful comments and critical review of the manuscript.

Disclosures
The authors have no financial conflicts of interest.

References

29. Gardella, S., C. Andrei, D. Ferrera, L. V. Lotti, M. E. Bianchi, and

28. Lim, J. Y., M. J. Park, K. I. Im, N. Kim, E. J. Jeon, E. J. Kim, M. L. Cho, and


26. Park, J. H., K. S. Seo, S. Tadi, B. H. Ahn, J. U. Lee, J. Y. Heo, J. Han,


**Supplemental Figure 1. Graft-versus-host disease (GVHD) Induction.** Schema of the experiments. BALB/c (H-2<sup>d</sup>) mice were administered 800 cGy of total body irradiation and transplants of $5 \times 10^6$ whole BM cells and $5 \times 10^6$ spleen cells from allo C57BL/6 (H-2<sup>b</sup>) donors. Syngeneic recipients (H-2<sup>d</sup>) received similar transplants with cells from BALB/c (H-2<sup>d</sup>) mice. Recipient were administered NecroX-7 by intravenous injections at 2-day intervals from 1 day before BMT to 14 days after BMT. The mouse groups were administered either 0.03 mg/kg NecroX-7, 0.1 mg/kg NecroX-7, 0.3 mg/kg NecroX-7, or PBS.
Supplemental Figure 2. The inhibitory effect of NecroX-7 on alloreactive T cell responses is not associated with apoptosis induction or decreased cell viability. (A) Cell apoptosis analyzed by flow cytometry. The lower left Annexin-V−/propidium iodide (PI)− represents normal healthy cells. The lower right Annexin-V+/PI− and upper right Annexin-V+/PI+ quadrant represent early and later apoptotic cells, respectively. The upper left quadrant, Annexin-V−/PI+ represent necrotic cells. (B) Cell viability as evaluated with the CCK-8 assay. Values of CCK-8 assay on cell viability after the treatment with NecroX-7. Bars are shown as means ± SEM from at least 3 independent experiments.