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Glycyrrhizin protects γ-irradiated mice from gut bacteria-associated infectious complications by improving miR-222-associated *Gas5* RNA reduction in macrophages of the bacterial translocation site

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Abstract

Gut bacteria-associated sepsis is a serious concern in patients with gastrointestinal acute radiation syndrome (GIARS). In our previous studies, gut bacteria-associated sepsis caused high mortality rates in mice exposed to 6–9 Gy of γ-rays (6–9 Gy GIARS-mice). IL-12^{+}CD38^{+} iNOS^{+} Mϕ (M1Mϕ) located in the bacterial translocation site (mesenteric lymph nodes, MLNs) of unirradiated mice were characterized as host defense antibacterial effector cells. However, cells isolated from the MLNs of GIARS-mice were mostly CCL1^{+}IL-10^{+}LIGHT^{+}miR-27a^{+} Mϕ (M2bMϕ, inhibitor cells for the M1Mϕ polarization). Reduced long noncoding RNA *Gas5* and increased *miR-222* expression in MLN-Mϕ influenced by the irradiation were shown to be associated with M2bMϕ polarization. In this study, the mortality of 7 Gy GIARS-mice was completely controlled after the administration of glycyrrhizin (GL), a major active ingredient in licorice root (*Glycyrrhiza glabra*). Bacterial translocation and subsequent sepsis were minimal in 7 Gy GIARS-mice treated with GL. Increased *Gas5* RNA level and decreased *miR-222* expression were shown in MLN-Mϕ isolated from 7 Gy GIARS-mice treated with GL, and these Mϕ did not display any properties of M2bMϕ. These results indicate that gut bacteria-associated sepsis in 7 Gy GIARS-mice was controlled by the GL through the inhibition of M2bMϕ polarization at the bacteria translocation site. Expression of *Ccl1*, a gene required for M2bMϕ survival, is silenced in the MLNs of 7 Gy GIARS-mice due to the *Gas5* RNA, which is increased in these cells after the suppression of *miR-222* (a *Gas5* RNA expression inhibitor) by the GL.

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Introduction

High mortality rates associated with gastrointestinal acute radiation syndrome (GIARS) are a serious concern in victims exposed to 6–15 Gy of γ-rays (1–4). In our previous studies (5–7), the mortality of mice exposed to 6–8 Gy of whole body γ-irradiation was associated with sepsis caused by bacterial translocation. Generally, gut bacteria-associated infectious complications do not develop in healthy individuals (5–7), because pathogens that invade from the gastrointestinal tracts are rapidly eliminated by the host defense effector cells at the bacterial translocation sites (mesenteric lymph nodes, MLNs, and lamina propria, LP) (3). M1Mϕ (IL-12$^+$CD38$^+$iNOS$^+$ Mϕ) have been identified as major host defense antibacterial effector cells against bacterial translocation (5–7). However, M2bMϕ (CCL1$^+$IL-10$^+$LIGHT $^+$miR-27a$^+$ Mϕ), that appear in association with irradiation at the bacterial translocation sites, suppress the M1Mϕ polarization (5). As such, GIARS-mice are much more susceptible to bacterial translocation and subsequent sepsis due to their weakened and/or lack of antibacterial innate immunity.

Molecular mechanisms and signaling pathways involved in the phenotypic polarization of Mϕ have been well-described (8–10). Recently, we have reported (11) that decreased expression of noncoding RNA *Gas5* is linked to M2bMϕ polarization influenced by LPS and immune complex or IL-1β. The expression of *Gas5* RNA level is shown to be minimal in MLN-Mϕ isolated from GIARS-mice (6). M1Mϕ are readily obtained by antigen stimulation from MLN-Mϕ isolated from GIARS-mice transduced with the *Gas5* gene using lentiviral vector (Gas5 lentivirus) (6). Also, the MLN-Mϕ isolated from GIARS-mice transduced with *Gas5* gene did not polarize to M2bMϕ again, even though they were stimulated with a combination of LPS and immune complex (6). *Ccl1* gene transcription is silenced by *Gas5* (12), and CCL1 has been well-known to be an essential chemokine required for M2bMϕ survival (13).

The effects of glycyrrhizin (GL) on various opportunistic infections have been well-described (14–20). GL is an extract from licorice roots with a structure of 20β-carboxy-11-oxo-30-norolean-12-en-3β-yl-2-O-β-D-glucopyranuronosyl-α-D-glucopyranosiduronic acid (21). GL has been used clinically for over 40 years in patients with chronic hepatitis in Japan (22, 23). Through the induction of anti-suppressor cells or inhibition of CCL2 production, GL suppresses M2a/cMϕ polarization (24, 25, 20), myeloid-derived suppressor cell function (18, 19), and Th2 cell generation (14–17, 26, 27). Through the modulation of these suppressor cell polarization, GL protects severely burned mice from infections stemming from *Candida albicans* (15, 16), S*taphylococcus aureus* (20), and *Pseudomonas aeruginosa* (18, 19, 20). Through the induction of IFN-γ, GL protects healthy individuals infected with the hepatitis virus (22, 28) and influenza virus (29). Also, the antitumor activities of GL have been well-described (24, 30–32). GL suppresses the growth of Meth A solid tumor (24) and B16 melanoma (30) through the stimulation of IFN-γ-associated host antitumor immunities. Furthermore, various immunomodulating activities of GL have been reported including inhibition of inflammation (33, 34), augmentation of NK cell activity (35), induction of antimicrobial peptide production by keratinocytes (18, 19, 36), and induction of IL-12, CCL3 and CCL5 from Mϕ and/or T cells (17, 20, 37).

In the current study, the effect of GL on the mortality of 7 Gy GIARS-mice with gut bacteria-associated sepsis was investigated. Bacterial translocation and subsequent sepsis were demonstrated in 7 Gy GIARS-mice starting one week after irradiation, and all of these mice died within 3 weeks of irradiation. However, the mortality rates of GIARS-mice treated with GL were dramatically reduced. GL reduced the mortality of 7 Gy GIARS-mice by controlling the M2bMϕ polarization in the bacterial translocation site. Host antibacterial effector cells against bacterial translocation and subsequent sepsis have been identified to be M1Mϕ (5, 7), and M2bMϕ have been characterized as inhibitor cells for the M1Mϕ polarization (38). Decreased expression of *Gas5* RNA, a silencer of *Ccl1* gene transcription (39), was restored in MLN-Mϕ of 7 Gy GIARS-mice treated with GL. CCL1 is an essential chemokine for M2bMϕ survival (13). The MLN-Mϕ from 7 Gy GIARS-mice treated with GL were shown to be non-M2bMϕ, and hence had the ability to easily polarize to M1Mϕ with antigen stimulation. A decrease in *Gas5* RNA expression in MLN-Mϕ from GIARS-mice was shown to be associated with a high expression of *miR-222* in the same Mϕ, but a high level of *miR-222* expression was not demonstrated in MLN-Mϕ of GIARS-mice treated with GL. These results indicate that GL protects 7 Gy GIARS-mice from bacterial translocation and subsequent sepsis through the modulation of M2bMϕ polarization in the bacterial translocation site by inhibiting *miR-222* expression followed by the restoration of the decreased expression of *Gas5* RNA.

Materials and Methods

Mice and γ-irradiation

BALB/c mice (9- to 12-wk-old, specific pathogen-free) were purchased from The Jackson Laboratory (Bar Harbor, ME). Data shown in survival experiments in Figure 1A are representative of two independent experiments using 44 male mice (10–12 mice per group), and data shown in the others are mean ± SD from three independent experiments performed with male mice (two experiments) and female mice (one experiment) consisting 6 to 9 mice per group. These mice were exposed to 7 Gy of whole body irradiation with a ^{137}Cs-ray (0.662 MeV) irradiator (Mark I Model 30; J.L. Shepherd & Associates, San Fernando, CA) at a dose rate of 1.05 Gy/min, which was reduced from the 5.08-Gy/min via lead attenuators. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch at Galveston (Institutional Animal Care and Use Committee approval #1305020).

Reagents and media

Advanced DMEM/F12 medium, murine CCL1 ELISA kit, TRIzol reagent, Ambion mirVana miRNA Isolation Kit, PrimeScript RT reagent kit, streptavidin magnetic beads, PEG-it Virus Precipitation Solution, Lipofectamine 2000 and RNAi MAX transfection reagents, *miR-222* mimic, negative control (NC) miRNA, and TaqMan microRNA probes for *miR-222* and *miR-361* quantification were purchased from Thermo Fisher Scientific (Waltham, MA). iTaq Universal SYBR Green Supermix was purchased from Bio-Rad Laboratories (Hercules, CA). Biotin-conjugated anti-mouse F4/80 antibody was obtained from eBioscience (San Diego, CA). MagCollect buffer was purchased from R&D Systems (Minneapolis, MN). Anti-Ly6G antibody was purchased from Biolegend (San Diego, CA). Recombinant murine

M-CSF was purchased from PeproTech (Rocky Hill, NJ). RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GE Healthcare Life Sciences, Pittsburgh, PA) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Gibco) (complete medium) was utilized for a cultivation of Mϕ. NMDI14, murine IgG, and LPS were purchased from Sigma-Aldrich (St. Louis, MO).

Glycyrrhizin (GL)

GL (ammonium salt) was supplied from Minophagen Pharmaceutical Co., Ltd, Tokyo, Japan. GL was dissolved in 5% EtOH containing saline or serum free medium (10 mg/mL), and diluted with saline at appropriate concentrations, and 0.2 mL of the solution was administered intraperitoneally (i.p.) to 7 Gy GIARS-mice. Final concentration of EtOH was less than 0.1%. In our early studies (29), the effect of various doses of GL on influenza A_2 virus infection in mice was examined. The protective effect of GL was demonstrated when infected mice were treated with greater than 2.5 mg of the compound per kg. This effect was dose dependent and observed with doses ranging from 2.5 to 10 mg/kg. The maximum protection of mice exposed to the virus was shown when 10 mg/kg or more doses of GL were administered. Similar dose-dependent protective/therapeutic effects of GL have been demonstrated in various immunocompromised mice infected with *Candida albicans* (15, 16) and *Pseudomonas aeruginosa* (18, 19). In these experiments, GL protected the infected mice through the modulation of impaired production of IL-12 (17) and IFN-γ (15) or excessive CCL2 production (25, 27). Based on our accumulated results, in this study, a 10 mg/kg dose of GL was administered to 7 Gy GIARS-mice.

Preparation of MLN-Mϕ and bone marrow-derived Mϕ

In the majority of experiments, Mϕ ($F4/80^+$ cells) were prepared from the MLNs of 7 Gy GIARS-mice 10 days after γ-irradiation. As previously described (5–7), single-cell suspensions were obtained by gently pressing MLNs in PBS supplemented with 2% FBS using a cell strainer, adjusted to 5×10^6 cells/mL in MagCollect buffer, and added with biotin-labeled anti-F4/80 antibody. Fifteen min after the incubation on ice, cells were washed twice, resuspended with the cold same buffer, and added with streptavidin-coated magnetic beads. Mϕ were separated from the cell suspension by a positive selection technique. A Mϕ-enriched population (>97% pure as $F4/80^+$ cells) was consistently obtained using this technique. In some experiments, MLN-Mϕ were cultured in complete medium supplemented with 10 μg/mL of GL for 24 h. For the functional analysis of *miR-222*, MLN-Mϕ (5×10^5 cells/mL) were transfected with *miR-222* mimic or control miRNA using Lipofectamine RNAi MAX according to the manufacturer's protocol (reverse transfection method).

Bone marrow-derived Mϕ were prepared as previously described (11). Briefly, bone marrow cells were isolated from mouse femoral and tibial bones by flushing the marrow out with PBS. Then, these cells (1×10^5 cells/mL) were cultured for 7 days in advanced DMEM/F12 medium supplemented with 10% FBS and 25 ng/mL M-CSF on a 6-cm petri dish. The medium was changed every 2 days during the cultivation. The purity of $F4/80^+$ cells 7 days after the cultivation was routinely more than 98%. For the M(LPS+IC) preparation, bone marrow-derived Mϕ (5×10^5 cells/mL) were harvested and recultured for 3 days in media

supplemented with 100 ng/mL of LPS on culture plates, previously coated with 100 μg/mL of murine IgG for 2 h at 37°C (38). These Mϕ preparations were harvested, and total RNAs extracted from these cells were assayed for the gene expression by real-time PCR as described below.

Preparation of lentiviral vector expressing Gas5 (Gas5 lentivirus)

Murine *Gas5* cDNA was amplified from pCMV-Sport6-Gas5 plasmid and cloned into pLenti7.3/V5-TOPO vector (pLenti7.3-Gas5). Lentiviruses were prepared using HEK293FT cells as described in the manufacturer's protocol. In brief, 3 μg of pLenti-Gas5 vector and 9 μg of packaging mix were cotransfected into HEK293FT cells using a transfection reagent Lipofectamine 2000. Seventy-two hours after transfection, supernatants were filtered (0.45-μm filter). To concentrate produced viruses in the supernatants, PEG-it Virus Precipitation Solution was added to every 4 volumes of the supernatants, and the mixture was incubated for 16 h at 4°C. Virus in the mixture was precipitated by centrifugation at 1500 x g for 30 min, resuspended in PBS, and stored at −80°C until further use. For the titration of the viruses, the suspension was serially diluted with DMEM supplemented with 10% FBS, and added to HEK293FT cells. Forty-eight hours after the cultivation, GFP^+ cells were counted by flow cytometry, which was expressed from pLenti7.3/V5-TOPO vector. The titer (transducing units/mL) were calculated as following formula: TU/mL = (% GFP^+ cells/100 x total number of cells in the well) / culture volume (mL) x dilution factor. Mock viruses were generated by the same procedure using otherwise identical vector lacking *Gas5* cDNA (negative control lentivirus, NC lentivirus).

Gene expression analyses

For the gene expression analysis, the total RNA was extracted from MLN-Mϕ with TRIzol reagent according to the manufacturer's instruction. The cDNAs were synthesized with SuperScript III First-Strand Synthesis System. Quantitative real-time PCR was performed using an iTaq Universal SYBR Green Supermix on a ViiA 7 Real-Time PCR System (Thermo Fisher Scientific) with the specific primer pairs to murine *Ccl1, Tnfsf14* (LIGHT), *Gas5* and *Gapdh*, as listed in Supplementary Table 1. The expression levels of *Ccl1* and *Gas5* were normalized to that of housekeeping gene *Gapdh*. For a quantification of *miR-222*, the total RNA was extracted from the same cells using Ambion mirVana miRNA Isolation Kit. The expression levels of *miR-222* and *miR-361* were quantified using TaqMan microRNA Assay. The kit uses gene-specific stem-loop reverse transcription primers and TaqMan probes to detect mature microRNA transcripts. PCR reaction was carried out on the ViiA 7 Real-Time PCR System. The expression levels of *miR-222* were normalized to that of *miR-361*.

Statistical analyses

Data were summarized as mean ± SD. Results were statistically analyzed by a Student *t* test or a one-way ANOVA. Survival curves were compared using a log-rank test. Differences were considered significant at the 0.05 level of significance.

Results

Effect of GL on the mortality of GIARS-mice

All GIARS-mice treated with saline died within 3 weeks of 7 Gy γ-irradiation (7 Gy GIARS-mice). When GL (10 mg/kg) was administered i.p. to these mice 1, 3, 5, 7, and 9 days after exposure to 7 Gy of γ-rays, all of them survived for more than 30 days after the irradiation (Fig. 1A). The same protective effect of GL on 7 Gy GIARS-mice was demonstrated when it was administered only on days 7 and 9 post-irradiation. When treated only on day 9 post-irradiation, 70% of 7 Gy GIARS-mice survived (Fig. 1A). In our previous studies (6), the mortality of 7 Gy GIARS-mice was associated with infectious complications stemming from gut microbiota. Therefore, we next examined the bacterial growth in the primary bacterial translocation site (MLNs) and other organs (liver, spleen, and kidneys) in 7 Gy GIARS-mice treated with or without GL. As shown in Figure 1B, progressive growth of bacteria was shown in all tested organs of GIARS-mice 8–12 days post-irradiation. However, the pathogen did not grow significantly in these organs of GIARS-mice treated with GL 7 and 9 days post-irradiation (Fig. 1B). These results indicate that bacterial translocation and subsequent sepsis do not develop in 7 Gy GIARS-mice treated with GL.

Suppressive effect of GL on the M2bMϕ polarization in the MLNs of 7 Gy GIARS-mice

MLN-Mϕ isolated from GIARS-mice have been previously characterized as M2bMϕ, which have a function to suppress the polarization of M1Mϕ (a major host defense antibacterial effector cell against bacterial translocation and subsequent sepsis) (5, 6). Therefore, the effect of GL on the M2bMϕ polarization in the MLNs of 7 Gy GIARS-mice was examined. $CCL1^{+}IL\text{-}10^{+}LIGHT^{+}miR\text{-}27a^{+}$ Mϕ (M2bMϕ) were detected in the MLNs of these mice 10 days post-irradiation. However, M2bMϕ were not isolated from the MLNs of these GIARS-mice treated with GL. $CCL1^{-}IL\text{-}10^{-}LIGHT^{-}$*miR-27a*$^{-}$ cells (non-M2b phenotype) were isolated from the MLNs of GIARS-mice treated with GL (Fig. 2A–C).

Effect of GL on Gas5 RNA expression in MLN-Mϕ of 7 Gy GIARS-mice

The importance of *Gas5* RNA for avoiding the M2bMϕ polarization has been described in our previous paper (11). *Gas5* is a long noncoding RNA with a function to silence *Ccl1* gene transcription (12), and CCL1 is an essential cytokine for the M2bMϕ survival (13). *Gas5* was consistently expressed in the MLN-Mϕ of normal mice, while it was not in the MLN-Mϕ of GIARS-mice. When 7 Gy GIARS-mice were treated with GL, the *Gas5* RNA level in their MLN-Mϕ was restored to a normal level (Fig. 3A). Similar effect of GL on *Gas5* RNA expression was demonstrated in cultures of MLN-Mϕ from 7 Gy GIARS-mice. Thus, MLN-Mϕ of 7 Gy GIARS mice, treated with 10 μg/mL of GL, expressed *Gas5* RNA at the level shown in normal mouse MLN-Mϕ (Fig. 3B). This effect of GL was also seen in M2bMϕ generated from bone marrow-derived Mϕ stimulated with LPS and immune complex in combination [M(LPS+IC)]. M(LPS+IC) have been described as a standard M2bMϕ preparation (38). The expression of *Ccl1* mRNA (Fig. 3C) and production of CCL1 (Fig. 3D) were markedly decreased in M(LPS+IC) 24 h after cultivation with 10 μg/mL of GL. Similarly, mRNA expression and production of CCL1 were decreased in M(LPS+IC) transduced with the *Gas5* gene via a lentiviral vector (*Gas5* lentivirus).

Reduction of miR-222 expression in MLN-Mϕ of 7 Gy GIARS-mice treated with GL

Because *miR-222* is known to directly bind to *Gas5* RNA and inhibit this RNA expression (39), we focused on this miRNA expression in MLN-Mϕ from 7 Gy GIARS-mice treated with or without GL. *miR-222* was increasingly expressed in MLN-Mϕ of GIARS-mice 1 to 15 days after the γ-irradiation (Fig. 4A). When GL was administered to 7 Gy GIARS-mice 7 and 9 days post-irradiation, *miR-222* expression dropped to the level shown in normal mouse MLN-Mϕ (Fig. 4A). Similar effect of GL on *miR-222* expression was demonstrated in cultures of MLN-Mϕ isolated from 7 Gy GIARS-mice 10 days post-irradiation (Fig. 4B, C). *Gas5* RNA reduction was shown to be mediated by *miR-222*, as evident from the *Gas5* RNA reduction seen in MLN-Mϕ from normal mice after the transfection of *miR-222* (Fig. 4D). The results shown in Fig 4A to 4D indicate that GL improves *Gas5* RNA expression in MLN-Mϕ of 7 Gy GIARS-mice through the reduction of *miR-222* expression.

Discussion

Although there are some measures of preventing mortality of victims with hematopoietic acute radiation syndrome exposed to 2–5 Gy of γ-ray, similar measures are less effective in preventing mortality of victims with gastrointestinal acute radiation syndrome (GIARS) exposed to 6–15 Gy of γ-rays (1–4). Thus, there is an urgent need for innovative strategies to treat GIARS patients. In our previous paper (6), the mortality of mice exposed to 6–9 Gy of γ-rays (6–9 Gy GIARS-mice) was shown to be associated with infectious complications stemming from gut microbiota translocation. Also, we have demonstrated that M1Mϕ located in the bacterial translocation sites (MLNs and lamina propria) of unirradiated mice are characterized as a major host defense antibacterial effector cell. However, Mϕ isolated from the MLNs of GIARS-mice were shown to be mostly M2bMϕ (inhibitor cells for M1Mϕ polarization) (5). Reduced *Gas5* expression and increased *miR-222* expression in the MLN-Mϕ of 6–9 Gy GIARS-mice were shown to be associated with M2bMϕ polarization (6). In the present study, the effect of GL on the mortality of mice exposed to 7 Gy of γ-rays (7 Gy GIARS-mice) was investigated. Furthermore, to make clear the mode of action of GL, the effects of the compound on M2bMϕ polarization in the MLNs of these mice was studied. Bacterial translocation from the gut to the MLNs was observed in 7 Gy GIARS-mice one week after irradiation, and all of these mice died within 3 weeks of irradiation. However, all of the 7 Gy GIARS-mice treated twice with GL, 7 and 9 days post-irradiation, survived. The growth of bacteria in the MLNs and other organs was minimal in these mice treated with GL. M1Mϕ (IL-12$^+$CD38$^+$ iNOS$^+$ Mϕ) in the bacterial translocation site (from the intestinal tracts to MLNs) have been described as major host defense antibacterial effector cells against invading pathogens (5–7). However, M2bMϕ (IL-10$^+$LIGHT$^+$CCL1$^+$ *miR-27a*$^+$ Mϕ) were predominant in the MLNs of 7 Gy GIARS-mice, due to the M1Mϕ polarization being suppressed by M2bMϕ (5, 6). As shown in Figure 2, GL was shown to be an inhibitor of M2bMϕ polarization. After GL treatment, MLN-Mϕ isolated from 7 Gy GIARS-mice did not show any properties of M2bMϕ. Also, MLN-Mϕ isolated from GL-treated GIARS-mice easily polarized to M1Mϕ with bacterial antigen stimulation (data not shown). On the other hand, GL was not effective in controlling the mortality for GIARS-mice exposed to 10 or more Gy (data not shown), is an indication that for severe intestinal damage induced by high doses of γ-rays, GL treatment may need to be modified to account for the higher dosage of

radiation. In fact, 10 or more Gy of γ-rays suppress the self-renewing function of intestinal stem cells that are important for the maintenance of crypt integrity and regeneration (40). In our recent studies (6), intestinal damages (detected by a decreased number of crypts and diminished length of villi in the ileum) were markedly healed in 10 Gy GIARS-mice after transplantation of intestinal lineage cells differentiated from embryonic stem cell-derived definitive endoderm. Thus, as our next steps, we are currently trying to control the mortality of 10–15 Gy GIARS-mice by the transplantation of intestinal lineage cells combined with the GL treatment.

Some adverse effects (e.g., sodium retention, hypokalemia, decrease ion aldosterone and renin) of GL have been known in humans; however, we have not seen abnormal electrolyte or hormone levels in 7 Gy GIARS-mice treated i.p. with 10 mg/kg of GL. According to the FDA guideline, murine dose of 10 mg/kg is calculated as 0.8 mg/kg for a human equivalent dose. More than 40 years, GL has been widely utilized for chronic hepatitis patients in Japan by drip infusion of 40 to 60 mL/day of Stronger Neo-Minophagen C (SNMC, Minophagen Pharmaceutical Co., Ltd.), a GL-containing preparation. Because 20 mL SNMC-INJECTION contains 40 mg of GL, the doses utilized for these patients are 1.3 to 2.0 mg/kg/day. Therefore, a dose of GL utilized in our murine studies is considered to be not high. In the following studies, we will determine the effect of GL on the M2bMϕ polarization in human experimental system.

Now we discuss how GL suppresses the M2bMϕ polarization in MLNs of GIARS-mice. CCL1 is an essential cytokine for the maintenance of M2bMϕ survival (13). The expression of *Gas5* RNA, a long noncoding RNA to silence *Ccl1* gene expression, was decreased in MLN-Mϕ of 7 Gy GIARS-mice, and it was restored in MLN-Mϕ of these mice after treatment with GL. The recovery of the *Gas5* gene expression in MLN-Mϕ of 7 Gy GIARS-mice treated with GL was confirmed when a standard preparation of M2bMϕ polarized from bone marrow-derived Mϕ [M(LPS+IC)] after being treated with GL. Also, CCL1 was not expressed/produced in M(LPS+IC) and the MLN-Mϕ of 7 Gy GIARS-mice after transduction with the *Gas5* gene via a lentiviral vector. These results indicate that GL suppress the M2bMϕ polarization by improving the *Gas5* level. *miR-222* is shown to directly bind to *Gas5* and to reduce its RNA level (39). We also demonstrated that *Gas5* RNA level was reduced in MLN-Mϕ from normal mice transfected with *miR-222* mimic. These results indicate that *miR-222* plays a significant role on the reduction of *Gas5* RNA expression during the M2bMϕ polarization process. *miR-222* expression that was increased in MLN-Mϕ of 7 Gy GIARS-mice was restored to a level shown in normal mice after the GL treatment. Also, reduced *miR-222* expression and increased *Gas5* RNA expression in MLN-Mϕ from GIARS-mice were demonstrated when these cells were cultured with GL. These results suggest that the effect of GL on the increased *Gas5* RNA expression and decreased *miR-222* expression in MLN-Mϕ from GIARS-mice may be the driving force that is suppressing the M2bMϕ polarization. Generally, the *Gas5* RNA level is regulated by nonsense-mediated RNA decay (NMD) (42, 43). *miR-222*-induced reduction of *Gas5* RNA was not seen in MLN-Mϕ treated with NMDI14, an inhibitor of NMD pathway (data not shown). On the other hand, certain cytokines (such as CCL2, IL-10, etc.) have been described to be involved in the polarization of M2a/M2cMϕ (44, 45). In the previous reports, GL has been reported as an inhibitor of M2a/M2cMϕ polarizations (18, 25, 27). Thus, GL

suppresses M2a/M2cMϕ polarizations by inhibiting CCL2 and IL-10 production. In this paper, the inhibitory effect of GL on M2bMϕ polarization and it's mode of action were demonstrated, as follows: 1) severe sepsis stemming from bacterial translocation were not shown in 7 Gy GIARS-mice treated with GL, and all these mice survived for 30 days or more after the irradiation, 2) a major antibacterial effector cell (M1Mϕ) against bacterial translocation appeared in MLNs of the irradiated mice treated with GL, and the polarization of M2bMϕ (an inhibitor cell on M1Mϕ polarization) in MLNs of these mice was not demonstrated, and 3) GL improved miR-222-associated *Gas5* RNA reduction in MLN-Mϕ of γ-irradiated mice. In addition to *miR-222*, certain miRNAs (*miR-21*, *miR-34a*, and *miR-135b*) have been reported to bind to *Gas5* (46–48). In 7 Gy GIARS-mice, these miRNAs have a possibility to be involved M2bMϕ polarization. This possibility will be tested in the following studies.

In recent 16S rRNA gene amplicon sequencing analysis (49), abundance of *Proteobacteria* has shown to be increased almost 1,000-fold in the feces of mice 4 days after 10 Gy of total body γ-irradiation and then it was returned to normal levels within 7 days of the irradiation. Abundance of *Clostridia* and *Bacteroidetes* was less affected in the feces of these irradiated mice. Because M2bMϕ polarization was started in the MLNs of 7 Gy GIARS-mice 7 days after the irradiation (5), radiation-associated microbiome changes in the intestine (during 4 to 7 days after the irradiation) seem to be not directly linked to this Mϕ polarization (7 or more days after the irradiation).

GL interacts with various immunocompetent cells. In 7 Gy GIARS-mice, however, neutrophils and lymphocytes are dramatically decreased within a few days of the irradiation. Therefore, the interaction of GL to these cells in GIARS-mice considered to be not practical. On the other hand, Mϕ and some groups of innate lymphoid cells are known to be resistant against this dose of γ-irradiation. When NSG mice adoptively transferred with 7 Gy GIARS-mouse Mϕ died within 3 days of *E. faecalis* oral infection, all of the same NSG mice survived after inoculation with Mϕ from 7 Gy GIARS-mice treated with GL (unpublished data). Therefore, we considered that Mϕ were responsible cells when 7 Gy GIARS-mice survived after the GL administration. In our recent studies, Mϕ isolated from various organs including MLNs of 7 Gy GIARS-mice did not show any M2bMϕ properties, and peripheral blood monocytes and peritoneal Mϕ from GIARS-mice treated with GL were shown to be IL-12^{+} cells. These results indicate that various Mϕ derived from 7 Gy GIARS-mice that were treated with GL are convertible to be M1Mϕ.

Because a high dose of GL has been reported to protect gastrointestinal (GI) mucosal injuries in mice with non-alcohol fatty liver diseases (NAFLD-mice) (50), the effect of GL on GI damages of 7 Gy GIARS-mice was examined in our laboratory. In the results, similar GI mucosal injuries (shortening of villus length and reduced number of crypts in the intestine) were seen in 7 Gy GIARS-mice before and after treatment with the compound at a dose of 10 mg/kg. From these results, we assume that the mitigative effect of GL on GI mucosal injuries is not directly involved in the reduced mortality rate of 7 Gy GIARS-mice treated with GL.

For M2bMϕ polarization in GIARS-mice, we have hypothesized the following 5 steps: (i) high mobility group box 1 (HMGB1) is released from damaged or dying cells influenced by the irradiation (51, 52), (ii) this protein induces *miR-222* expression in Mϕ (53–55), (iii) increased *miR-222* activate the NMD pathway (39), (iv) the activation of this pathway causes the decreased *Gas5* RNA expression (Fig. 4D), and (v) Mϕ with reduced *Gas5* RNA polarize to the M2b phenotype (11). Thus, it is indicated that resident Mϕ are polarized into M2bMϕ through the few steps after initiation by the increased level of HMGB1. GL has been proved to directly bind to HMBG1 and interfere with the binding of HMGB1 to DNA in living cells (56, 57). Also, the interaction of HMGB1 with its receptors (RAGE and TLR4) is shown to be blocked by GL (58). We have also demonstrated that serum HMGB1 levels were not increased in 7 Gy GIARS-mice treated with GL (unpublished data). When 7 Gy GIARS-mice were treated i.p. with anti-HMGB1 antibody, Mϕ from these mice did not show any M2bMϕ properties, just as GIARS-mice treated with GL. Therefore, we think that the effect of GL on the improved antibacterial resistance against bacterial translocation is exhibited through the inhibition of M2bMϕ polarization, that is initiated by HMGB1 released from damaged or dying cells influenced by irradiation, and GL suppresses M2bMϕ polarization through the HMGB1 blocking.

The antibacterial effects of GL against various bacterial infections through the modulation of mannose receptor expressing M2Mϕ (e.g., M2aMϕ and M2cMϕ) have already been demonstrated in mice 2–5 days after burn injury (20, 25). By controlling M2a/cMϕ polarization, the suppression of CCL2 and/or IL-10 production from neutrophils, Mϕ and/or T cells have been shown to be involved in the antibacterial effects of GL (20). Due to the plasticity of Mϕ, M2a/cMϕ switch to a non-M2a/cMϕ when IL-4 or IL-10 is absent (59). Therefore, the lifespan of M2a/cMϕ is relatively short. In contrast, M2bMϕ live longer without any exogenous growth factors, because M2bMϕ themselves produce CCL1 (13). In this study, the M2bMϕ polarization was shown to be inhibited by GL through the restoration of impaired *Gas5* RNA expression in MLN-Mϕ of 7 Gy GIARS-mice. So far, the pathologic roles of M2bMϕ have been described in various diseases including infectious diseases (bacterial, viral, and parasitic infection), autoimmune diseases (systemic lupus erythematous and lupus nephritis), diseases in nervous systems (spinal code injury and Alzheimer's disease), glycolipid metabolic disorders (obesity and type 2 diabetes mellitus diabetes), cardiovascular diseases (atherosclerosis and myocardial disease), and cancer (hepatocellular carcinoma) (60). Based on these findings, GL may have a function to control these diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key points:

Gut bacteria-associated sepsis in irradiated mice was controlled by GL.

This compound inhibited M2bMϕ polarization at the bacteria translocation site.

Expression of *Gas5* (↓) and *miR-222* (↑) in Mϕ was improved by GL.

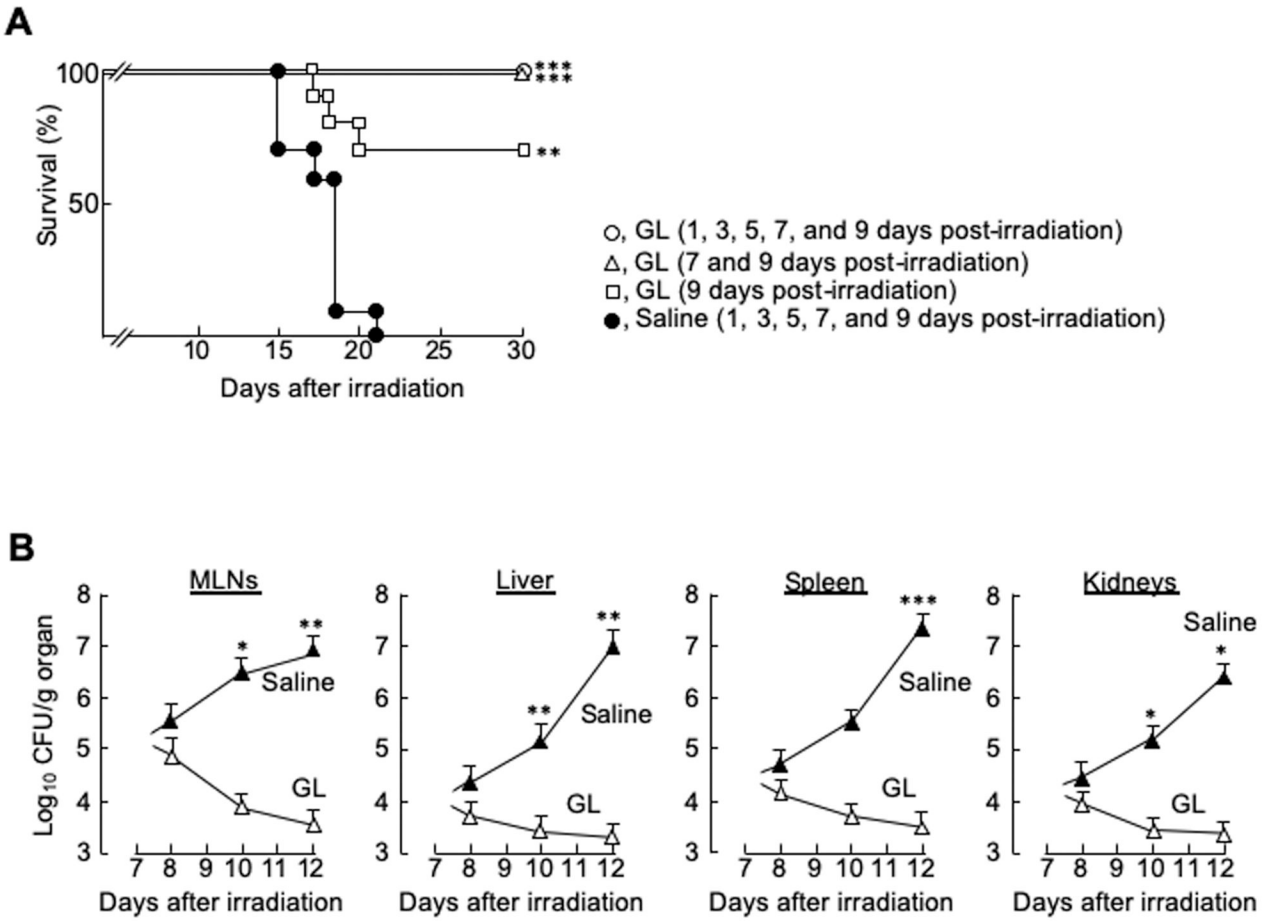

Figure 1. Effect of GL on the survival of 7 Gy GIARS-mice.

(A) Seven Gy GIARS-mice were treated i.p. with 10 mg/kg of GL 1, 3, 5, 7, and 9 days post-irradiation (○, 12 mice), 7 and 9 days post-irradiation (△, 10 mice), or 9 days post-irradiation (□, 10 mice). As a control, GIARS-mice were treated with saline (i.p., 0.2 mL/mouse) 1, 3, 5, 7, and 9 days post-irradiation (●, 12 mice). These mice were observed every 12 h to determine their survival rate. Data shown are representative of two independent experiments using male mice. $^{**}p < 0.01$, $^{***}p < 0.001$ by log-rank test. (B) Numbers of bacteria in various organs of GIARS-mice treated with GL (7 and 9 days post-irradiation, △, 18 mice; 6 mice/each time point) or saline (7 and 9 days post-irradiation, ▲, 18 mice; 6 mice/each time point) were counted 8 to 12 days after the γ-irradiation by colony counting. Data are displayed as mean ± SD from three independent experiments using either male or female mice. $^{*}p < 0.05$, $^{**}p < 0.01$ by Student *t* test.

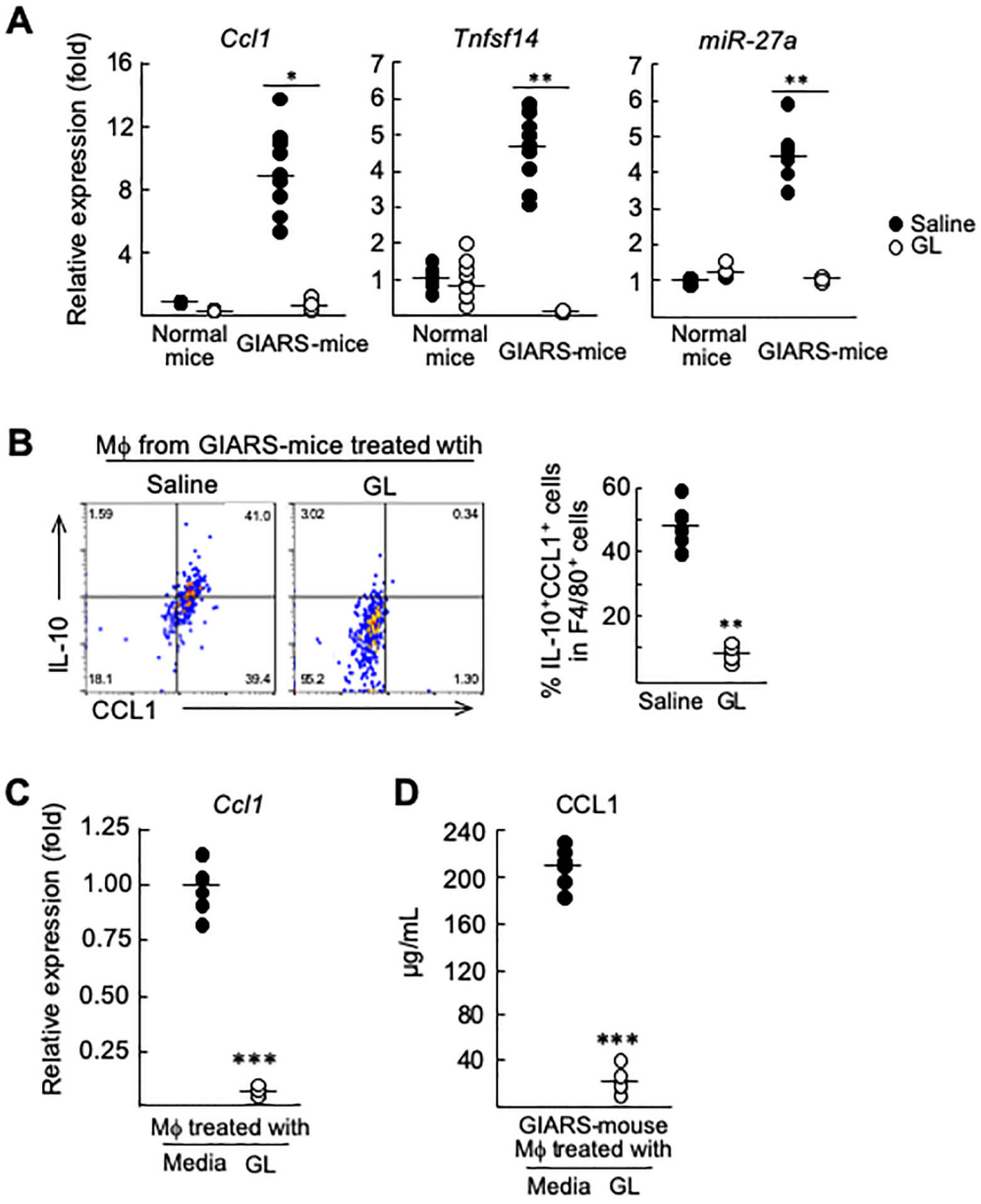

Figure 2. M2bMϕ (IL-10$^+$CCL1$^+$LIGHT$^+$*miR-27a*$^+$ Mϕ) in MLNs of 7 Gy GIARS-mice treated with GL.

Seven Gy GIARS-mice were treated i.p. with GL 7 and 9 days post-irradiation. MLN-Mϕ, isolated from normal mice (9 mice) or GIARS-mice (10 days post-irradiation, 8 mice), were analyzed for the expression of *Ccl1*, *Tnfsf14*, and *miR-27a* (A). Percentage of IL-10$^+$CCL1$^+$ cells in these MLN-Mϕ was analyzed by flow cytometry (B). Representative plots in the 3 independent experiments using either male or female mice are displayed (left). Data from three independent experiments are shown in right. Also, MLN-Mϕ from 7 Gy GIARS-mice (6 mice) were cultured with GL (10 μg/mL) or media for 24 h. Then, Mϕ harvested were analyzed for *Ccl1* mRNA expression (C). Culture fluids of these cells were also assayed for CCL1 by ELISA (D). Data are displayed as mean ± SD from three independent experiments using either male or female mice. *$p < 0.05$, **$p < 0.01$ by Student *t* test.

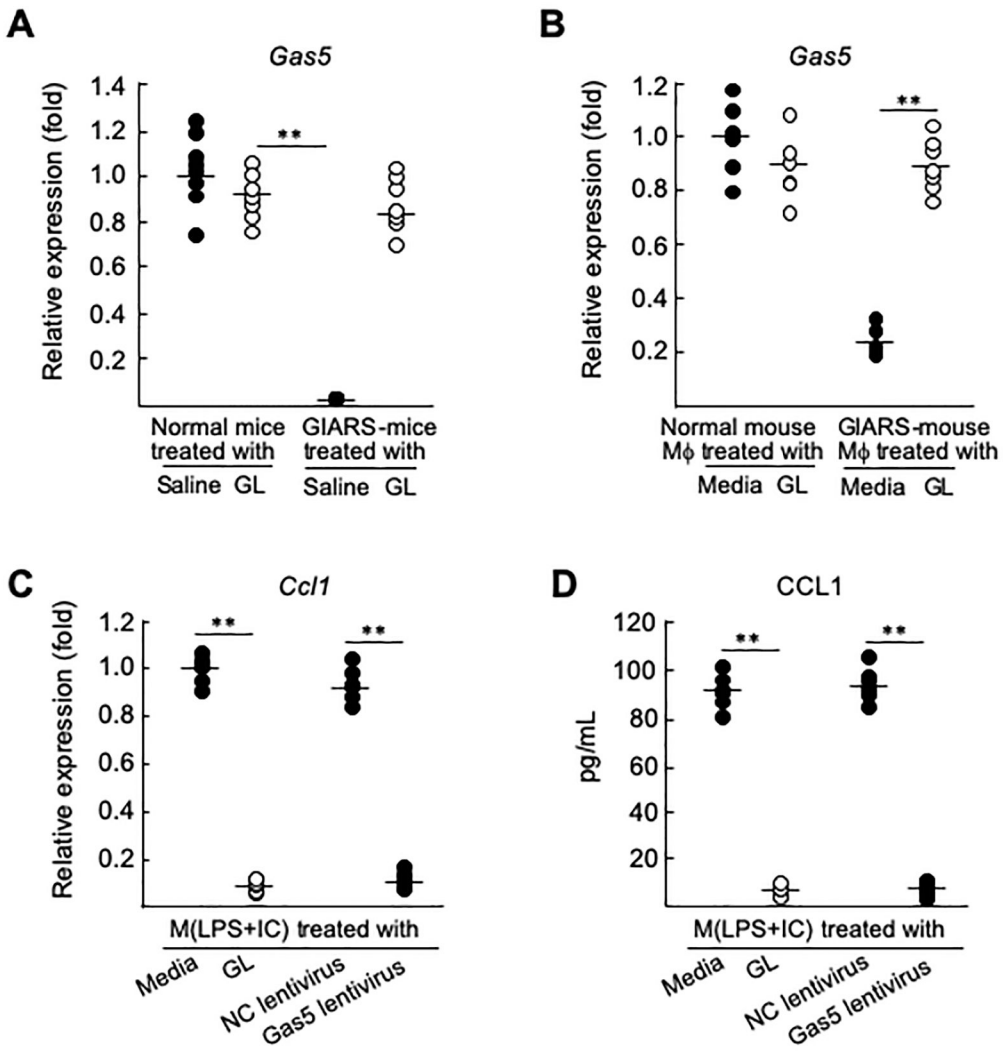

Figure 3. Property of MLN-Mϕ from 7 Gy GIARS-mice treated with GL.

(A) Seven Gy GIARS-mice were treated with GL (10 mg/kg, i.p., 7 and 9 days post-irradiation, 10 mice). As controls, normal mice (10 mice) and 7 Gy GIARS-mice (8 mice) were treated with saline (0.2 mL/mouse) in the same fashion. Ten days post-irradiation, MLN-Mϕ isolated from these mice were analyzed for *Gas5* RNA expression. (B) MLN-Mϕ obtained in (A) were cultured for 24 h with media supplemented with GL (10 μg/mL). MLN-Mϕ harvested were analyzed for *Gas5* RNA expression. (C, D) M(LPS+IC) (bone marrow-derived Mϕ stimulated with LPS and IC in combination, 5×10^5 cells/mL) were cultured in media supplemented with or without GL (10 μg/mL) or added with 1×10^7 TU/mL of negative control (NC) lentivirus or Gas5 lentivirus for 24 h ($n = 6$ per group). Then, Mϕ harvested were tested for *Ccl1* mRNA expression (C) or CCL1 production (D). For the production of CCL1, harvested Mϕ were recultured with fresh media for 48 h and culture fluids obtained were assayed for CCL1. Data are displayed as mean ± SD from three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by Student t test.

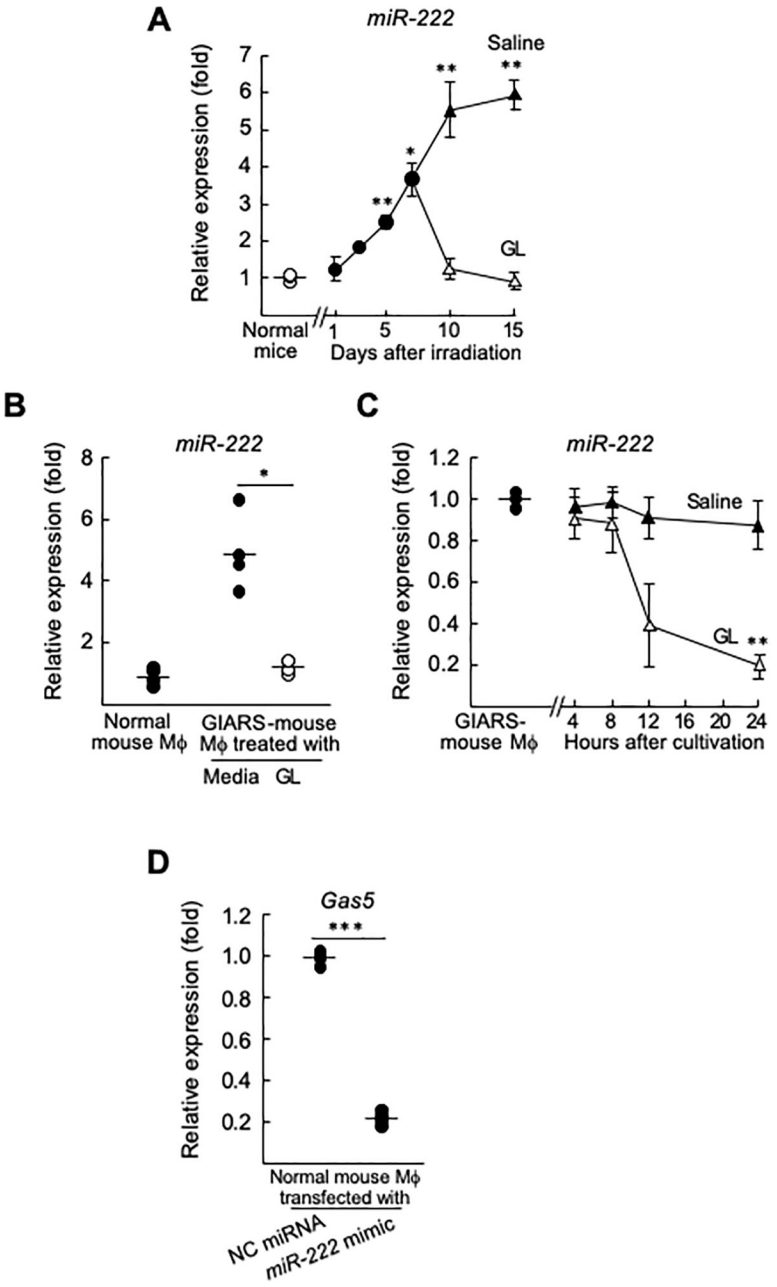

Figure 4. miR-222 reduction in MLN-Mϕ of GL-treated 7 Gy GIARS-mice.

(A) Seven Gy GIARS-mice were treated with GL (7 and 9 days post-irradiation, △, 8 mice) or saline (7 and 9 days post-irradiation, ▲, 8 mice). Then, MLN-Mϕ isolated from these mice 10 days post-irradiation were analyzed for *miR-222* expression. (B, C) MLN-Mϕ (1×10^6 cells/mL) from normal or GIARS-mice (10 days post-irradiation) were cultured with GL (10 μg/mL) for 24 h (B) or 4 to 24 h (C) ($n = 6$ per group), then Mϕ harvested were analyzed for *miR-222* expression. (D) Normal mouse MLN-Mϕ, transfected with *miR-222* mimic or negative control (NC) miRNA ($n = 6$ per group), were analyzed for *Gas5* RNA expression.

Data are displayed as mean ± SD from three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by Student t test.