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Brief Article

Reciprocal expression of enteric antimicrobial proteins in intestinal graft-versus-host disease

Short title: Enteric antimicrobial proteins in GVHD

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Abstract

We recently demonstrated that expression of α-defensins, the major antimicrobial peptides produced by Paneth cells was severely suppressed in mice with graft-versus-host disease (GVHD). In this study, we found that antibacterial lectin, regenerating islet-derived IIIγ (RegIIIγ) was upregulated in villous enterocytes, thus demonstrating the reciprocal control of enteric antimicrobial proteins in GVHD. Upregulation of RegIIIγ was mediated by a mechanism independent upon radiation-induced intestinal tract damage. MyD88-mediated signaling in intestinal epithelium was required for RegIIIγ upregulation in GVHD and antibiotic therapy downregulated RegIIIγ expression. These results suggest that MyD88-mediated sensing of the intestinal microbes disregulated in GVHD induces RegIIIγ upregulation in GVHD and argue a role for RegIIIγ in the pathogenesis of GVHD.

Introduction

Allogeneic hematopoietic stem cell transplantation (SCT), a curative therapy for a number of hematologic diseases, is complicated by graft-versus-host disease (GVHD). Particularly, intestinal GVHD is critical for determining the outcome of allogeneic BMT(1). Recently, regenerating islet-derived 3α (RegIIIα) is identified as a specific biomarker for intestinal GVHD in human using a large-scale and quantitative proteomic discovery approach(2, 3). Reg genes constitute a multigene family, which is categorized into four subclasses. RegIIIγ, a homologue of human RegIIIα in mice, is preferentially expressed in the small intestine. RegIIIγ have canonical C-type lectin domains that bind to the peptidoglycan, which is an essential component of the bacterial cell wall and thus has direct antimicrobial activity, specifically against Gram-positive bacteria and protects the epithelial barrier function of the intestinal mucosa (4).

The intestinal microbial communities are actively regulated by Paneth cells through their secretion of antimicrobial peptides. Among them, α-defensins are the most potent antimicrobial peptides that account for 70% of the bactericidal peptide activity released from Paneth cells (5, 6). We recently found that Paneth cells were targeted by GVHD, resulting in marked reduction in the expression of α-defensins(7). Thus, it is puzzling why blood levels of RegIIIα levels are elevated, while α-defensins are downregulated in GVHD. In this study, we evaluated enteric expression of RegIIIγ at the cellular level in mouse models of bone marrow transplantation (BMT) and found

that the major producers of RegIIIγ were villous enterocytes, not Paneth cells in GVHD. Upregulation of RegIIIγ in GVHD was dependent upon MyD88-mediated sensing of the intestinal microflora.

Material and Methods

Mice. Female C57BL/6 (B6: H-2^b), B6D2F1 (H-$2^{b/d}$), B6-Ly5.1 (H-2^b, CD45.1^+), BALB.B (H-2^b), and C3H.Sw (H-2^b) mice were purchased from Charles River Japan, KBT Oriental, or Japan SLC. B6-background Myeloid differentiation factor 88 (MyD88)-deficient (MyD$88^{-/-}$) mice(8) were kindly provided by Dr. Kiyoshi Takeda at Osaka University. All animal experiments were performed under the auspices of the Institutional Animal Care and Research Advisory Committee.

BMT. Mice were transplanted as previously described(9). In brief, after lethal X-ray total body irradiation (TBI) delivered in 2 doses at 4h intervals, mice were intravenously injected with 5×10^6 T-cell depleted-bone marrow (TCD-BM) cells with or without 2×10^6 splenic T cells on day 0. Isolation of T cells and T-cell depletion were performed using the T cell isolation kit and anti-CD90-MicroBeads, respectively, and the AutoMACS (Miltenyi Biotec) according to the manufacturer's instructions. In unirradiated model of BMT, B6D2F1 mice were intravenously injected with 12×10^7 splenocytes(7, 10). PMV regimen is consisted of polymyxin B (10^5 U/kg), metronidazole (30 mg/kg), and vancomycin (30 mg/kg). Mice were maintained in specific pathogen-free condition and received normal chow and autoclaved hyperchlorinated water (PH 4) for the first 3 wks post-BMT and filtered water thereafter. The degree of clinical GVHD was assessed weekly by a scoring system which sums changes in five clinical parameters: weight loss, posture, activity, fur texture, and skin

integrity (maximum index = 10) as described previously(9).

Histological and immunohistochemical analysis. For pathological analysis, samples of the small intestine were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned, slide mounted, and stained with H&E. Immunohistochemistry was performed as described (11) using rabbit anti-defensinα_1 and anti- RegIIIγ (Funakoshi). Histofine Simple Stain MAX PO (Rat) kits and subsequently DAB solution (Nichirei Biosciences) was used to generate brown-colored signals. Slides were then counterstained with hematoxylin. Pictures from tissue sections were taken at room temperature using a digital camera (DP72; Olympus) mounted on a microscope (BX51; Olympus).

Preparation and analysis of isolated mouse crypts. Individual crypts were isolated from the small intestine as previously described(6). Following fixation and permeabilization, isolated crypts were incubated for 1h with FITC-conjugated anti-Lysozyme (10µg/ml; Dako), followed by incubation for 1h with Allexa Fluor 594-conjugated phalloidin (1U/ml; Invitrogen). Tetramethyl DAPI (5µg/ml; Invitrogen) was used to stain the nucleus. Samples were mounted in Aqua Poly/Mount (Polysciences) and examined with a confocal laser-scanning microscope (LSM510; Carl Zeiss).

ELISA. Serum levels of RegIIIγ and IL-22 were measured by using the ELISA Kit for RegIIIγ (USCN) and the ELISA Kit for mouse IL-22 (BioLegend), respectively.

Quantitative real-time PCR analysis. Total RNA was purified using the RNeasy Kit (QIAGEN). cDNA was synthesized using a QuantiTect Reverse Transcription Kit (QIAGEN). PCR reactions and analyses were performed with ABI PRISM 7900HT SDS 2.1 (Applied Biosystems) using TaqMan Universal PCR master mix (Applied Biosystems), and TaqMan Gene Expression Assays (Defa1: Mm02524428_g1, RegIIIg Mm01181783_g1, and Gapdh: Mm99999915_g1, Applied Biosystems). The relative amount of each mRNA was determined using the standard curve method and was normalized to the level of GAPDH in each sample.

Statistical analysis. Mann-Whitney U tests were used to compare data. All tests were performed with the SigmaPlot Version 10.0 software. $P < .05$ was considered statistically significant.

Results

Reciprocal control of α-defensins and RegIIIγ expression in GVHD

Lethally irradiated B6D2F1 (H-$2^{b/d}$) mice received 5 × 10^6 TCD-BM alone (control group) or TCD-BM plus 2 × 10^6 T cells (GVHD group) from major histocompatibility complex (MHC)-mismatched B6 (H-2^b) donors on day 0. The allogeneic animals developed severe GVHD, as previously demonstrated(7, 10) (data not shown). Pathological analysis of the small intestine 7d after BMT showed mostly normal architecture in controls, while blunting of villi was observed in the GVHD group (**Fig. 1A**). Confocal cross-sectioning of individual crypts isolated from the small intestine demonstrated Paneth cell loss in mice with GVHD, as previously shown(7) (**Fig. 1B**).

α-Defensins are the major antimicrobial peptides produced by Paneth cells (6). Immunohistochemical analysis showed that defensin α_1 expression was limited in Paneth cells in the crypts of naïve mice (**Fig. 1C**). Expression of defensin α_1 was preserved in controls but was severely suppressed in mice with GVHD 7d after BMT (**Fig. 1C**). In contrast, RegIIIγ expression was markedly increased in villous enterocytes in the GVHD group (**Fig. 1D**). It should be noted that major producers of RegIIIγ were not Paneth cells, and there was little expression of defensin α_1 in enterocytes in GVHD.

To confirm the differential expression of α-defensins and RegIIIγ, their expression levels in the terminal ileum were evaluated by quantitative real-time PCR analysis. In the GVHD group, expression of *defensin-α_1* (*Defa1*) was markedly reduced (**Fig. 1E**), while RegIIIγ expression was significantly increased (**Fig. 1F**).

RegIIIγ upregulation in GVHD by a mechanism independent upon radiation-induced intestinal tract damage

We determined if increased expression of RegIIIγ in the small intestine correlated with increased serum levels of RegIIIγ. Serum levels of RegIIIγ were not increased in the control group after BMT, whereas those were significantly and constantly elevated in the GVHD group (**Fig. 2A**). We next addressed whether RegIIIγ upregulation could be related to radiation-induced intestinal tract damage using an unirradiated B6 → B6D2F1 model as previously described(7, 10). Again, serum levels of RegIIIγ were significantly and constantly elevated in the GVHD group to the similar levels observed in the irradiated model (**Fig. 2B**).

RegIIIγ upregulation in MHC-matched models of GVHD

We then evaluated if upregulation of RegIIIγ could be observed in clinically relevant, MHC-matched, but minor histocompatibility antigen-mismatched models of

BMT. Again, serum levels of RegIIIγ were significantly elevated in the B6 → BALB.B model (**Fig. 2C**) and the C3H.Sw → B6 model of BMT (**Fig. 2D**).

RegIIIγ is induced by the stimulation of intestinal microbes through MyD88-mediated signaling in intestinal epithelium in GVHD

We investigated mechanisms of upregulation of enteric expression of RegIIIγ in GVHD. Since recognition of commensal microflora by toll-like receptors (TLRs) is required for intestinal homeostasis and RegIIIγ expression is controlled by microorganism-associated molecular patterns that activate MyD88 pathway(12-15), we hypothesized that bacterial stimuli through MyD88 pathway could mediate the upregulation of RegIIIγ in GVHD. To test this hypothesis, wild-type (WT) or MyD88$^{-/-}$ B6 mice were used as recipients in the C3H.Sw → B6 model of BMT. Clinical GVHD scores were not significantly different between WT and MyD88$^{-/-}$ mice on days 28 and 35 after BMT **(Supplemental Figure A)**. Nonetheless, serum levels of RegIIIγ were significantly lower in MyD88$^{-/-}$ mice than in WT mice on d28 and d35 after BMT (**Fig. 2E**).

We and others have shown that GVHD induces dramatic alteration in the intestinal microbiota (7, 16, 17). We therefore hypothesized that upregulated expression of RegIIIγ might be a natural mechanism of adaptation aimed to restore normal intestinal ecology. To evaluate whether modifying the enteric flora using oral antibiotics

could inhibit upregulation of RegIIIγ, mice were treated with broad-spectrum antibiotic combination as described with a slight modification(14). PMV was administered by daily oral gavage from d-14 in the C3H.Sw → B6 model of BMT. It reduced clinical GVHD scores, as previously described(7) **(Supplemental Figure B)**. Notably, PMV regimen prevented upregulation of RegIIIγ on d28 after BMT (**Fig. 2F**).

Induction of RegIIIγ also requires IL-22-mediated signals from innate lymphoid cells(18, 19). However, serum levels of IL-22 were significantly lower in the GVHD group than in controls on d28 and d35 after BMT in the C3H.Sw → B6 model (**Supplemental Figure C**). Gut decontamination with the PMV regimen or the use of MyD88$^{-/-}$ B6 mice did not change serum levels of IL-22 (data not shown).

Discussion

Epithelial antimicrobial proteins have an essential role in allowing epithelial surfaces to cope with microbial challenges. They include defensins, cathelicidins, C-type lectins such as the Reg family(20). In this study, we found that enteric expression of α-defensins and RegIIIγ was reciprocally controlled in GVHD. This was due to the difference in the major producers of these molecules in GVHD. It has been shown that α-defensins are exclusively produced by Paneth cells, whereas RegIIIγ is produced by both villous enterocytes and Paneth cells in steady state(13, 21). In GVHD, enterocyte production of RegIIIγ was markedly increased, whereas Paneth cell

production of α-defensins was severely suppressed.

Intestinal GVHD is characterized by severe villous atrophy and crypt degeneration. Crypt cell apoptosis is one of the initial lesions and the cardinal features of the intestinal GVHD(22, 23). Many experimental and clinical evidence favors the idea that crypt cells are the principal focus of the attack by donor T cells and inflammatory cytokines in GVHD(24). Intestinal stem cells (ISCs) and their niche, Paneth cells reside in the crypts and we have shown that both ISCs and Paneth cells are targeted by GVHD(7, 10). In contrast, there is little evidence of direct damage to mature villous enterocytes in mild GVHD and damage to mature enterocyte appears only at severe stage, suggesting that these events are secondary to the alterations in crypt cell turnover(22-25).

Serum levels of RegIIIγ were increased in an unirradiated model of BMT, demonstrating a mechanism independent upon radiation-induced intestinal tract damage. These results are consistent with our recent observation that GVHD induces Paneth cell injury and subsequent dysbiosis by a mechanism independent upon conditioning(7). A recent clinical study demonstrated that low Paneth cell numbers at onset of intestinal GVHD is associated with high risk for non-relapse mortality(26).

We have shown that MyD88-mediated signaling in host non-hematopoietic cells is required for upregulation of RegIIIγ in villous enterocytes in GVHD. Since severity of GVHD was not altered in MyD88$^{-/-}$ mice as has been shown(27),

downregulation of RegIIIγ was not secondary to amelioration of GVHD. These results suggest a potential lack of reliability of RegIIIγ as a marker for GVHD and argue against a role for it in the pathophysiology of GVHD. Administration of the broad-spectrum antibiotic combination PMV markedly decreased serum levels of RegIIIγ in GVHD. These results are consistent with previous studies demonstrating that RegIIIγ expression is controlled by microorganism-associated molecular patterns that activate MyD88 pathway in non-hematopoietic cells in steady state and in bacterial infection(12-15, 17). Sensitivity of RegIIIγ as a biomarker of intestinal GVHD may be reduced when intensive and broad antibiotics are administered.

We and others have shown that development of intestinal GVHD induced marked dysbiosis in the intestinal flora in mice and human(7, 16, 17, 28). Although dominantly expanded bacteria in GVHD differs at institutions(7, 16, 17), we found the prominent outgrowth of Gram-negative bacteria, *Escherichia coli* (*E. coli*) in the intestinal flora in GVHD(7), thus supporting the previous findings that Gram-negative bacteria, but not Gram-positive bacteria, induces RegIIIγ expression(14, 29). Thus, it is tempting to assume that enhanced production of RegIIIγ is a natural adaptation mechanism for the dysbiosis in GVHD. Paneth cell metaplasia is also observed in the colon of GVHD patients probably as an adaptation mechanism for Panrth cell loss and the dysbiosis(30).

We have shown that Paneth cell-derived α-defensin production is markedly inhibited in GVHD that have selective bactericidal activity mostly against Gram-negative bacteria(7, 31, 32). On the other hand, the antibacterial activity of RegIIIγ is likely restricted to Gram-positive bacteria because of their accessibility to peptidoglycan on the cell surface of Gram-positive bacteria(4). Thus, bacteria belonging to different classes may battle against each other by stimulating innate antimicrobial mechanisms that selectively inactivate specific bacteria in the mammalian gut, and dramatic decrease in a ratio of α-defensins to RegIIIγ production may be in tune with overwhelming outgrowth of Gram-negative bacteria.

RegIIIγ mRNA expression also requires IL-22-mediated signals from ILCs(18, 19). However, IL-22 was reduced in GVHD in our study. Hanash et al. recently demonstrated that IL-22 producing innate lymphoid cells were eliminated in GVHD; however, RegIIIγ was still expressed in GVHD in IL-$22^{-/-}$ mice, suggesting that RegIIIγ expression was upregulated by IL-22 independent pathways in the presence of minimum amounts of IL-22 in GVHD(33).

Recently, RegIIIα is identified as a specific biomarker for intestinal GVHD in human using a large-scale and quantitative proteomic discovery approach(2, 3). Our results support these studies and give mechanistic insights. These new insights will help to understand pathophysiology of GVHD in the context of host-microbe interaction and

to establish new therapeutic strategies that can improve clinical outcome of allogeneic SCT.

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Figure Legends

Figure 1. Reciprocal control of expression of Paneth cell-derived α-defensins and epithelial cell-derived RegIIIγ in GVHD

Lethally irradiated B6D2F1 mice were transplanted with TCD BM cells without (control group) or with T cells (GVHD group) from MHC-mismatched B6 donors. Small intestines were isolated from mice 7d after BMT. **(A)** Histology of the small intestine stained with H&E. **(B)** Confocal cross-sectioning of the isolated small intestinal crypt. Lysozyme (green) is expressed by Paneth cells. Tetramethyl DAPI (blue) stains the nucleus and phalloidin (red) stains F-actin. Magnification: 1000×. Bars, 10 μm. Immunohistochemical staining for defensin α_1 (brown) **(C)** and RegIIIγ (brown) **(D)**. Magnification: 100×. Bars, 100 μm. RNA was extracted from the small intestines on day 7 and quantitative real-time PCR analysis for *Defa1* **(E)** and *RegIIIγ* **(F)** compared to GAPDH was performed (n = 6 / group). Data are representative of two similar experiments and are shown as means ± SE. **P* < .05.

Figure 2. Increased serum levels of RegIIIγ in GVHD is inhibited by intestinal decontamination or in the absence of MyD88 signaling pathway in hosts

Serum levels of RegIIIγ were measured after BMT (n = 6 / group). Data are representative of two similar experiments and are shown as means ± SE. **P* < .05. **(A)** Irradiated B6 →B6D2F1 model. **(B)** Unirradiated B6 →B6D2F1 model. **(C)** Irradiated

B6 → BALB.B model. **(D)** Irradiated C3H.Sw → B6 model. **(E)** Irradiated C3H.Sw → B6 model. B6 mice were either WT or MyD88$^{-/-}$ B6 mice. **(F)** Irradiated C3H.Sw → B6 model. A cohort of mice were treated with with PMV regimen by daily oral gavage from d-14 of BMT.

Figures

Figure 1.

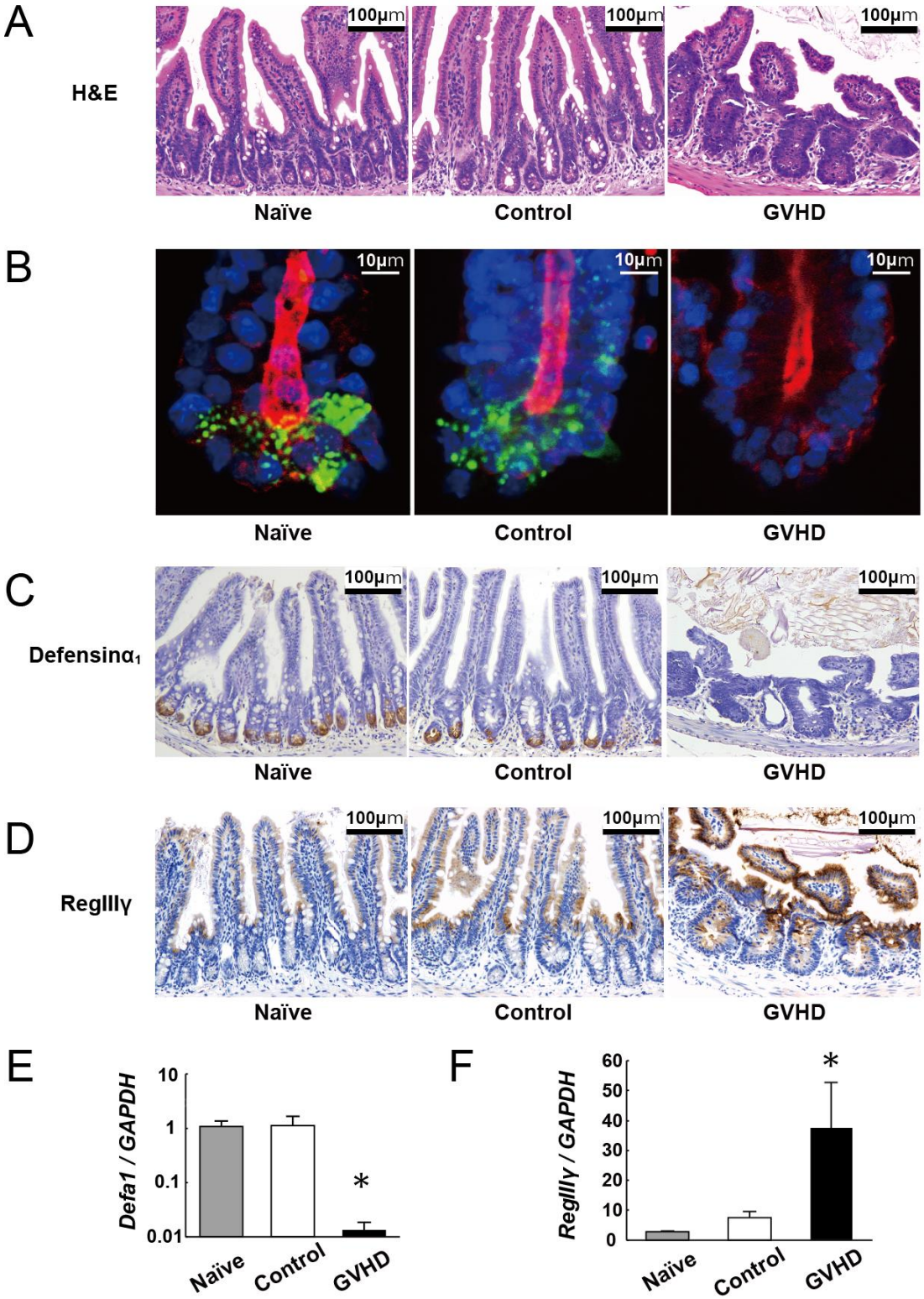
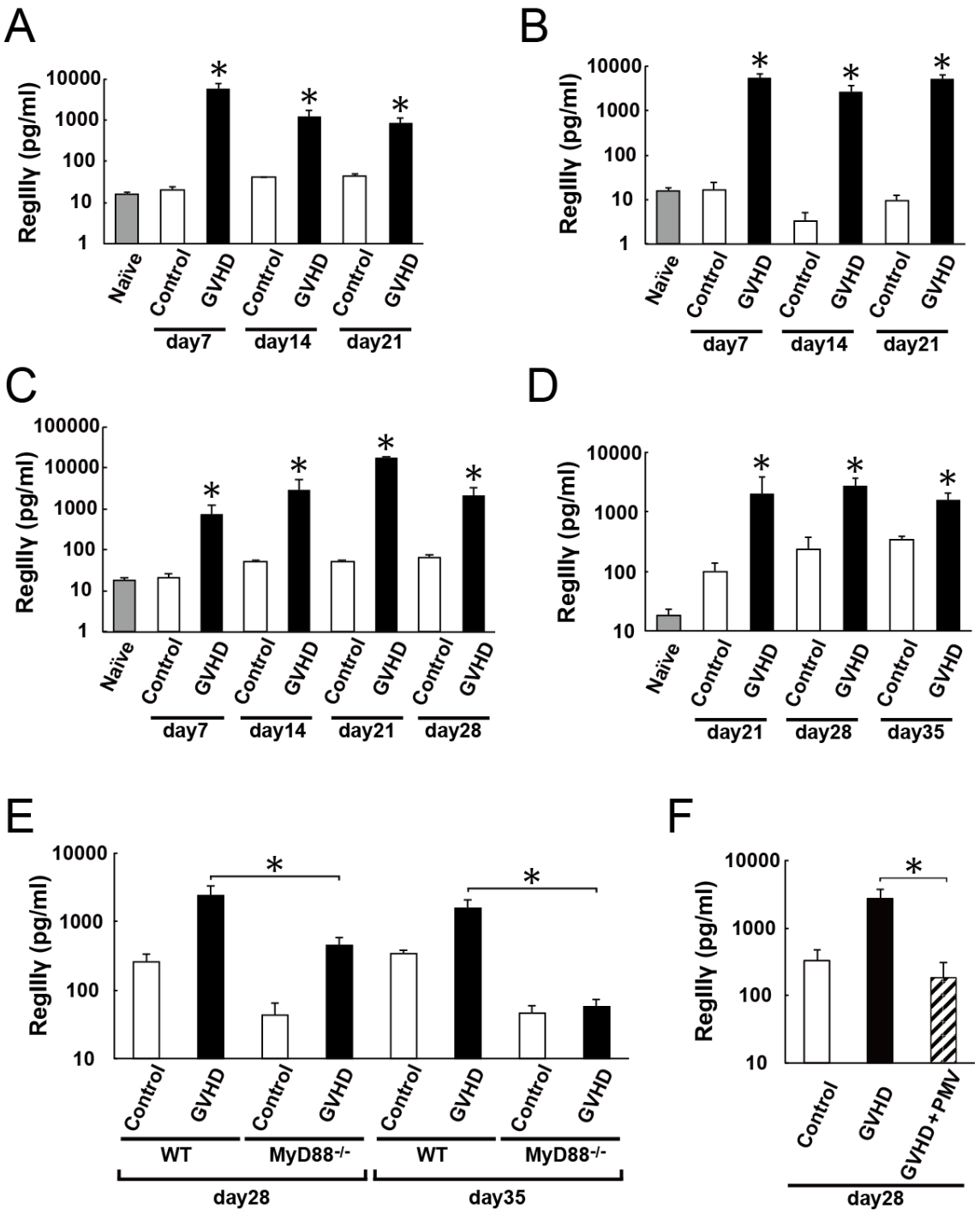


Figure 2.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