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Galectin-1–Driven Tolerogenic Programs Aggravate *Yersinia enterocolitica* Infection by Repressing Antibacterial Immunity

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Yersinia enterocolitica **is an enteropathogenic bacterium that causes gastrointestinal disorders, as well as extraintestinal manifestations. To subvert the host's immune response, *Y. enterocolitica* uses a type III secretion system consisting of an injectisome and effector proteins, called *Yersinia* outer proteins (Yops), that modulate activation, signaling, and survival of immune cells. In this article, we show that galectin-1 (Gal-1), an immunoregulatory lectin widely expressed in mucosal tissues, contributes to *Y. enterocolitica* pathogenicity by undermining protective antibacterial responses. We found higher expression of Gal-1 in the spleen and Peyer's patches of mice infected orogastrically with *Y. enterocolitica* serotype O:8 compared with noninfected hosts. This effect was prevented when mice were infected with *Y. enterocolitica* lacking YopP or YopH, two critical effectors involved in bacterial immune evasion. Consistent with a regulatory role for this lectin during *Y. enterocolitica* pathogenesis, mice lacking Gal-1 showed increased weight and survival, lower bacterial load, and attenuated intestinal pathology compared with wild-type mice. These protective effects involved modulation of NF-κB activation, TNF production, and NO synthesis in mucosal tissue and macrophages, as well as systemic dysregulation of IL-17 and IFN-γ responses. In vivo neutralization of these proinflammatory cytokines impaired bacterial clearance and eliminated host protection conferred by Gal-1 deficiency. Finally, supplementation of recombinant Gal-1 in mice lacking Gal-1 or treatment of wild-type mice with a neutralizing anti-Gal-1 mAb confirmed the immune inhibitory role of this endogenous lectin during *Y. enterocolitica* infection. Thus, targeting Gal-1–glycan interactions may contribute to reinforce antibacterial responses by reprogramming innate and adaptive immune mechanisms.** ***The Journal of Immunology*****, 2017, 199: 000–000.**

Yersinia enterocolitica is a Gram-negative enteropathogenic bacterium that causes human gastroenteritis, as well as extraintestinal manifestations, such as reactive arthritis and sepsis (1). During the course of infection, *Y. enterocolitica* reaches the intestinal tract, invades M cells of Peyer's patches (PPs), and disseminates to the mesenteric lymph nodes (MLNs), spleen, and liver (1). Although innate immune cells, including NK cells, dendritic cells (DCs), and macrophages, confer early protection during the course of *Y. enterocolitica* infection, Th1 and Th17 effector cells ultimately provide antibacterial immunity depending on the magnitude of the inoculum, entry route, and engagement of selected pattern recognition receptors (2–8). However, despite activation of a broad spectrum of immune-protective mechanisms, *Y. enterocolitica* has evolved a complex machinery to subvert antibacterial responses (9).

The pathogenicity of *Y. enterocolitica* relies on chromosome-encoded and plasmid-encoded virulence factors (9), including a type III secretion system composed of a syringe-like needle structure (injectisome) and effector proteins, termed *Yersinia* outer proteins (Yops), that are directly injected into host cells (9–11). Translocation of Yops (YopP, YopH, YopE, YopT, YopM, and YopO) into target cells contributes to disarm innate and adaptive immunity through multiple mechanisms. Although YopP/J inhibits NF-κB signaling, suppresses proinflammatory cytokines, modulates Ag uptake, and induces macrophage and DC apoptosis (12–16), YopH counteracts T cell activation (17, 18), and YopE, YopT, and YopO inhibit phagocytosis by disrupting actin cytoskeleton (11). However, in contrast to the intrinsic bacterial factors governing *Y. enterocolitica* pathogenesis, host factors co-opted by *Y. enterocolitica* to subvert antibacterial immunity are largely unknown.

Galectin-1 (Gal-1), a member of a family of β-galactoside–binding proteins, has emerged as an immune regulatory checkpoint that influences innate and adaptive immune compartments (19). This endogenous lectin, abundantly expressed in inflamed and mucosal tissues, controls excessive immune responses and promotes resolution of autoimmune pathology (20–22), favors immune escape in cancer

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R.C.D., M.S.D.G., and G.A.R. conceived and designed the experiments; R.C.D., R.J.E., S.P.M.-H., and J.C.S. performed experiments; R.C.D., R.J.E., S.P.M.-H., M.S.D.G., and G.A.R. analyzed data; R.C.D., M.S.D.G., I.A., G.A.R., and S.P.M.-H. contributed reagents/materials/analysis tools; and R.C.D., M.S.D.G., S.P.M.-H., and G.A.R. wrote the manuscript.

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Abbreviations used in this article: AG, aminoguanidine; DC, dendritic cell; Gal-1, galectin-1; iNOS, inducible NO synthase; *Lgals1*$^{-/-}$, Gal-1–knockout; MLN, mesenteric lymph node; PP, Peyer's patch; rGal-1, recombinant Gal-1; Treg, regulatory T cell; WT, wild-type; Yop, *Yersinia* outer protein.

(23–26), suppresses allergic reactions (27), and fuels tolerogenic programs during parasitic infection (28). The mechanisms underlying these broad immunoregulatory effects involve selective apoptosis of Th1 and Th17 effector cells (22), promotion of tolerogenic DCs (29), deactivation of M1-type macrophages (30–32), promotion of an IL-10–dependent immunoregulatory signature (20, 33–35), and expansion of $Foxp3^+$ regulatory T cells (Tregs) (36). At the molecular level, Gal-1 acts by cross-linking a preferred set of glycosylated receptors, promoting their cell surface retention, and reorganizing and modulating their signaling threshold (37, 38).

Although little is known about the immunoregulatory role of Gal-1 during bacterial infection, emerging studies highlighted the relevance of this lectin during corneal immunopathology induced by *Pseudomonas aeruginosa* (39), gut inflammation induced by *Citrobacter rodentium* (40), and airway epithelial pathology induced by pneumococcal neuraminidases (41). Thus, given the broad immune-inhibitory activity of Gal-1 (19), its abundant expression in mucosal tissues (42), and its emerging roles during infection (37, 43, 44), we hypothesized that this endogenous lectin could serve as a homeostatic checkpoint and a mediator of immune evasion during *Y. enterocolitica* infection. Our results suggest that blocking Gal-1 may help to attenuate *Y. enterocolitica* pathogenicity and intestinal pathology by reinforcing immune-protective mechanisms.

Materials and Methods

Mice

C57BL/6 Gal-1–knockout ($Lgals1^{-/-}$) mice were kindly provided by F. Poirier (Institute Jacques Monod, Paris, France). C57BL/6 wild-type (WT) mice were purchased from the Animal Facilities of the National University of La Plata, La Plata, Argentina. Breeding colonies were established at the Animal Facilities of the National University of San Luis. Mice were kept under specific pathogen–free conditions in a cabinet (Ehret, Emmendingen, Germany) and provided with sterile food and water ad libitum. Male or female mice (6–8 wk-old) were used for all experiments. Studies were performed according to institutional and national guidelines.

Bacterial culture and infection

Y. enterocolitica serotype 0:8 (pYV^+, WA-314; kindly provided by G. Kapperud, Oslo, Norway) was used for infection. *Y. enterocolitica* WA-314 deficient in YopH (pYV^+, WA-C pYV *yopH* Nal^r Kan^r) (45) and *Y. enterocolitica* WA-314 deficient in YopP (pYV^+, WA-C pYV Nal^r Kan^r) (45) were developed in I.A.'s laboratory. Bacteria were cultured as described earlier (5). Mice were starved for 2 h and infected orogastrically with $2–5 \times 10^8$ bacteria in 0.2 ml of sterile PBS (pH 7.4) using a gastric tube. Some groups were inoculated with 6 mg/kg aminoguanidine (AG), an inducible NO synthase (iNOS) inhibitor. Control mice were treated with PBS. The number of inoculated bacteria was controlled by plating serial dilutions of the inoculated suspension on Trypticase soy agar. Survival and weight were determined in infected mice. At 5, 14, and 21 d postinfection, serum samples from each mouse were collected according to standard methods. The spleen, PPs, and MLNs were aseptically removed and homogenized in PBS. Then, duplicates of 0.05 ml of serial dilutions of spleen and MLN homogenates were plated on Trypticase soy agar (46), whereas PP homogenates were plated on MacConkey–Igarsan agar. CFU were counted after incubation at 27°C for 48 h. The limit of detectable CFU was 25 ($\log_{10}$ 25 = 1.4) (47). All experiments were repeated at least three times, revealing comparable results.

Stimulation of peritoneal macrophages

Resident peritoneal macrophages were obtained from mice using 5–10 ml of sterile pyrogen-free saline, washed twice by centrifugation at $200 \times g$ for 10 min at 4°C, and resuspended in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS, 5 mM L-glutamine, 50 μM 2-ME, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin. This cellular suspension was then added to each well of a 24-well culture plate to obtain 2×10^6 cells per well. Cells were incubated at 37°C in a 5% CO_2 atmosphere for 24 h and, after washing three times with saline, adherent cells were incubated without (Medium) or with *Y. enterocolitica* WA-314 (*Y. enterocolitica*) (multiplicity of infection, 10:1) in the absence or presence of 5 μM ERK1/2 inhibitor (PD98059) or p38 inhibitor (SB203580; both from Calbiochem, San Diego, CA). Macrophages were incubated for 1 h at 37°C in a 5% CO_2 atmosphere, and 5 μl gentamicin was added. Cells were incubated overnight, and culture supernatants were obtained and immediately used for NO determination or stored at −20°C until Gal-1 determination.

NO and urea determination

Nitrite production, as a stable end product of NO metabolism, was measured in culture supernatants from macrophages that were treated or not for 12 h using the Griess reaction assay. Briefly, 100 μl of culture supernatant was mixed with 100 μl of Griess reagent in a 96-well flat-bottom plate and incubated for 10 min at room temperature. OD_{550} was determined by a plate reader (Bio-Rad, New York, NY). Urea was determined in supernatants of macrophage cultures and in tissue homogenates using the Urea Color 2R kit (Wiener, Rosario, Argentina), following the manufacturer's instructions.

Apoptosis assay

Macrophages isolated from WT or $Lgals1^{-/-}$ mice were infected with *Y. enterocolitica* and incubated for 2 h at 37°C in a 5% CO_2 atmosphere. DMSO was used as a positive control for apoptosis. Cells (1×10^6) were washed twice with PBS and suspended in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$ [pH 7.4]). Macrophages were incubated with Annexin V-FITC (Sigma, San Diego, CA) for 15 min at room temperature in the dark. Cells were washed twice and resuspended in 500 μl of binding buffer. Then, 2.5 μl of propidium iodide was added, and macrophages were analyzed by flow cytometry using a FACSCalibur cytometer (Becton, Dickinson and Company).

SDS-PAGE and Western blot

Homogenates of PPs and spleen, as well as macrophage lysates, were processed for SDS-PAGE and Western blot. Briefly, tissue homogenates were washed with cold PBS, and cells were prepared with lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 10 mM EDTA, 1% [v/v] Nonidet P-40) in the presence of a mixture of protease inhibitors (1:100). After 20 min of incubation on ice, lysates were centrifuged for 15 min at $12{,}000 \times g$ at 4°C. To concentrate proteins, tissue homogenates were precipitated with 4 vol. acetone at −20°C for 6 h. Then, precipitated proteins were centrifuged at $6400 \times g$ for 30 min at 4°C, supernatants were removed, and the pellet was dried. Finally, pellets were dissolved in PBS supplemented with protease inhibitors, and protein concentration was determined using a Bradford assay. SDS-PAGE and Western blot analysis were performed as previously described (23). Equal amounts of protein were resolved using 15% SDS-PAGE. Separated proteins were electroblotted onto PVDF Membranes (Amersham Biosciences, London, U.K.) and probed with rabbit anti–Gal-1 (1:5000 dilution) Ab that was generated as described (48) or goat anti–β-actin (1:3000 dilution) polyclonal Ab. Membranes were incubated with a peroxidase-labeled anti-rabbit IgG or anti-goat IgG and developed using an enhanced chemiluminescence detection kit (Amersham Biosciences) and Kodak BioMax films (Rochester, NY). Films were analyzed using ImageJ software and the intensity of each band was recorded and documented as relative expression.

ELISAs for cytokine determination

Cytokines (IL-17, IFN-γ, IL-6, and TNF) were assessed 5 d postinfection in sera and tissue samples of infected and uninfected WT and $Lgals1^{-/-}$ mice using capture ELISA kits (eBioscience, San Diego, CA), according to the manufacturer's instructions. Cytokine concentrations were normalized to the protein content. Limits of detection were 4 pg/ml for IL-17 and IL-6, 15 pg/ml for IFN-γ, and 8 pg/ml for TNF.

In vivo cytokine blockade

IFN-γ and IL-17 were neutralized in vivo using a hamster anti-mouse IFN-γ mAb (MAB4851) and a rat anti-mouse IL-17 mAb (MAB421; both from R&D Systems), as described (5). The same doses of isotype-control mAbs were injected as controls. Briefly, $Lgals1^{-/-}$ mice were treated in vivo with anti–IFN-γ, anti–IL-17, or isotype-control mAb i.p. (3.2 mg/kg) on days 0 and 3 postinfection. The percentage of mice surviving in each group was recorded. On day 5 postinfection, spleen, PPs, and MLNs were obtained, and CFU were counted as described (5).

In vivo administration of recombinant Gal-1 and neutralizing anti–Gal-1 mAb

Recombinant Gal-1 (rGal-1) was produced and purified as outlined previously (31). An anti–Gal-1–neutralizing mAb was produced, evaluated,

and validated as described (24, 49–51). Four animal groups were used for in vivo rescue experiments: *Lgals1*$^{-/-}$ mice injected i.p. with rGal-1 (3.2 mg/kg) daily for 5 d starting on infection day (day 0), as described (22); *Lgals1*$^{-/-}$ mice injected i.p. with vehicle control; WT mice treated i.p. with anti–Gal-1–neutralizing mAb (5 mg/kg) in 0.2 ml of PBS on the day of infection (day 0) and at day 3 postinfection; and WT mice treated with an isotype-control mAb, as described (24, 49). The percentage of surviving mice, as well as animal weight, were recorded. Mice were killed at day 5 postinfection, and CFU were counted in homogenates of spleen, PPs, and MLNs. Levels of IL-17, IFN-γ, IL-6, and TNF were determined in serum or in PPs, as described above.

NF-κB assay

NF-κB activation was determined in PPs of WT mice treated with anti–Gal-1 or isotype-control mAb and in *Lgals1*$^{-/-}$ mice treated with rGal-1 or vehicle control using a nonradioactive, sensitive method for detection of NF-κB DNA-binding activity in nuclear extracts, following the manufacturer's recommended protocol [NF-κB (p65) Transcription Factor Assay Kit; Cayman Chemical, MI]. Results were expressed as absorbance per 10^6 cells.

Histopathological assessment

For histopathological examination, intestine was collected at day 5 postinfection from mice belonging to each group, fixed in 10% buffered formalin phosphate, embedded in paraffin sections, and stained with H&E.

Statistical analysis

Differences between the groups were tested for significance using the Mann–Whitney *U* test or one-way ANOVA with the Dunnett multiple-comparison test, as appropriate. A p value <0.05 was considered statistically significant. Survival curves were generated using Kaplan–Meier survival analysis. Results are expressed as the mean ± SEM. All statistical analyses were carried out using Prism version 5.0 (GraphPad, La Jolla, CA).

Results

Endogenous Gal-1 controls Y. enterocolitica *infection*

To investigate the relevance of Gal-1 in *Y. enterocolitica*–induced immunopathogenesis, we first analyzed expression of this lectin during the course of *Y. enterocolitica* infection in vivo. We observed substantial upregulation of Gal-1 in PPs and spleen of *Y. enterocolitica*–infected C57BL/6 WT mice compared with noninfected C57BL/6 WT mice ($p < 0.01$ in spleen at day 5, $p < 0.001$ in PPs at day 5, $p < 0.05$ in spleen and PPs at day 7) (Fig. 1A–D). This effect was prevented when mice were infected with *Y. enterocolitica* lacking the effector protein YopP ($p < 0.01$) or YopH ($p < 0.05$) (Fig. 1E). Moreover, in vitro infection of macrophages with *Y. enterocolitica* led to significant upregulation

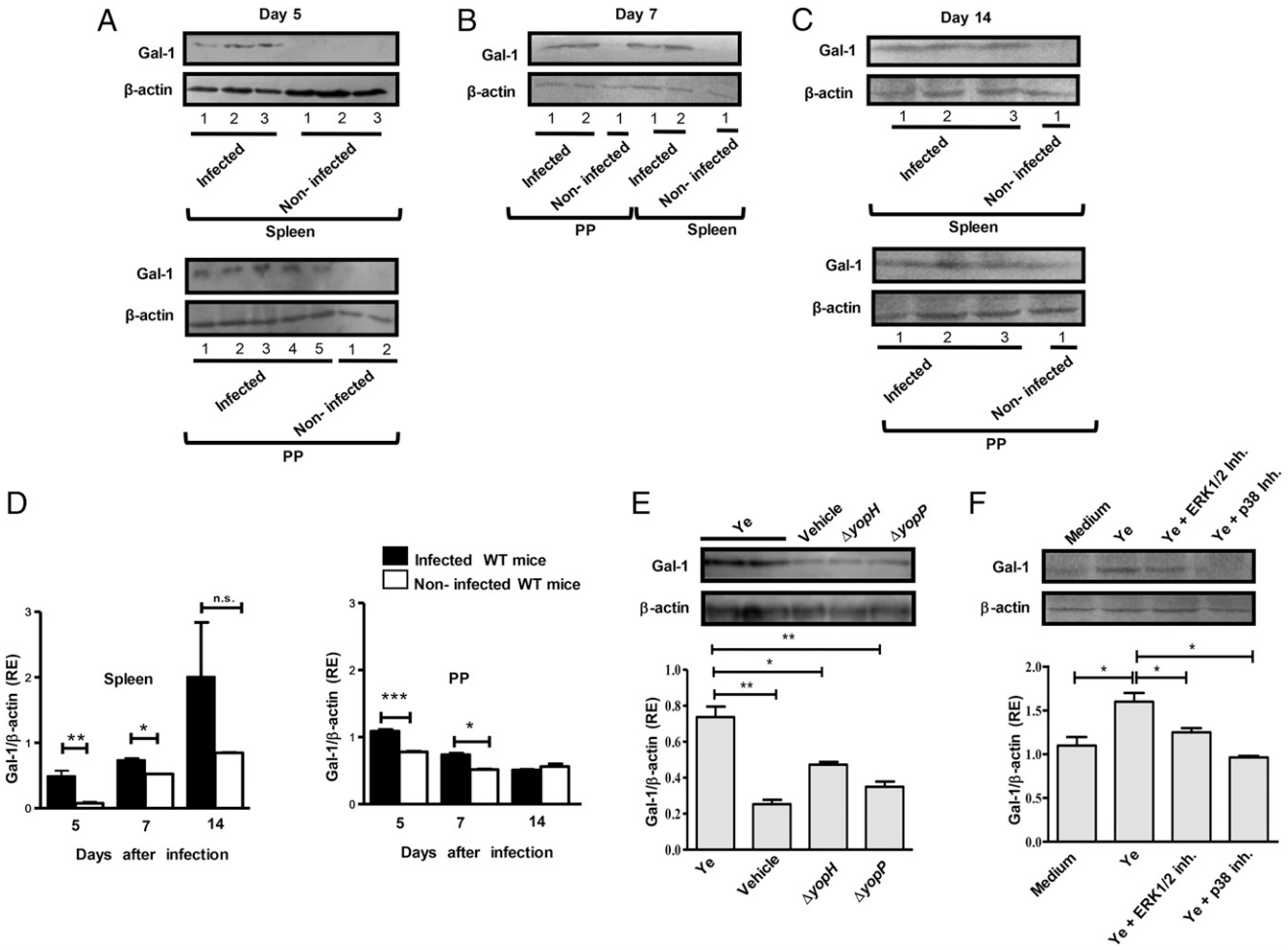

FIGURE 1. *Y. enterocolitica* modulates Gal-1 expression in vivo. WT mice were orogastrically infected or not with 2–5 × 10^8 bacteria. Western blot analysis of Gal-1 expression in tissue homogenates, including PPs and spleen obtained from mice at days 5 (**A**), 7 (**B**), and 14 (**C**) postinfection. Numbers (A–C) represent individual mice. (**D**) Densitometric analysis of Gal-1 relative to β-actin in tissues of infected and uninfected mice. Data are representative of three experiments with three to five mice per group. (**E**) Western blot analysis of Gal-1 expression in lysates of splenocytes obtained 5 d after in vivo infection with *Y. enterocolitica* or *Y. enterocolitica* lacking YopP (Δ*yopP*) or YopH (Δ*yopH*). The relative expression of Gal-1 compared with β-actin was determined. A representative of two independent experiments is shown. (**F**) Western blot analysis of Gal-1 expression in macrophages infected or not (Medium) in vitro with *Y. enterocolitica* in the absence or presence of ERK1/2 or p38 inhibitors. Data are representative of two experiments with three to five mice per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$.

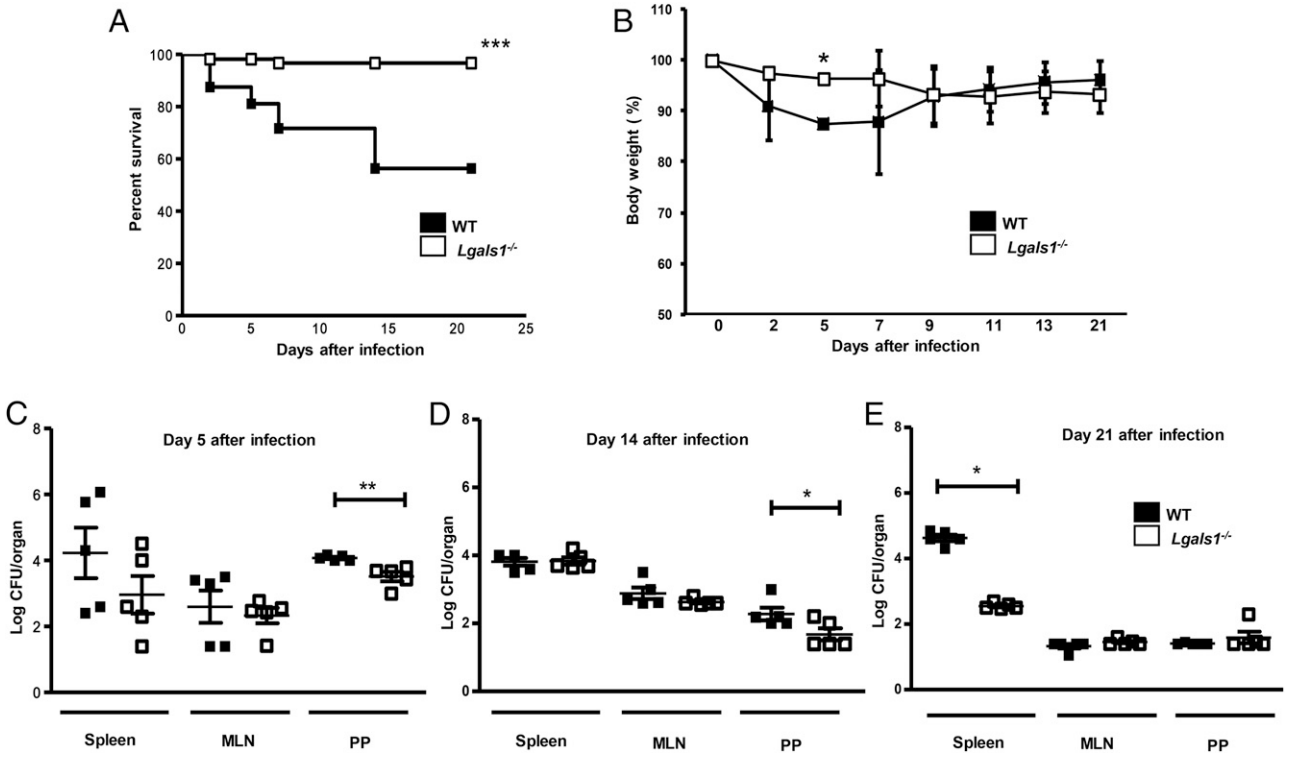

FIGURE 2. Lack of Gal-1 attenuates *Y. enterocolitica* infection. *Lgals1*$^{-/-}$ or WT mice were orogastrically infected with 2–5 $\times$ 10^8 bacteria, and their survival (**A**) and weight (**B**) were determined. Survival curves were generated using Kaplan–Meier survival analysis representing three independent experiments (15 WT and 15 *Lgals1*$^{-/-}$ mice). Survival kinetics was compared using the Mantel–Haenszel log-rank test. (**C**–**E**) *Lgals1*$^{-/-}$ or WT mice were orogastrically infected with 2–5 $\times$ 10^8 bacteria. At days 5 (C), 14 (D), and 21 (E) postinfection, CFU were counted in spleens, MLNs, and PPs. The limit of detectable CFU was 25 ($\log_{10}$ 25 = 1.4). Data are representative of three experiments with five mice per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

of Gal-1 expression ($p < 0.05$); this effect was prevented when ERK1/2 or p38 signaling was interrupted (Fig. 1F, $p < 0.05$). Thus, bacterial infection induces local and systemic upregulation of Gal-1 through mechanisms involving Yops and MAPK signaling.

To evaluate whether Gal-1 expression influences the clinical outcome of *Y. enterocolitica* infection, we compared the survival of infected WT and *Lgals1*$^{-/-}$ mice. We found a significantly higher survival rate in *Lgals1*$^{-/-}$ mice compared with their WT counterparts (Fig. 2A, $p < 0.001$). Moreover, mice lacking Gal-1

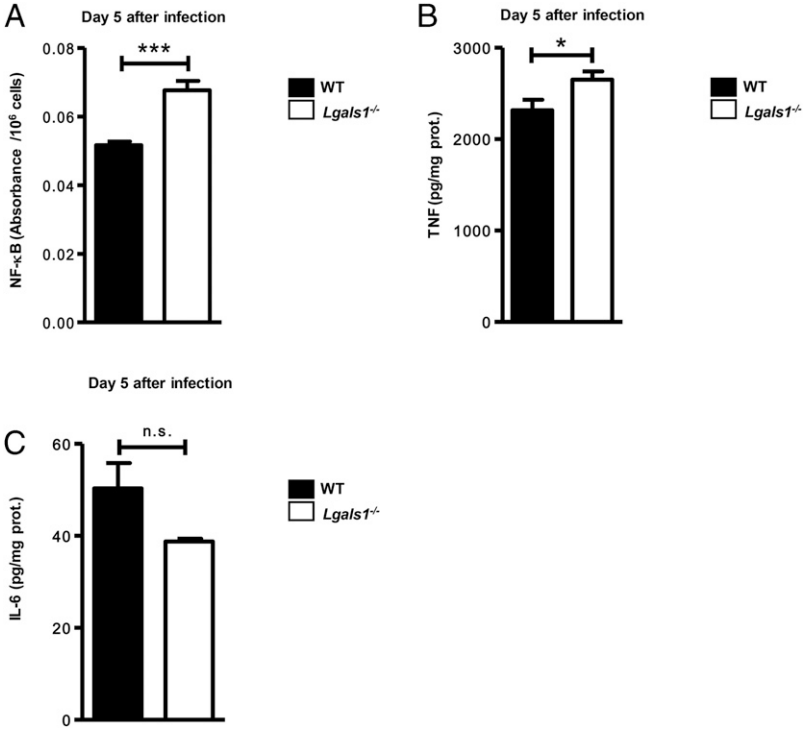

FIGURE 3. Gal-1 controls NF-κB activation and TNF production during *Y. enterocolitica* infection. Homogenates of PPs were obtained from *Lgals1*$^{-/-}$ mice or WT mice after 5 d of infection. Nuclear translocation of NF-κB (**A**), TNF production (**B**), and IL-6 production (**C**) were determined, as described in *Materials and Methods* (n = 5 mice per group). Data are the mean ± SEM of three experiments. *$p < 0.05$, ***$p < 0.001$.

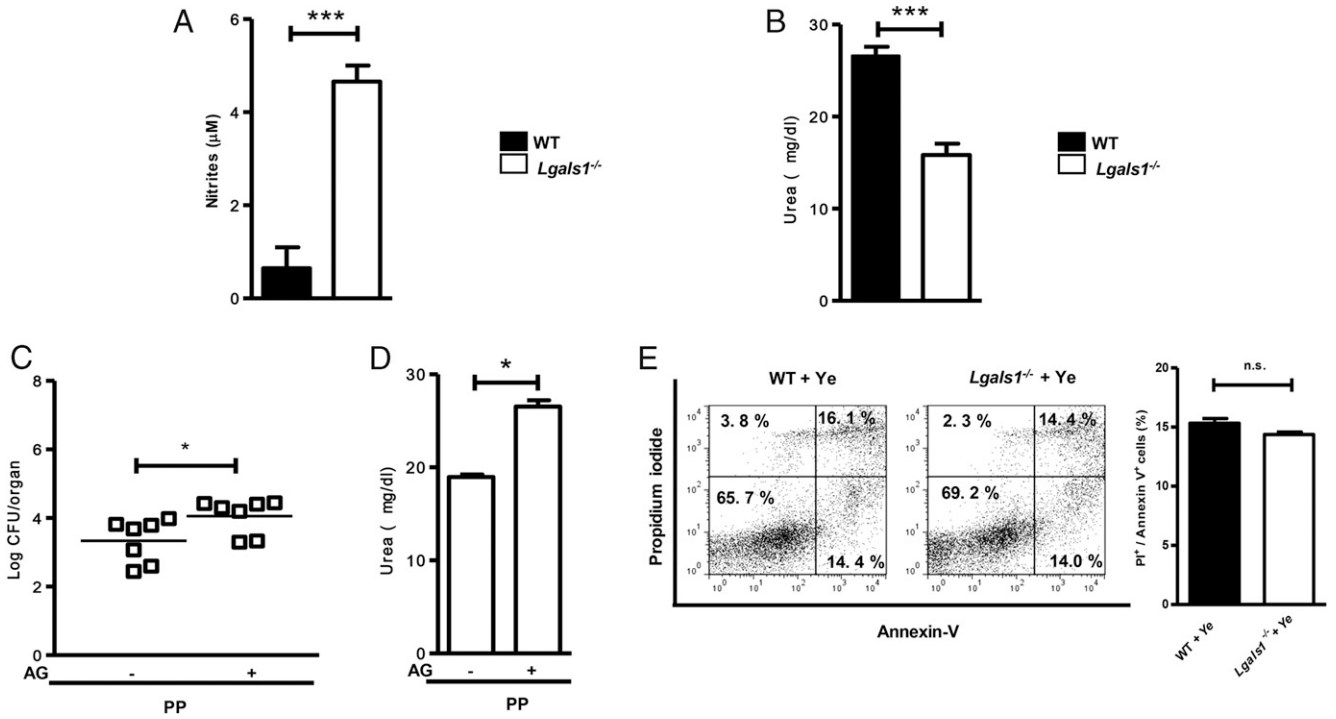

FIGURE 4. Gal-1 differentially modulates NO and urea production in response to in vitro or in vivo infection. Determination of nitrites (**A**) and urea (**B**) in culture supernatants from in vitro–infected peritoneal macrophages isolated from *Lgals1*$^{-/-}$ or WT mice. (**C** and **D**) *Lgals1*$^{-/-}$ mice were orogastrically infected with 2–5 $\times$ 10^8 bacteria and treated with AG (iNOS inhibitor). (C) CFU in PP homogenates at day 5 after *Y. enterocolitica* infection. Limit of detectable CFU was 25 ($\log_{10}$ 25 = 1.4). (D) Determination of urea in PP homogenates at day 5 after *Y. enterocolitica* infection. (**E**) Flow cytometry of annexin-V and propidium iodide staining in macrophages isolated from *Lgals1*$^{-/-}$ or WT mice infected in vitro with *Y. enterocolitica*. The percentage of annexin-V$^+$ propidium iodide$^+$ cells is shown (right panel). Data are the mean $\pm$ SEM (A, B, D, and E, right panel) or are representative (C and E, left panel) of three independent experiments with three to seven mice per group. *$p < 0.05$, *** $p < 0.001$.

displayed higher weight compared with WT mice, reaching statistical significance at day 5 postinfection (Fig. 2B, $p < 0.05$). Furthermore, lower numbers of CFU were detected in PPs, but not in MLNs or spleen, of *Lgals1*$^{-/-}$ mice versus WT mice at 5 and 14 d postinfection ($p < 0.001$, $p < 0.05$, respectively) (Fig. 2C, 2D). However, at 21 d postinfection, differences between Gal-1 mutant and WT mice were evident only in the spleen ($p < 0.05$) (Fig. 2E), suggesting a spatiotemporal regulation of Gal-1 activity that might act locally (at the mucosal level) during the early stages of infection and shift toward a systemic effect at later time periods. Thus, lack of endogenous Gal-1 confers protection against *Y. enterocolitica* infection.

Endogenous Gal-1 controls innate immune pathways during Y. enterocolitica *infection*

To identify possible mechanisms underlying the protective effects of Gal-1 deficiency, we first explored central innate immune pathways involved in *Y. enterocolitica* clearance (52). Five days after *Y. enterocolitica* infection, PP homogenates isolated from *Lgals1*$^{-/-}$ mice showed increased nuclear translocation of the NF-κB transcription factor (Fig. 3A, $p < 0.001$) and higher amounts of TNF (Fig. 3B, $p < 0.001$) compared with PPs from WT mice, suggesting that Gal-1 may act by attenuating mucosal proinflammatory responses. However, no differences were observed in IL-6 production in PPs between WT and mutant strains (Fig. 3C).

Gal-1 modulates L-arginine metabolism in peritoneal macrophages and microglia by skewing the balance from classically activated M1 macrophages (synthesizing high levels of NO) toward alternatively activated M2 macrophages (expressing high levels of arginase) (30, 32). To explore whether modulation of L-arginine metabolism might account for the immunoregulatory activity of this lectin during *Y. enterocolitica* infection, we analyzed the synthesis of NO and urea (a metabolic product of arginase enzymatic activity) in peritoneal macrophages obtained from Gal-1–deficient and WT mice. *Lgals1*$^{-/-}$ macrophages produced considerably higher amounts of NO and lower amounts of urea than did WT mice when infected in vitro with *Y. enterocolitica* (Fig. 4A, 4B, $p < 0.001$). This effect was recapitulated in peritoneal macrophages isolated from in vivo–infected *Lgals1*$^{-/-}$ and WT mice (data not shown). Consistent with these findings, treatment of *Y. enterocolitica*–infected *Lgals1*$^{-/-}$ mice with AG, a selective inhibitor of iNOS activity, prevented the protective effect triggered by Gal-1 deficiency (Fig. 4C, $p < 0.05$). Remarkably, inhibition of NO synthesis led to increased bacterial load ($p < 0.05$) and higher amounts of urea ($p < 0.05$) in PPs from *Y. enterocolitica*–infected *Lgals1*$^{-/-}$ mice (Fig. 4C, 4D). However, no significant differences in apoptosis were detected between in vitro–infected *Lgals1*$^{-/-}$ and WT macrophages (Fig. 4E). Thus, in response to *Y. enterocolitica* infection, endogenous Gal-1 limits antibacterial responses, at least in part, by restraining mucosal proinflammatory circuits and counteracting the classical L-arginine–NO pathway.

Lack of Gal-1 confers protection against Y. enterocolitica *infection through systemic induction of IFN-γ– and IL-17–mediated responses*

The critical role of Th1 and Th17 cells in controlling *Y. enterocolitica* infection (4, 5, 53) and the enhanced sensitivity of these proinflammatory cells to the regulatory effects of Gal-1 (22, 35, 54, 55) prompted us to investigate the contribution of IFN-γ and IL-17 to host protection conferred by Gal-1 deficiency. We found significantly higher levels of IFN-γ and IL-17 in sera collected

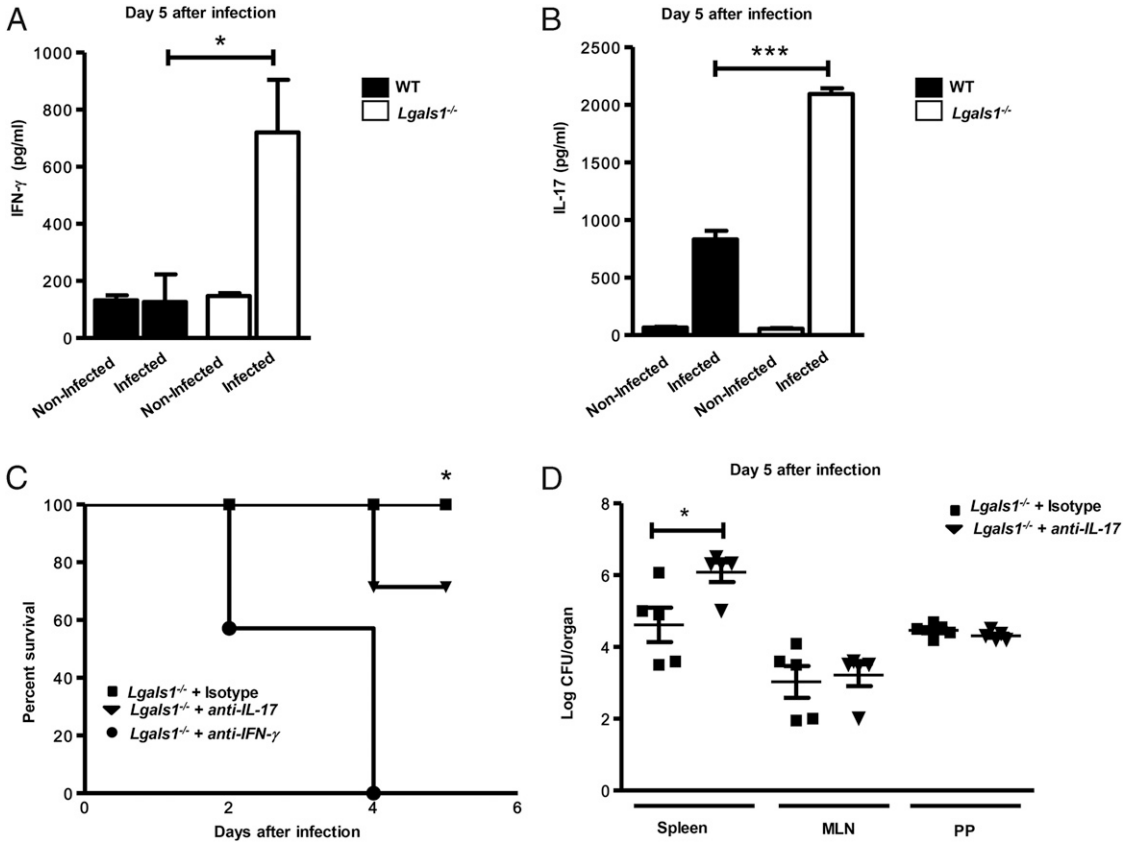

FIGURE 5. Lack of Gal-1 confers protection against *Y. enterocolitica* infection through systemic induction of IFN-γ– and IL-17–mediated responses. *Lgals1*$^{-/-}$ and WT mice were orogastrically infected or not with 2–5 × 10^8 bacteria, and IFN-γ (**A**) and IL-17 (**B**) were determined in sera 5 d later. Bars represent the mean ± SEM of three experiments performed in duplicate. (**C** and **D**) *Lgals1*$^{-/-}$ mice were treated in vivo with anti–IFN-γ– or anti–IL-17–neutralizing mAb (3.2 mg/kg) on days 0 and 3 postinfection. (C) Percentage of mice surviving in each group. (D) Determination of CFU in homogenates of spleen, MLNs, and PPs. Limit of detectable CFU was 25 ($\log_{10}$ 25 = 1.4). Data are representative of three independent experiments (n = 3–5 mice per group). *$p < 0.05$, ***$p < 0.001$.

from *Lgals1*$^{-/-}$ mice compared with WT mice ($p < 0.05$, $p < 0.001$, respectively) 5 d postinfection (Fig. 5A, 5B). These differences were not observed in sera from uninfected mice, regardless of their genotype (Fig. 5A, 5B), suggesting that Gal-1 deficiency may unleash proinflammatory responses following antigenic challenge.

To investigate the relevance of IFN-γ and IL-17 in host protection conferred by Gal-1 deficiency, we treated *Y. enterocolitica*–infected *Lgals-1*$^{-/-}$ mice with anti–IFN-γ– or anti–IL-17–neutralizing mAb and monitored the clinical course of infection. Remarkably, protection against *Y. enterocolitica* infection was completely abrogated in mice receiving an anti–IFN-γ–neutralizing mAb, regardless of Gal-1 deficiency (100% of *Lgals1*$^{-/-}$ infected mice died at day 4 postinfection), whereas *Y. enterocolitica*–infected *Lgals1*$^{-/-}$ mice treated with anti–IL-17 mAb showed only a partial effect (Fig. 5C, $p < 0.05$). Of note, administration of an isotype-control mAb did not affect mice survival, and it preserved the protective effect observed in the absence of Gal-1 (Fig. 5C, 5D). Furthermore, spleens from *Lgals-1*$^{-/-}$ infected mice treated with anti-cytokine mAb showed higher bacterial load compared with those receiving isotype-control mAb (Fig. 5D, $p < 0.05$). These results demonstrate the different roles of IFN-γ and IL-17 in *Y. enterocolitica* clearance and host protection conferred by Gal-1 deficiency.

*Exogenous administration of rGal-1 thwarts protective anti–*Y. enterocolitica *responses triggered by Gal-1 deficiency*

To confirm the direct role of Gal-1 in hindering immunity against *Y. enterocolitica* infection, we explored whether exogenous rGal-1 could override the protective effect observed in *Lgals1*$^{-/-}$ hosts and rescue the mutant phenotype. Compared with *Lgals1*$^{-/-}$ mice receiving vehicle control, *Y. enterocolitica*–infected *Lgals1*$^{-/-}$ mice treated with rGal-1 (3.2 mg/kg daily for 5 d starting on the day of infection) showed reduced protection, as revealed by the increased number of CFU in spleen, PPs, and MLNs (Fig 6A, $p < 0.05$ at day 5 postinfection). Moreover, rGal-1 supplementation in *Lgals-1*$^{-/-}$ mice restored NF-κB activation and TNF synthesis in PPs to levels similar to those attained in WT hosts (Fig. 6B, 6C, $p < 0.001$, $p < 0.05$, respectively). Furthermore, rGal-1 administration suppressed IL-6 production in PPs (Fig. 6D, $p < 0.05$). However, administration of the exogenous lectin was not sufficient to restrain heightened IFN-γ and IL-17 responses triggered in the context of Gal-1 deficiency (Fig. 6E, 6F), suggesting that endogenous Gal-1 might be crucial to counterbalance Th1–Th17 dysregulation as has been reported (22). Nevertheless, exogenous rGal-1 was sufficient to thwart the antibacterial protective effect and counteract proinflammatory mechanisms unleashed in the absence of the endogenous lectin.

Ab-mediated Gal-1 blockade protects mice from Y. enterocolitica *infection*

In the search for a potential adjuvant strategy that could reinforce antibacterial responses, we investigated the effect of Ab-mediated Gal-1 blockade during the course of *Y. enterocolitica* infection. Remarkably, WT mice treated with anti–Gal-1–neutralizing mAb (5 mg/kg given at days 0 and 3 postinfection) showed lower bacterial load in PPs, MLNs, and spleen compared with WT mice treated with isotype-control mAb (Fig. 7A, $p < 0.01$ in PPs, $p < 0.05$

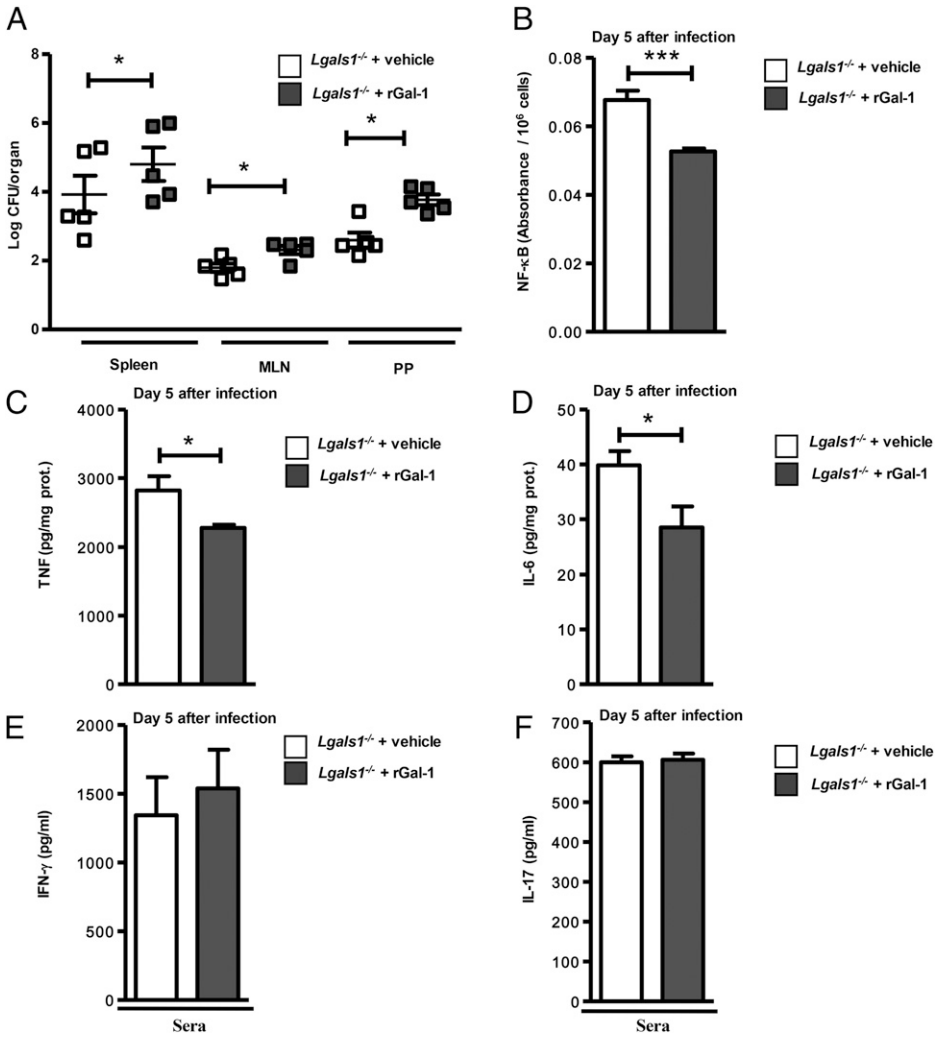

FIGURE 6. Exogenous administration of rGal-1 thwarts protective anti–*Y. enterocolitica* responses triggered by Gal-1 deficiency. (**A**–**F**) *Lgals1*$^{-/-}$ mice were treated i.p. with rGal-1 or vehicle control daily for 5 d starting on the day of infection. (A) Mice were sacrificed at day 5, and CFU were determined in homogenates of spleen, MLNs, and PPs. Limit of detectable CFU was 25 ($\log_{10}$ 25 = 1.4). Data are representative of three independent experiments (n = 5 mice per group). NF-κB translocation (B), TNF production (C), and IL-6 synthesis (D) in PPs from *Lgals1*$^{-/-}$ mice treated with rGal-1 or vehicle control (n = 5 mice per group). Data represent the mean ± SEM of two independent experiments. Determination of IFN-γ (E) and IL-17 (F) at day 5 postinfection in sera from *Lgals1*$^{-/-}$ mice treated with rGal-1 or vehicle control. Data are mean ± SEM of three independent experiments (n = 5–8 mice per group). *$p < 0.05$, ***$p < 0.001$.

in MLNs and spleen). Moreover, Ab-mediated Gal-1 blockade induced greater NF-κB activation in PPs from *Y. enterocolitica*–infected WT mice compared with isotype-treated groups (Fig. 7B, $p < 0.01$), showing comparable levels as those attained in PPs from *Y. enterocolitica*–infected *Lgals1*$^{-/-}$ mice (Fig. 3A). Histopathological assessment revealed a more prominent mononuclear cell infiltrate in PPs from anti–Gal-1–treated *Y. enterocolitica*–infected WT mice, similar to that observed in *Y. enterocolitica*–infected *Lgals1*$^{-/-}$ animals (Fig. 7C). Finally, higher levels of IFN-γ and IL-17 were detected in WT mice receiving the anti–Gal-1 mAb (Fig. 7D, 7E, $p < 0.05$) compared with those given isotype control. Collectively, these results validate the therapeutic potential of Gal-1 blockade as an adjuvant mechanism to reinforce host protective immunity during bacterial infection.

Discussion

Negative-regulatory pathways function as homeostatic signals that control unresolved inflammation and curb tissue damage while limiting protective immune responses (56). These pathways include the so-called "inhibitory checkpoints," which may be usurped by pathogens to subvert innate and adaptive immune mechanisms (56). Galectins, a family of soluble glycan-binding proteins, have emerged as regulatory checkpoints that are secreted to the extracellular medium through an unconventional pathway and control immune cell fate by interacting with relevant glycosylated receptors, including CD45, CD43, T-cell Ig and mucin-domain containing-3, CTLA-4, and lymphocyte activation-gene-3 (19). Gal-1, a prototype member of this family, is expressed by a wide variety of cell types, including macrophages (57), activated T cells (58), activated B cells (59), and DCs (29), and is highly represented in privileged and mucosal tissue (20, 42). This endogenous lectin contributes to disarm host immune mechanisms by promoting contraction of the Th1/Th17 compartment (22), favoring expansion of Foxp3^{+} and Foxp3^{-} Tregs (20, 28, 35, 36), deactivating macrophages and microglia (30–32), and inducing tolerogenic DCs (29). These regulatory circuits, hierarchically driven by Gal-1, have been investigated in settings of cancer (23, 36, 60), fetal loss (61, 62), autoimmunity (20–22), and parasite infection (28).

In this study, we identified a central role for Gal-1 in *Y. enterocolitica* immunopathogenesis using loss-of-function and gain-of-function experiments. Oral *Y. enterocolitica* administration induced considerable upregulation of Gal-1 in PPs and spleen through a YopP/YopH-dependent mechanism. Lack of Gal-1 conferred protection against *Y. enterocolitica* infection by reinforcing innate and adaptive immune programs, including NF-κB activation, TNF and NO production, and heightened expression of IFN-γ and IL-17. This protective effect was substantiated by supplementation of rGal-1 into *Y. enterocolitica*–infected *Lgals1*$^{-/-}$ mice or by treatment of infected WT mice with a neutralizing anti–Gal-1 mAb. Similarly, in a model of corneal immunopathology induced by *P. aeruginosa*, local treatment with rGal-1 attenuated corneal inflammation through mechanisms involving reduced Th17 responses and expansion of Th2 cells (39). Moreover, previous studies showed that influenza infection predisposes airway epithelial cells

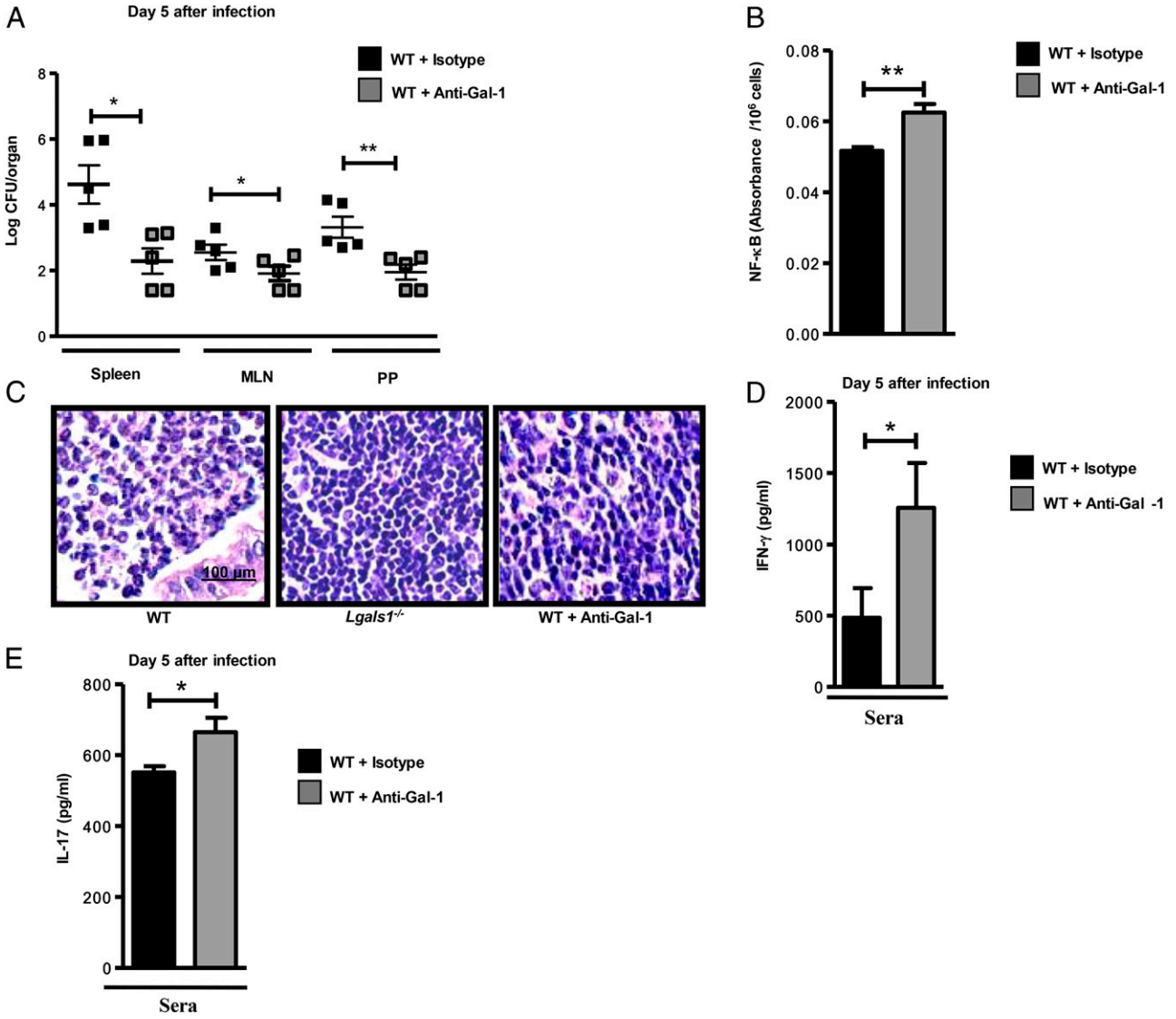

FIGURE 7. Ab-mediated Gal-1 blockade protects mice from *Y. enterocolitica* infection. (**A**–**E**) WT mice were infected with *Y. enterocolitica* and treated with anti–Gal-1–neutralizing mAb or isotype control (5 mg/kg at days 0 and 3 postinfection). (A) Mice were sacrificed at day 5 after *Y. enterocolitica* infection, and CFU were recorded in spleen, MLN, and PP homogenates. Limit of detectable CFU was 25 ($\log_{10}$ 25 = 1.4). One representative of three independent experiments is shown (n = 5 mice per group). (B) Translocation of NF-κB in PPs from WT mice treated with anti–Gal-1 mAb or isotype control (n = 5 mice per group). Results are the mean ± SEM of two independent experiments. (C) Histopathological analysis (H&E stain original magnification ×100) of PPs from WT mice, *Lgals1*$^{-/-}$ mice, and anti–Gal-1–treated WT mice 5 d after *Y. enterocolitica* infection. Determination of IFN-γ (D) and IL-17 (E) 5 d postinfection in sera from WT mice treated with anti–Gal-1–neutralizing mAb or isotype control. Data are the mean ± SEM of three independent experiments (n = 5–8 mice per group). *$p < 0.05$, **$p < 0.01$.

to *Streptococcus pneumoniae* infection via release of neuraminidases with subsequent exposure of Gal-1–specific glycan epitopes (41), suggesting a broader role for Gal-1–glycan interactions in bacterial infection. In this regard, Gal-1 may also facilitate infection by other pathogens, including HIV (63), dengue virus (64), and *Trichomonas vaginalis* (65), whereas it confers protection against Nipah virus (66) and can differentially regulate *Trypanosoma cruzi* infection at different stages of the disease (28, 67). These data highlight the diverse roles of Gal-1 in microbial invasion and pathogenesis through immune- and nonimmune-mediated mechanisms.

Glycosylation controls a variety of signaling programs, including those operating in immune cell signaling and host–pathogen interactions (38). Because bacteria and eukaryotic cells possess *O*- and *N*-linked glycans (68, 69), glycan-binding proteins may serve as a bridge that regulates bacterial infection, internalization, and immunity. Whether YopP/YopH associate with endogenous Gal-1 to control *Y. enterocolitica* pathogenesis should be further explored. Following this direction, future efforts should be aimed at analyzing glycosylation profiles of *Yersinia* virulence factors and their specific interactions with endogenous galectins. Interestingly, recent studies showed that epithelial cell glycosylation is regulated by gut microbes and by group 3 innate lymphoid cells (70), suggesting the possibility that, during infection, *Y. enterocolitica* could also alter the glycosylation profile of enterocytes, M cells, macrophages, and/or T cells, favoring exposure of Gal-1–specific glycan epitopes. In this regard, we previously showed that differential glycosylation of Th1, Th17, and Th2 cells may selectively regulate their viability by unmasking or masking Gal-1–specific ligands (22). Accordingly, we found higher levels of IFN-γ and IL-17 in serum from *Y. enterocolitica*–infected *Lgals1*$^{-/-}$ mice, as well as in WT mice treated with a neutralizing anti–Gal-1 mAb; this effect was a critical determinant of host protection, because specific blockade of these

proinflammatory cytokines increased local and systemic bacterial load, regardless of the absence of this lectin. However, supplementation of rGal-1 into infected Gal-1–deficient hosts did not rescue systemic Th1/Th17 dysregulation, suggesting that a combination of exogenous and endogenous Gal-1 may be required to achieve full suppression, as has been previously shown in experimental autoimmune settings (20, 22). Although Gal-1 deficiency promoted upregulation of IFN-γ and IL-17 and prolonged mice survival, systemic blockade of IFN-γ completely rescued this phenotype, whereas IL-17 neutralization induced a partial effect (Fig. 5A–C). Interestingly, previous studies reported selective protective roles for IFN-γ (5, 71) and IL-17 (4, 5, 53) during *Y. enterocolitica* infection, depending on preferential TLR activation and predominant routes of infection (4). Moreover, recent studies showed that *Y. pseudotuberculosis* supports Th17 expansion, whereas it limits Treg differentiation by directly interfering with TCR signaling (72).

Remarkably, host protection induced by Gal-1 deficiency involved increased NO synthesis and decreased urea production in PPs of *Y. enterocolitica*–infected mice. These data are in agreement with previous findings on the ability of exogenous Gal-1 to skew the balance from classically activated M1 macrophages/microglia toward an alternatively activated M2 phenotype (30, 32). These results are relevant in the context of *Yersinia* infection because *Y. enterocolitica* and *Y. pseudotuberculosis* control NO production in murine macrophages, with YopJ, YopE, and YopH being responsible for such effects (8, 73, 74). In this regard, although *Y. enterocolitica* can trigger apoptosis of infected macrophages (11, 12, 15, 16), Gal-1 did not seem to mediate this effect (Fig. 4E). Moreover, lack of Gal-1 promoted greater activation of the NF-κB transcription factor and release of TNF, an NF-κB–regulated cytokine, in mucosal tissue of *Y. enterocolitica*–infected mice. Supporting these findings, a regulatory loop has been reported by which NF-κB and MAPKs induce Gal-1 expression in T cells; this, in turn, leads to inhibition of NF-κB signaling (58, 75). However, recent studies in a setting of osteoarthritis demonstrated that exogenous Gal-1 could also activate an NF-κB–regulated gene network in chondrocytes (76), suggesting that this lectin may differentially control pro- or anti-inflammatory gene expression in different cell types and physiologic/pathologic settings. In this context, recent studies suggested that TNFR p55 acts as a regulatory switch that limits *Y. enterocolitica*–induced reactive arthritis by modulating IL-6, NO, and NF-κB pathways and controlling Treg expansion (5, 8, 77). Because these molecular and cellular pathways are preferred targets of the immunoregulatory activity of Gal-1, a possible cross-talk between TNFR p55 signaling and this endogenous lectin might contribute to hierarchically dissect the immunopathogenesis of *Y. enterocolitica* infection and the underlying tissue damage. Interestingly, although endogenous Gal-1 controlled NF-κB activation and TNF synthesis, it had no apparent role in IL-6 production during the time period analyzed (Fig. 3). However, TNF and IL-6 were downregulated when exogenous Gal-1 was administered to *Lgals1*$^{-/-}$ mice (Fig. 6C, 6D). This distinctive effect could be related to different kinetics of cytokine synthesis and regulation in response to endogenous versus exogenous Gal-1.

In conclusion, our study highlights the involvement of host Gal-1 as a critical mediator of the immune inhibitory activity of *Y. enterocolitica* in vivo. By targeting innate and adaptive immune compartments, Gal-1 limits bacterial clearance and curtails mice survival. Disruption of Gal-1–glycan interactions, using a neutralizing anti–Gal-1 mAb, conferred protection against *Y. enterocolitica* infection by reprogramming antibacterial immunity. This study may have broad therapeutic implications for strengthening immune responses in a wide range of enteropathogenic microbial infections.

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Disclosures

The authors have no financial conflicts of interest.

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