Galectin-1 Suppresses Experimental Colitis in Mice

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Background & Aims: **Uncontrolled T-cell activation plays a critical role in the pathogenesis of inflammatory bowel diseases. Therefore, pharmacologic strategies directed to restore the normal responsiveness of the immune system by deleting inappropriately activated T cells could be efficacious in the treatment of these pathologic conditions. Galectin-1 is an endogenous lectin expressed in lymphoid organs that plays a role in the maintenance of central and peripheral tolerance. The aim of the present study was to evaluate the therapeutic effects of galectin-1 on T-helper cell type 1-mediated experimental colitis induced by intrarectal administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS) in mice.** ***Methods:*** **Cells and tissues from mice with TNBS colitis receiving treatment with several doses of human recombinant galectin-1 (hrGAL-1) were analyzed for morphology, cytokine production, and apoptosis.** ***Results:*** **Prophylactic and therapeutic administration of rhGAL-1 resulted in a striking improvement in the clinical and histopathologic aspects of the disease. hrGAL-1 reduced the number of hapten-activated spleen T cells, decreased inflammatory cytokine production, and profoundly reduced the ability of lamina propria T cells to produce IFNγ in vitro. Moreover, hrGAL-1 led to the appearance of apoptotic mononuclear cells in colon tissue when administered in vivo and induced selective apoptosis of TNBS-activated lamina propria T cells in vitro.** ***Conclusion:*** **Collectively, these data show that hrGAL-1 exerts protective and immunomodulatory activity in TNBS-induced colitis and it might be effective in the treatment of inflammatory bowel diseases.**

Inflammatory bowel diseases (IBD), encompassing Crohn's disease (CD) and ulcerative colitis, are idiopathic chronic inflammatory conditions occurring with increasing frequency in western populations.[1] Although the etiology of IBD remains unknown, there is circumstantial evidence to link this condition to a failure of the mucosal immune system to attenuate the immune response to endogenous antigens, such as the normal enteric bacterial flora.[2] Support for this view has come from animal models of colitis, including the hapten model of colonic inflammation induced by intrarectal delivery of 2,4,6-trinitrobenzene sulfonic acid (TNBS), which consistently exhibit an imbalance of regulatory cytokines, most notably an excessive production of T helper cell (Th)-1–derived cytokines.[3,4]

Because activation-induced cell death of antigen-activated lamina propria (LP) T cells represents the crucial mechanism involved in the attenuation of the mucosal immune response, it has been postulated that a defective activation-induced cell death may be the key factor for the inappropriate mucosal T-cell accumulation observed in IBD and experimental colitis.[5] This scenario is supported by the recent demonstration that LP T cells isolated from CD patients are resistant to multiple apoptotic signals[6–8] and that the beneficial effects of anti-TNFα antibodies in CD are mediated by promoting mucosal T-cell death.[9] In addition, treatment with anti–IL-12 and anti–IL-6 receptor monoclonal antibodies (mAbs) suppresses experimental colitis in mice by inducing LP T-cell death.[3,8] Therefore, deletion of inappropriately activated LP T cells might be a therapeutic target to counteract locally overshooting immune system in IBD.

Galectin-1, a 14.5-kilodalton homodimer, is a member of a family of β-galactoside binding proteins that share growth regulatory and immunomodulatory activities.[10,11] Galectin-1 is constitutively expressed by smooth cardiac and skeletal muscles, thymus, kidney, and placenta; a number of galectin-1 receptors have been identified, including laminin, fibronectin, and the hematopoietic cell surface membrane proteins CD43 and

Abbreviations used in this paper: ELISA, enzyme-linked immunosorbent assay; FITC, fluorescence isothiocyanate; hrGAL, human recombinant galectin; IBD, irritable bowel disease; IFNγ, interferon gamma; IL, interleukin; IL2R, interleukin 2 receptor; LP, lamina propria; mAb, monoclonal antibody; MPO, myeloperoxidase; PCR, polymerase chain reaction; Th, T-helper; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TNFα tumor necrosis factor alpha.

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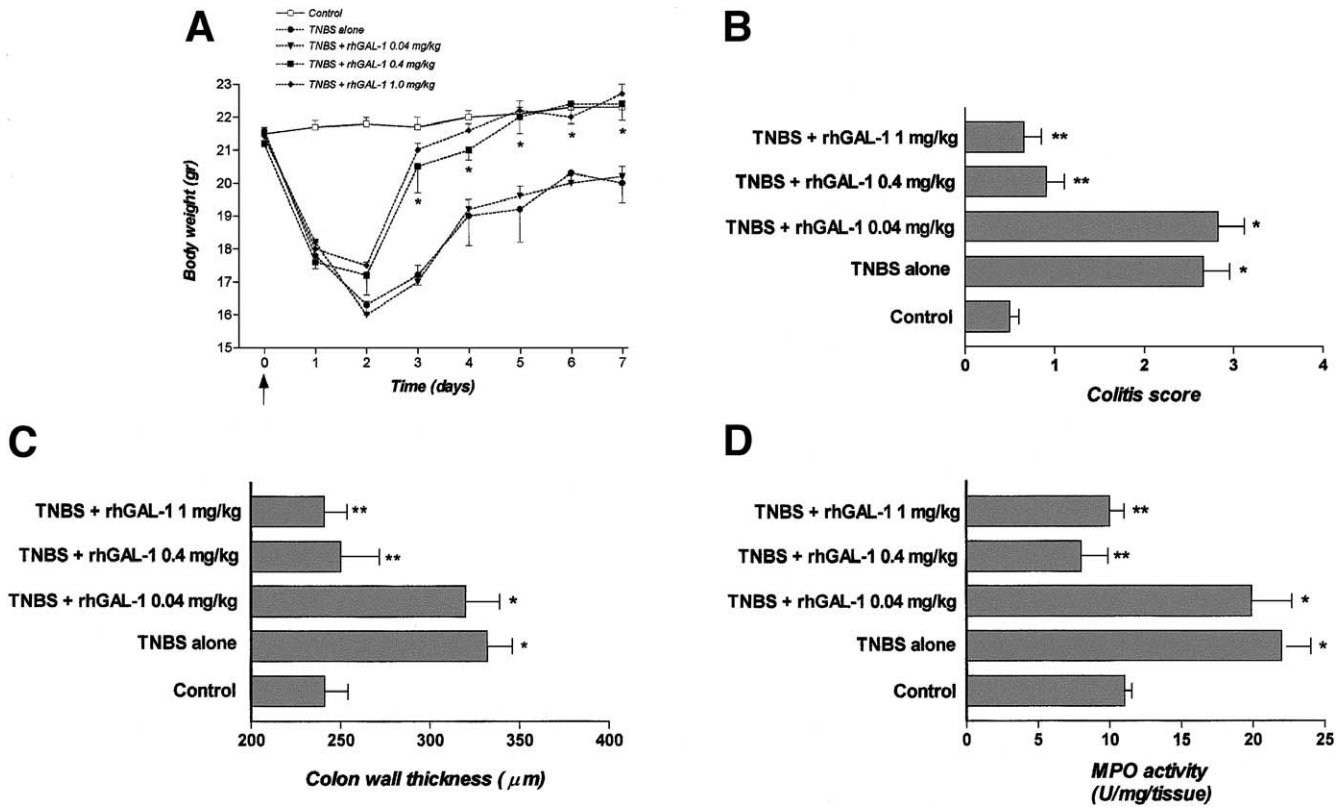

Figure 1. Early administration of rhGAL-1 protects against development of TNBS-induced colitis. Balb/c mice were given 2 mg TNBS intracolonically and rh-GAL1 was administered IV daily for 7 days, starting at the same time of intrarectal instillation of TNBS. (*A*) Wasting disease in mice with TNBS colitis is improved by rhGAL-1 in a dose-dependent manner. Each point represents the mean ± SE of 8–12 mice. $*P < 0.05$ versus TNBS alone and mice treated with 0.04 mg/kg rhGAL-1. (*B, C,* and *D*) Severity of TNBS-induced inflammation (microscopic score and MPO activity) is reduced by rhGAL-1 administration in a dose-dependent manner. *Bars* represent the mean ± SE of 8–12 mice. $*P < 0.05$ versus control (ethanol-treated) mice; $**P < 0.05$ versus mice treated with TNBS alone and mice treated with 0.04 mg/kg rhGAL-1.

CD45.[12,13] Galectin-1 has been shown to induce apoptosis of a subset of negatively selected $CD4^{lo}CD8^{lo}$ immature thymocytes and activated mature T lymphocytes, suggesting that this endogenous lectin is involved in generating and maintaining central and peripheral tolerance.[12–18] Recently, we have provided evidence that galectin-1 exerts immunomodulatory activity in vivo, suppressing disease in 2 experimental models of T-cell mediated diseases: collagen-induced arthritis in mice and concanavalin A-induced hepatitis in mice.[19,20] In these models, galectin-1 administration resulted in a selective elimination of antigen-activated T cells and in a Th1 to Th2 shift that induced a remission state in the evolution of the ongoing inflammatory disease.[19,20] We have now extended this finding by demonstrating that galectin-1 exerts therapeutic activity in TNBS-induced colitis in mice by eliminating the uncontrolled Th1 response to the hapten. Because this experimental model has a number of clinical, histologic, and immunologic analogies with CD, these data suggest the potential use of galectin-1 for the treatment of this disease.

Materials and Methods

Animals

Balb/c mice were obtained from Charles River (Monza, Italy). They were group-housed under controlled temperature (22°C) and photoperiod (12:12-hour light-dark cycle). The mice were allowed unrestricted access to standard mouse chow and tap water. They were allowed to acclimate to these conditions for at least 5 days before inclusion in an experiment. Protocols were approved by the Animal Study Committees of the University of Perugia according to governmental guidelines for animal care.

Induction of Colitis and Study Design

Colitis was induced in Balb/c mice as previously described.[3,21] Briefly, one-day fasted mice were anesthetized with halothane and O_2 and a 3.5F catheter was carefully inserted into the colon via the anus until approximately the splenic flexure (4 cm from the anus). To induce colitis, 2 mg of the hapten reagent TNBS (Sigma Chemical Co, Milan, Italy) in 50% ethanol (to break the intestinal epithelial barrier) was slowly administered into the lumen via the catheter fitted onto

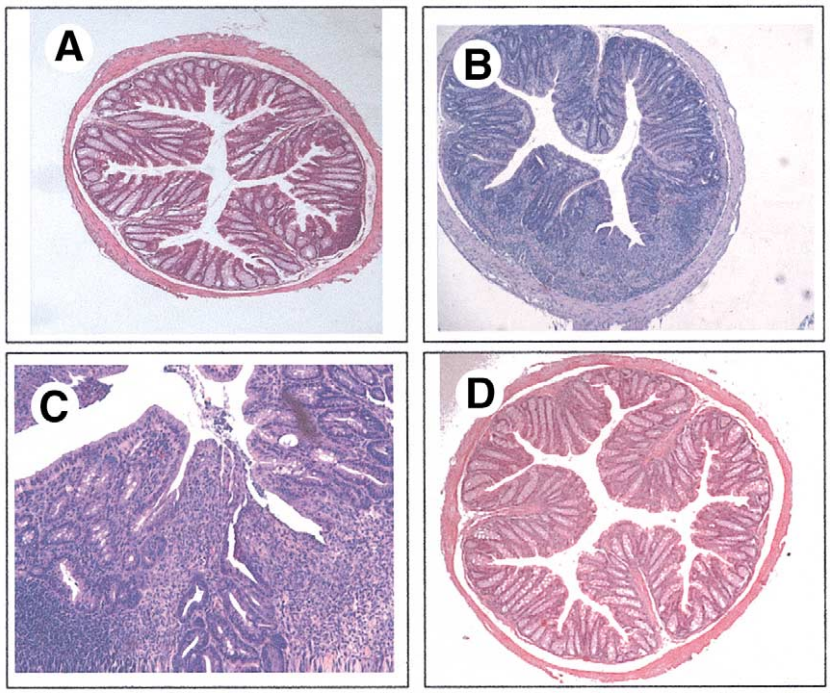

Figure 2. Early administration of rhGAL-1 protects against the development of TNBS-induced colitis. Histologic analysis (H&E staining) of the colon from mice killed 7 days after intrarectal instillation of TNBS, with or without treatment with rhGAL-1. (*A*) H&E-stained paraffin section of control (ethanol-treated) mouse (original magnification, 100×). (*B*) (Original magnification, 100×.) (*C*) (Original magnification, 400×.) H&E-stained paraffin section from a mouse killed 7 days after TNBS administration alone, showing thickening of the colon wall and massive inflammatory infiltrate in the lamina propria. (*D*) (Original magnification, 100×.) Effect of daily administration of 1 mg/kg rhGAL-1 on histologic colitis induced by TNBS. Subepithelial edema with no inflammatory infiltrate in the mucosa and submucosa is shown.

a 1-mL syringe (injection volume, 100 μL). In control experiments, mice received 50% ethanol alone using the same technique. The effect of recombinant human galectin-1 (rhGAL-1) on TNBS-induced colitis was investigated in 2 different protocols. To assess whether early administration of rhGAL-1 protects against development of colitis (prophylactic protocol), mice receiving 2 mg TNBS were randomized to receive saline or rhGAL-1. rhGAL-1 was given IV following TNBS injection and daily thereafter for 7 days at the doses of 0.04, 0.4, and 1 mg/kg. Mice were killed at day 7 and their colon analyzed. To address whether rhGAL-1 treatment was beneficial in treating established colitis (therapeutic protocol), administration of rhGAL-1 was started 2 weeks after colitis induction. Mice were treated IV daily for 1 week with 1 mg/kg

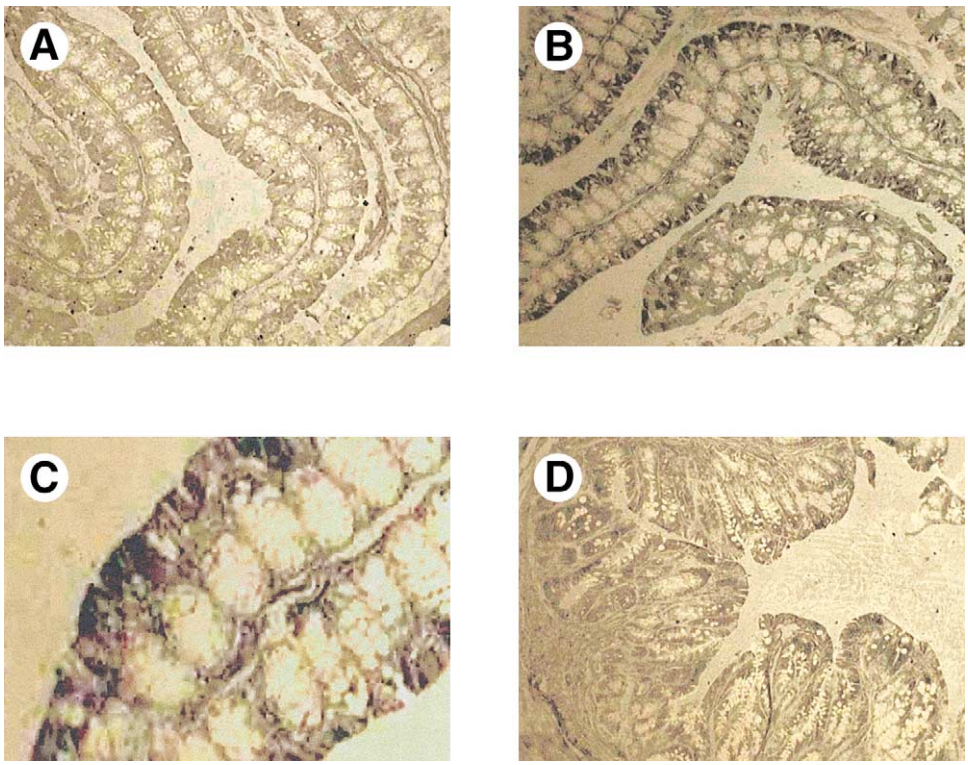

Figure 4. Immunodetection of endogenous galectin-1 by immunogold staining. (*A*) No labeling was detected in colon sections using a normal rabbit serum (original magnification, 100×). (*B*) (Original magnification, 100×.) and (*C*) (Original magnification, 400×.) A strong staining was detected, mainly at the level of the epithelial cells, using a rabbit anti–galectin-1 polyclonal antibody. (*D*) Galectin-1 staining was less pronounced in colon sections from a mouse killed 7 days after intrarectal instillation of TNBS.

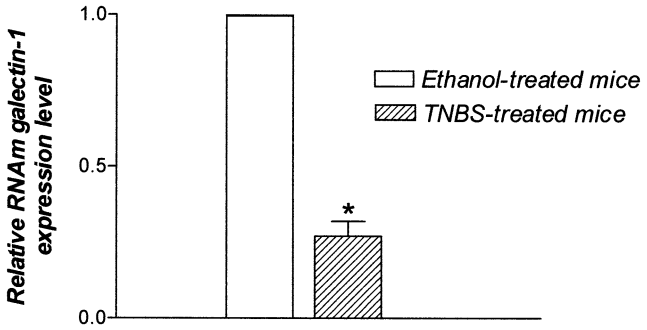

Figure 3. Real-time quantitative PCR of galectin-1 expression in colonic tissues. Intrarectal TNBS instillation resulted in a 4-fold decrease in galectin-1 colonic mRNA content. The means of triplicate determinations are shown. Data was normalized against 18S ribosomal RNA. $*P < 0.05$ versus control (ethanol-treated) mice.

rhGAL-1 or saline. At day 21 mice were sacrificed and their colon analyzed.

Real-Time Reverse Transcription-Polymerase Chain Reaction (PCR)

Mice were sacrificed and the colons were removed, immediately snap-frozen on liquid nitrogen, and stored at −80°C until used. Total RNA was isolated by using the TRIzol reagent (Life Technologies, Milan, Italy) as previously described.[21] PCR was performed using specific primers, designed using software PRIMER3 (Whitehead Institute, Cambridge, MA) with published sequence data from the National Center Biotechnology Information database. Primers were synthesized by Sigma Genosys (The Woodlands, TX). For mouse RNA 18s, the sense primer was 5′-ACA CGG ACA GGA TTG ACA GAT T -3′ and antisense 5′- CGT TCG TTA TCG GAA TTA ACC A-3′. For mouse galectin-1, the sense primer was: 5′ -TGA ACC TGG GAA AAG ACA GC- 3′ and the antisense was 5′ -TCA GCC TGG TCA AAG GTG AT- 3′. In control experiments with 3 replicates, no false positives were detected. Amplification reactions contained 5 μL cDNA, 12.5 μL of the 2 × Quanti tect SYBR Green reverse transcription-PCR Master Mix (Qiagen, Milan, Italy), and 0.75 μL of each of the specific primers. Primer concentrations in a final volume of 25 μL were 300 nmol/L for galectin-1 and 100 nmol/L for β-actin (housekeeping gene). All reactions were performed in triplicate in an iCycler iQ system (Biorad, Hercules, CA) and the thermal cycling conditions were: 15 minutes at 95°C, followed by 50 cycles of 95°C for 10 seconds and 60°C for 30 seconds. We used the expression of RNA 18s to normalize the expression data of galectin-1 cDNA. RNA 18s was used to correct for the differences in the amount of total RNA added to a reaction and to compensate for different levels of inhibition during reverse transcription of RNA and during PCR.

Immunohistochemistry for Endogenous Galectin-1 Expression

For immunohistochemical staining, paraffin sections were mounted on glass slide coated with 1% polylisine, deparaffinized with xilene, rehydrated and incubated with a rabbit anti–galectin-1 polyclonal antibody (diluted 1:1000 in 1% bovine serum albumin-PBS) for 24 hours at 4°C. The polyclonal anti–galectin-1 antibody, obtained as previously described,[22] was specific because it did not recognize other galectins, such as galectin-3.[23] Samples without primary antibody served as negative control. After 3 washes with PBS, slides were incubated with a 1:6 dilution of the anti-rabbit IgG-gold complex for 1 hour at room temperature. Colloidal gold particles (average diameter, 16 nm) were prepared as previously described, using sodium citrate as a reducing agent.[24] A silver enhancement kit (Sigma Chemical Co, St. Louis, MO) was used to visualize the gold particles at the light-microscopic level. After washing with tridistilled water, the sections were mounted on glass slides and studied in a Axioskop Zeiss (Germany). Photographs were taken with a Hitachi CDD Color Camera (Japan).

Histological Grading of Colitis

For histologic examination, tissues were removed, fixed in 10% buffered formalin phosphate, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). The degree of inflammation on microscopic cross sections of the colon was graded semiquantitatively from 0 to 4 (0, no signs of inflammation; 1, very low level; 2, low level of leukocyte infiltration; 3, high level of leukocyte infiltration, high vascular density, thickening of the colon wall; and 4, transmural infiltration, loss of goblet cells, high vascular density, thickening of the colon wall). Grading was performed in a blinded fashion by 2 experienced pathologists. Thickness of the colon wall was determined on cross sections by measuring the distance from the serosal surface to the luminal surface at 2-mm intervals along the entire length of each section by using an Olympus BX60 microscope (Olympus Co, Tokyo, Japan). Images were captured by a digital camera (SPOT-2, Diagnostic Instruments Inc, Burroughs, MI) and analyzed by a specific software (Delta Sistemi, Rome, Italy).

Cytokine and Myeloperoxidase (MPO) Assays

Immunoreactive murine TNFα, IFNγ, IL-12, and IL-1β were quantified in plasma and colon homogenates by using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D Systems, Abingdon, UK). Neutrophil infiltration in the colon was monitored by measuring tissue MPO activity using a spectrophotometric assay with tetramethylbenzidine as substrate, according to a previously published method.[25]

Flow-Cytometric Analysis of Spleen T Cells

Mice were killed 7 days after TNBS or ethanol injection, and the spleen removed. After monocyte elimination by adhesion and lysis of red cells with 0.15 mol/L NH_4Cl and 1mmol/L $KHCO_3$, lymphocytes were resuspended in RPMI/

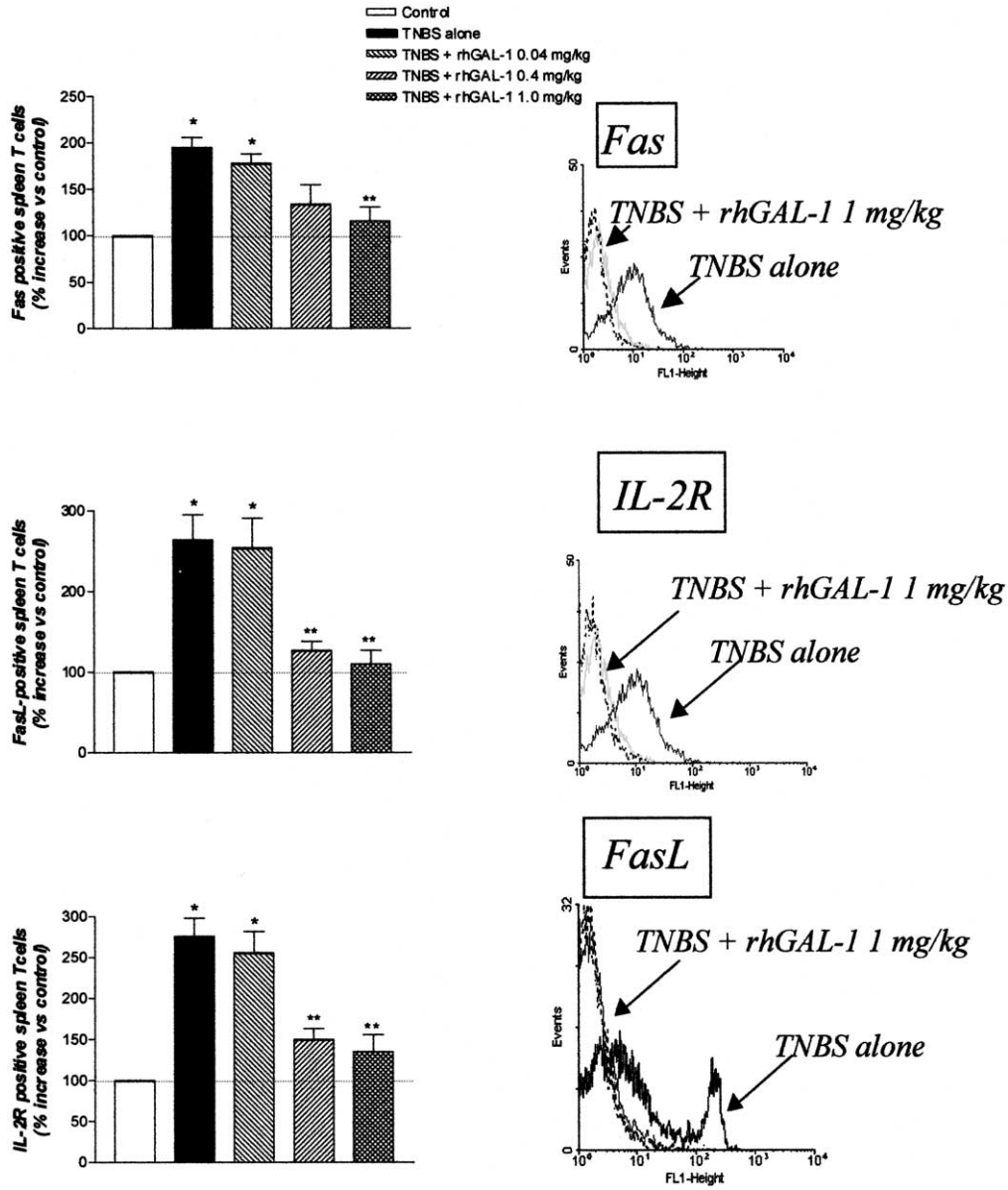

Figure 5. Early administration of rhGAL-1 markedly reduces the percentage of TNBS-activated spleen T cells. Cell surface analysis by flow cytometry of antigens characteristic of T-cell activation (Fas, FasL, and IL2R). Spleen T cells were isolated from mice killed 7 days after intrarectal instillation of TNBS in 50% ethanol or ethanol alone. rhGAL-1 or saline were administered IV daily. Bars represents mean ± SE of 5 mice/group. Representative cytofluorimetric studies are shown: cells were incubated with (solid line) or without (dotted line) FITC-labeled anti-mouse Fas, IL-2R, and FasL mAbs. $*P < 0.05$ versus control (ethanol-treated) mice; $**P < 0.05$ versus mice treated with TNBS alone and mice treated with 0.04mg/kg rhGAL-1.

fetal calf serum. T cells were then separated from B cells using nylon wool columns, as previously described.[26] After this procedure, about 90% of lymphocytes were CD3-positive T cells. Lymphocytes were then incubated with fluorescence isothiocyanate (FITC)-conjugated anti-Fas, anti-FasL, and anti-IL2R mAbs for 30 minutes at 4°C. Cells were then washed twice and resuspended in PBS/formaldehyde (0.5%). Stained cells were then analyzed on a FACScan cytofluorimeter (Becton Dickinson, San Jose, CA), and cells were gated using forward versus side scatter to exclude dead cells and debris.

Isolation of LP T Cells

Colonic LP T cells were prepared as previously described.[27] In brief, after excision of all visible lymphoid follicles, colonic tissue was treated with 1 mmol/L EDTA in PBS for 20 minutes to remove the epithelium. The tissue was then digested with collagenase (type IV; Sigma) for 20 minutes in a shacking incubator at 37°C; this step was repeated twice. The released cells were then layered on a 40–100% Percoll gradient (Pharmacia, Uppsala, Sweden) and spun at 1.800 rpm

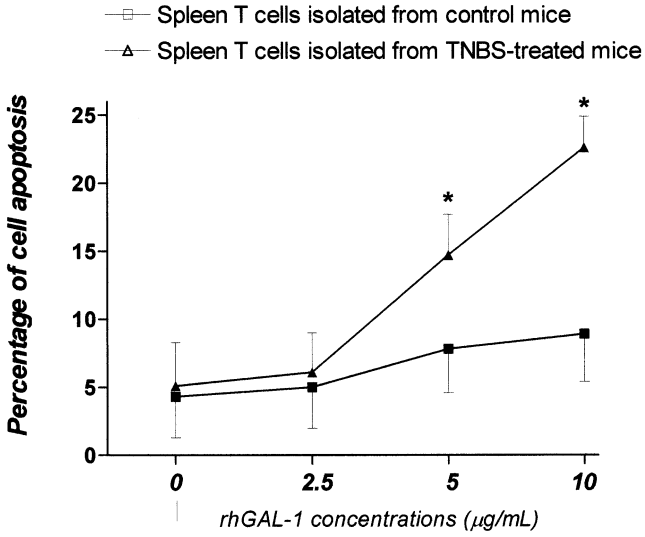

Figure 6. rhGAL-1 causes selective apoptosis of spleen T cells activated in vivo by intrarectal instillation of TNBS. Spleen T cells were isolated from mice killed 7 days after intrarectal instillation of TNBS in 50% ethanol or ethanol alone, and incubated for 8 hours with 2.5, 5, or 10 μg/mL rhGAL-1. At the end of the incubation period, cells were collected and processed for apoptotic cell detection. $*P < 0.05$ versus T cells isolated from control (ethanol-treated) mice.

to obtain the lymphocyte-enriched populations at the 40–100% interface. An enriched $CD4^+$ T-cell population was obtained by positive selection using FACSorter (Becton Dickinson). Flow cytometric analysis showed that the resultant cell population contained about 90% $CD4^+$ T lymphocytes.

Culture of LP $CD4^+$ T Cells for Assay of Cytokine Production

LP $CD4^+$ T cells from different treatment regimens were suspended in complete medium (RPMI 1640, 10% heat-inactivated fetal calf serum, 3 mmol/L L-glutamine, 10 mmol/L Hepes buffer, 10 μg/mL penicillin, 100 U/mL streptomycin, and 0.05 mmol/L 2-ME) and cultured at a concentration of 10^5 cells/mL. To measure cytokine production, LP $CD4^+$ T cells were placed for 48 hours onto uncoated culture wells (to measure production by unstimulated cells) or onto wells containing immobilized murine anti-CD3ϵ mAb (Pharmingen, San Diego, CA) and 1 μg/mL soluble anti-CD28 antibody (Pharmingen) (to measure production by stimulated cells). At the end of the incubation period, culture supernatants were harvested and assayed for cytokine concentration by using specific ELISA kits (R&D systems).

Evaluation of T-cell Apoptosis In Vitro

To investigate the effect of rhGAL-1 on T-cell apoptosis, freshly isolated spleen and LP T cells were incubated for 8 hours in complete medium with 3 mmol/L dithiothereitol, with increasing doses of rhGAL-1. At the end of the incubation period, apoptotic cells were identified at FACScan cytofluorimeter (Becton Dickinson) using annexin V and propidium iodide (PI) according to the manufacturer's instructions (R&D Systems). The percent of cell death was calculated by determining the percent of viable cells:

$$[\%\ \text{of viable} = (\%\ \text{of annexin V}^-\ \text{and PI}^-,\ \text{rhGAL-1 treated})/(\%\ \text{of annexin V}^-\ \text{and PI}^-,\ \text{control treated}) \times 100\ \text{and}\ \%\ \text{of death} = 100 - \%\ \text{of viable}].$$

To assess the mechanism underlying T-cell apoptosis induced by rhGAL-1, caspase 8 and caspase 9 activities were measured 2 and 8 hours after the incubation with rhGAL-1 by using specific caspase fluorometric protease assay according to manufacturer's instructions (ApoAlert, Clontech, Palo Alto, CA).

In Situ TUNEL Staining

For detection of apoptotic cells in tissue, terminal deoxynucleotidyl transferase-mediated deoxyuridine trisphosphate nick-end labeling (TUNEL) staining was performed in paraffin sections of colon from control (ethanol-treated) mice, mice with fully established TNBS-induced colitis, and mice with TNBS-induced colitis treated with 1mg/kg rhGAL-1. Colon sections were deparaffinized and the proteins present in the sections digested with 20 μg/mL proteinase K for 15 minutes at room temperature. Following 4 washes in distilled water, endogenous peroxidase was quenched with 2.0% H_2O_2 at room temperature and sections were washed 2 times with PBS. The labeling of degraded DNA specific to apoptotic cells was performed with a peroxidase-conjugated in situ apoptosis detection kit (ApopTag, Intergen, NY), according to the manufacturer's instructions. Detection of labeled ends was performed with anti-digoxigenin peroxidase antibody and substrate.

Drugs and Reagents

Routine buffer reagents were obtained from Sigma Chemical Co (Milan, Italy). All other chemicals were obtained from the sources indicated or Sigma Chemical.

Statistical Analysis

All values are expressed as mean ± SEM of n mice per group. Comparisons of more than 2 groups were made with a 1-way analysis of variance with post-hoc Tukey's test. Comparison of 2 groups was made using Student's t test for unpaired data when appropriate. Differences were considered statistically significant if P was < 0.05.

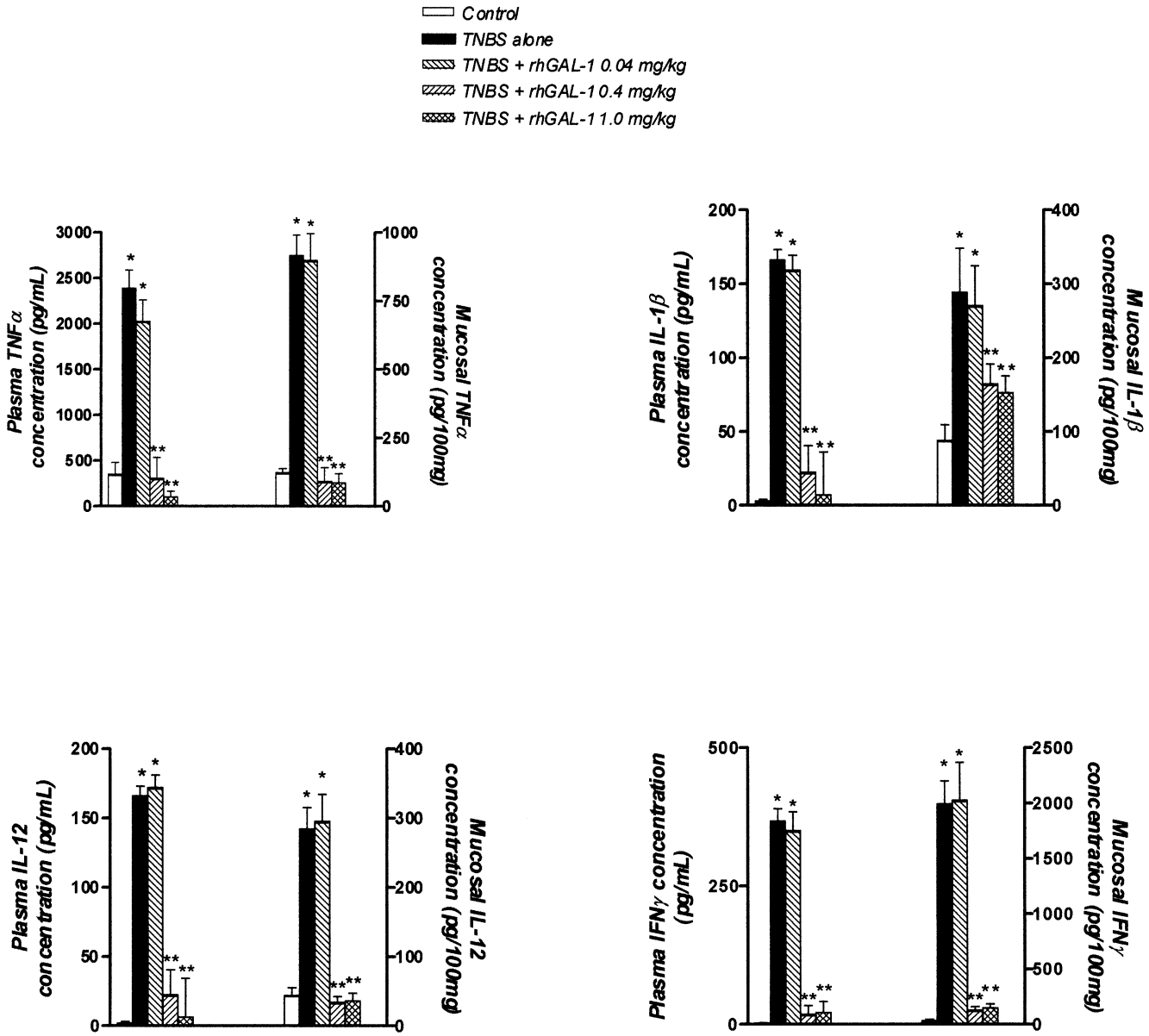

Figure 7. Early administration of rhGAL-1 suppresses Th1 cytokine production. Plasma and colon specimens were taken from mice killed 7 days after intrarectal instillation of TNBS in 50% ethanol or ethanol alone. rhGAL-1 or saline were administered IV daily for 7 days. *Bars* represent mean ± SE of 5 mice/group. Cytokines were measured on plasma and colon homogenates using specific ELISA as described in *Materials and Methods*. *$P < 0.05$ versus control (ethanol-treated) mice; **$P < 0.05$ versus mice treated with TNBS alone and mice treated with 0.04 mg/kg rhGAL-1.

Results

rhGAL-1 Protects Against Development of TNBS-Induced Colitis (Prophylactic Protocol)

Effect of early administration of rhGAL-1 on clinical and histologic signs of colitis. Intracolonic administration of TNBS in Balb/c mice induced a severe illness characterized by loss of body weight that remained approximately 15% lower than controls during all post-treatment periods (Figure 1*A*). Early administration of rhGAL-1 resulted in a dose-dependent prevention of wasting syndrome induced by TNBS administration (Figure 1*A*). The 2 higher doses of rhGAL-1 prevented the TNBS-induced wasting syndrome at similar extent ($P > 0.05$ between 0.4 and 1mg/kg). Distal colons of mice sacrificed 7 days after TNBS administration appeared hyperemic and thickened, with adhesion to other segments of intestine. On microscopic examination the

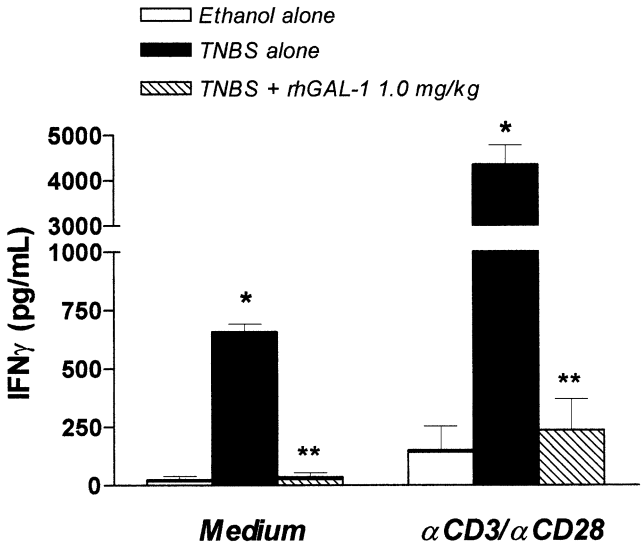

Figure 8. Early administration of rhGAL-1 abrogates IFNγ production by isolated LP $CD4^+$ T cells. Cells were isolated from mice killed 7 days after intrarectal administration of TNBS, with or without treatment with 1 mg/kg rhGAL-1. rhGAL-1 was administered IV daily for 7 days. *Bars* represent mean ± SE of 3 experiments (4–6 mice per group). $*P < 0.05$ versus control (ethanol-treated) mice; $**P < 0.05$ versus mice treated with TNBS alone.

colons of TNBS-treated mice demonstrated a marked mononuclear cell infiltration associated with loss of goblet cells (Figure 2*B* and *C*). Some parts of the mucosal layer lost crypts and these areas were replaced by lymphocytes and macrophages (Figure 2*C*). The histologic grading of colon sections increased from 0.5 ± 0.1 in control mice to 2.7 ± 0.3 in TNBS-treated mice ($P < 0.01$) (Figure 1*B*). In line with these histologic alterations, intrarectal instillation of TNBS resulted in a significant increase in colon mucosal wall thickness and MPO activity (Figure 1*C* and *D*). Early administration of rhGAL-1 reduced the extent of colonic damage induced by TNBS in a dose-dependent manner, as measured by the reduction in histologic injury score and colonic MPO content (Figure 1*B*, *C*, and *D*; Figure 2*D* and *E*).

Colonic galectin-1 expression. High levels of galectin-1 mRNA was detected in the colon of control mice. Real-time quantitative PCR demonstrated that rectal TNBS instillation caused a 4-fold decrease in colonic galectin-1 mRNA expression (Figure 3). As assessed by immunohistochemistry, normal colon constitutively expresses high levels of galectin-1, which was mainly localized at the level of the superficial epithelial cells (Figure 4*B*). In line with the results of quantitative real-time PCR, galectin-1 expression was markedly reduced in colon sections from mice with TNBS-induced colitis (Figure 4*D*).

Effect of early administration of rhGAL-1 on spleen T cells. As assessed by the expression on cell membranes of the activation-induced molecules Fas, FasL, and IL2R, colonic instillation of TNBS caused a significant increase in the number of activated spleen T cells (Figure 5). Early administration of rhGAL-1 reduced the percentage of activated spleen T cells in a dose-dependent manner (Figure 5). To investigate if the reduction in the percentage of activated spleen T cells observed in rhGAL-1–treated mice was related to the deletion of TNBS-activated T cells, spleen T cells were isolated from TNBS or ethanol-treated mice and cultured with increasing doses of rhGAL-1. As shown in Figure 6, rhGAL-1 increased the percentage of apoptosis only in spleen T cells isolated from TNBS-treated mice in a dose-dependent manner, while it had no effect on T cells isolated from control mice. rhGAL-1–induced cell death was reverted by the addition of lactose, indicating that T-cell apoptosis was mediated by carbohydrate-specific interactions (data not shown). These data suggest that intrarectal administration of TNBS results in activation of a subset of TNBS-specific T cells that are highly susceptible to the proapoptotic activity of rhGAL-1.

Effect of early administration of rhGAL-1 on TNBS-induced pro-inflammatory cytokine release. As shown in Figure 7, colonic instillation of TNBS resulted in a marked increase in mucosal and plasma concentrations of several Th1 cytokines, such as TNFα, IL-1β, IL-12, and IFNγ. In agreement with the reduction of colonic inflammation, early administration of rhGAL-1 dose-dependently reduced plasma levels of TNFα, IL-1β, IL-12, and IFNγ and the concentration of these proinflammatory cytokines in the colonic mucosa (Figure 7).

Effect of early administration of rhGAL-1 on IFNγ production from LP $CD4^+$ T lymphocytes. To further investigate the mechanisms underlying the therapeutic activity of rhGAL-1, we assessed the effect of this lectin on IFNγ generation from isolated colonic LP T cells. As illustrated in Figure 8, we found that purified $CD4^+$ LP T cells prepared from colonic tissue of mice treated with TNBS alone released significantly higher concentrations of IFNγ than control (ethanol-treated) mice, suggesting that LP T cells are directly activated by the haptenizing agent. On the other hand, LP $CD4^+$ T cells isolated from rhGAL-1-treated mice released amounts of IFNγ similar to those secreted by LP $CD4^+$ T cells harvested from control mice, suggesting that this lectin acts directly on the subset of LP $CD4^+$ T cells activated by TNBS-administration (Figure 8). Similarly, in vivo administration of rhGAL-1 almost completely abolished IFNγ release induced by incubating LP $CD4^+$ T cells with a

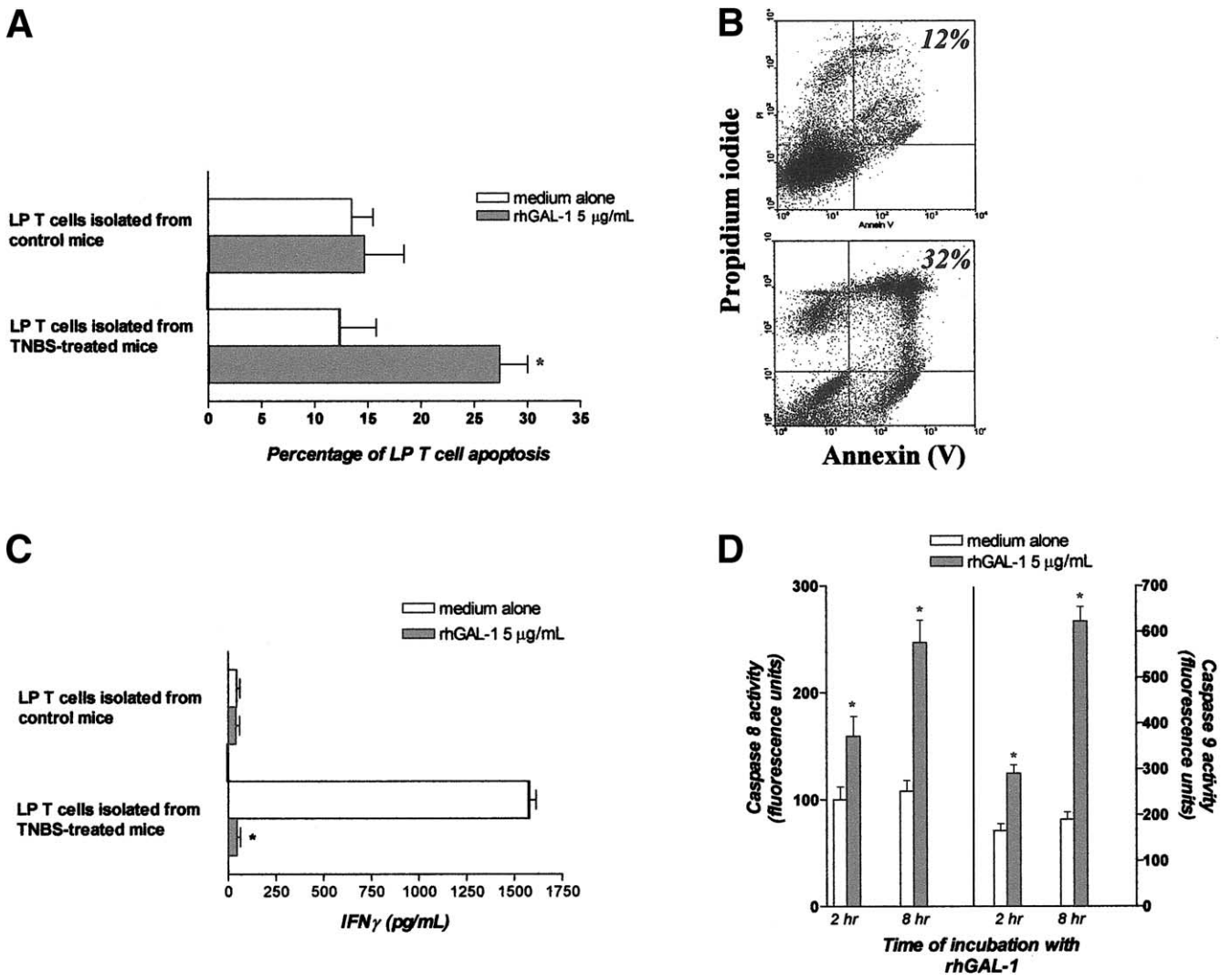

Figure 9. rhGAL-1 causes selective apoptosis of isolated LP $CD4^+$ T cells activated in vivo by TNBS. Cells were isolated from mice killed 7 days after intrarectal instillation of TNBS in 50% ethanol or ethanol alone and incubated for 8 hours with or without 5 μg/mL rhGAL-1. Bars represent mean ± SE of 3 experiments (4–6 mice per group). (*A*) Only LP T cells isolated from TNBS-treated mice were sensitive to the apoptotic effect of galectin-1, while LP T cells harvested from control mice were resistant. $*P < 0.05$ versus medium alone. (*B*) Representative scatter plots of LP T cells isolated from control mice (*upper insert*) and from TNBS-treated mice (*lower insert*) incubated for 8 hours with 5 μg/mL rhGAL-1. LP T cells isolated from TNBS-treated mice and treated with galectin-1 demonstrate a significant increase in the fraction of annexin V^+/propidium $iodide^+$ cells compared with LP T cells isolated from control mice. (*C*) rhGAL-1 almost completely abrogated IFNγ release from LP T cells isolated from TNBS-treated mice, paralleling the effect exerted by the lectin on T-cell death. (*D*) rhGal-1–induced cell death was related to the activation of caspase cascade. $*P < 0.05$ versus medium.

mixture of anti-CD3/anti-CD28 mAbs (Figure 8), indicating that this lectin acts not merely by blocking the stimulus for IFNγ production but by depleting the TNBS-sensitive T-cell clone. To confirm this hypothesis we incubated LP $CD4^+$ T cells isolated from ethanol or TNBS-treated mice with 5 μg/mL rhGAL-1. Interestingly, rhGAL-1 causes T-cell apoptosis only in LP T cells prepared from TNBS-treated mice, and cell death strictly paralleled the reduction in IFNγ release (Figure 9*A* and *B*). Again, galectin-1–induced cell death was reverted by the addition of lactose, suggesting that this effect was mediated by carbohydrate-specific interactions (data not shown). LP T-cell apoptosis was associated with a marked increase in both caspase 8 and 9 activity, suggesting that the activation of caspase cascade plays a critical role in galectin-1–induced cell death (Figure 9*C*).

rhGAL-1 Abolishes Fully Established TNBS-Induced Colitis (Therapeutic Protocol)

We then investigated whether rhGAL-1 was effective in suppressing established TNBS-induced colitis. As shown in Figure 10, administration of 1 mg/kg

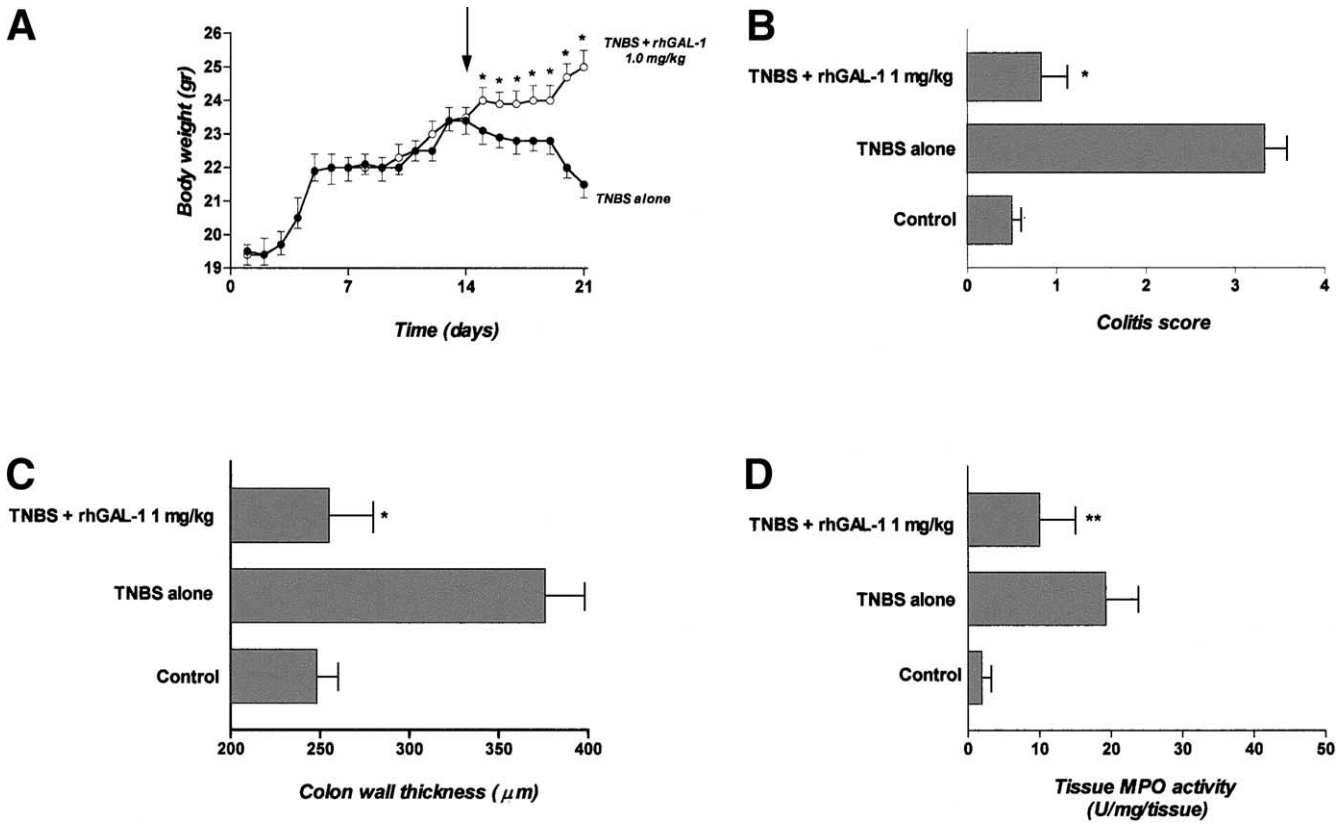

Figure 10. Therapeutic administration of rhGAL-1 abrogates fully established colitis induced by TNBS administration. rhGAL-1 was injected IV at the dose of 1mg/kg daily starting 14 days after intrarectal instillation of TNBS. Mice were killed 21 days after TNBS administration. (*A*) Wasting disease in mice with established TNBS colitis is improved by rhGAL-1. Each point represents average weight data pooled from 10 mice. $*P <$ 0.05 versus mice treated with TNBS alone. (*B, C,* and *D*) Severity of TNBS-induced inflammation (microscopic damage and MPO activity) is reduced by therapeutic administration of rhGAL-1. $*P < 0.05$ versus mice treated with TNBS alone; $**P < 0.05$ versus control (ethanol-treated) mice and versus mice treated with TNBS alone.

rhGAL-1 from day 14 to day 21 reverted the wasting syndrome induced by TNBS (Figure 10*A*), as well as the colonic inflammatory changes, assessed by the histologic score (Figure 10*B* and *C*) and colonic MPO activity (Figure 10*D*). Moreover, rhGAL-1 almost completely abolished plasma and mucosal release of all proinflammatory cytokines tested (IFNγ, TNFα, IL-1β, and IL-12) (Figure 11).

To further investigate the mechanisms underlying the protective therapeutic effect of galectin-1, we have then assessed the number of apoptotic mononuclear cells in tissue sections by an in situ TUNEL technique (see Material and Methods). In mice with fully established colitis, the epithelial architecture was found to be disorganized, with a high level of mononuclear cell infiltrate (Figure 12*A* and *B*). When mice with established colitis were treated with rhGAL-1, the epithelial architecture was almost completely recovered, with a significant reduction in the mononuclear cell infiltrate. In the area with residual mononuclear cell infiltration, TUNEL technique revealed that most of these cells were apoptotic (Figure 12*C*, *D*, and *E*).

Discussion

In the present study we provide evidence supporting the concept of an in vivo therapeutic role for galectin-1 in a murine model of chronic IBD. Perhaps one of the most striking aspects of our results is that the TNBS-induced colitis can be successfully treated with galectin-1 even when the lesions are fully established. Thus, when we administered galectin-1 in the later phases of the disease, we found an abrogation of the TNBS-induced wasting disease with a marked reduction in the histologic and immunologic signs of inflammatory activity.

There is circumstantial evidence to link a dysregulated Th1 response with IBD.[28–31] Although the mechanisms underlying the aberrant Th1 response are unknown, it has been speculated that a deficit in activated mucosal T-cell apoptosis contributes to the perpetuation of chronic intestinal inflammation by extending the life span of antigen-primed lymphocytes.[5] This view has been supported by the demonstration that mAbs directed against IL-12 and IL-6R suppress experimental colitis in mice by restoring LP T-cell susceptibility to apoptosis.[4,8]

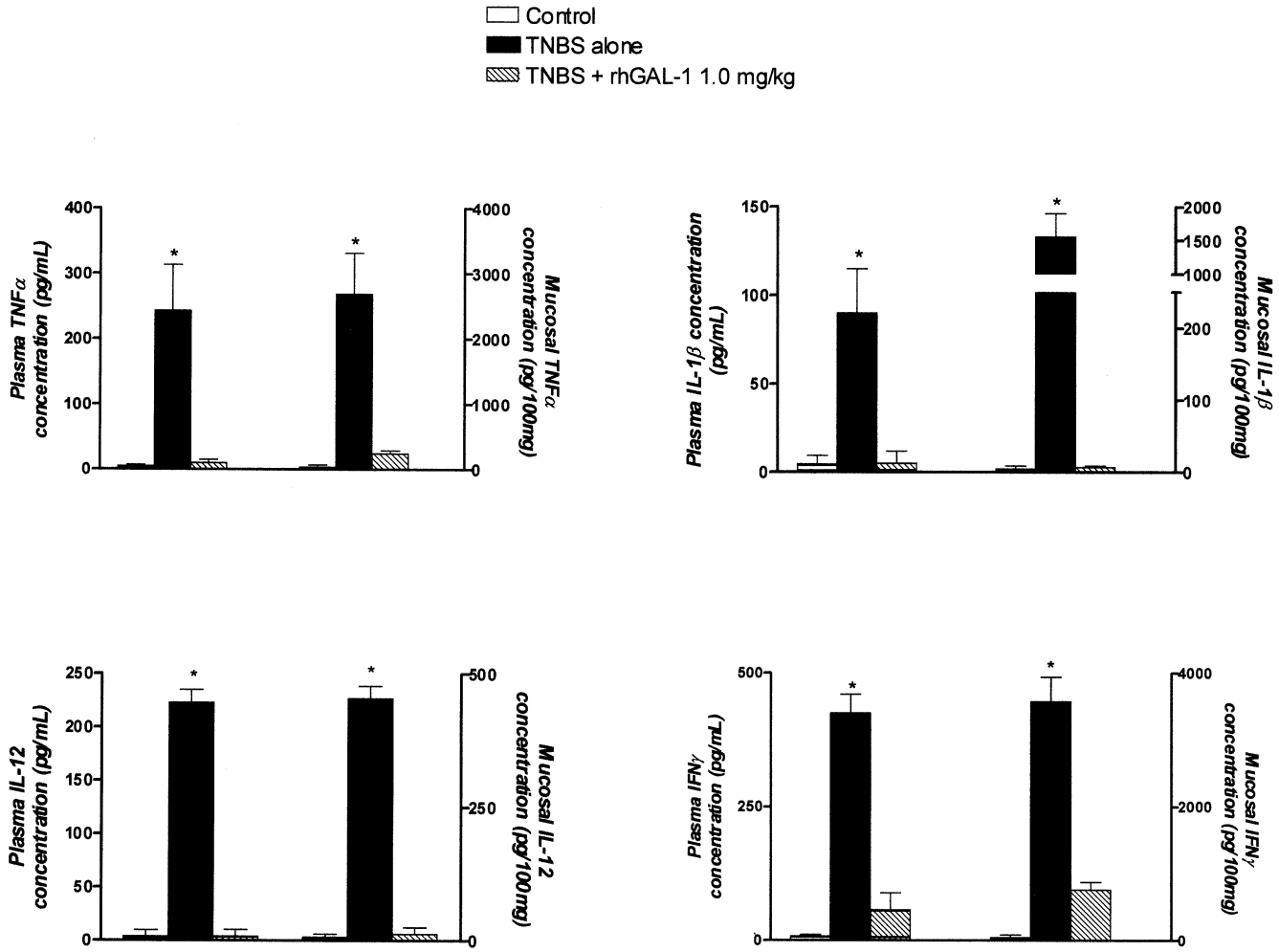

Figure 11. Therapeutic administration of rhGAL-1 suppresses Th1 cytokine production. Cytokines were measured on plasma and colon homogenates taken from mice killed 21 days after intrarectal instillation of TNBS in 50% ethanol or ethanol alone and processed for TNFα, IFNψ, IL-1β, and IL-12 measurement using specific ELISA kits. rhGAL-1 or saline was injected IV at the dose of 1 mg/kg daily, starting 14 days after TNBS administration. *Bars* represent mean ± SE of 5 mice/group. $*P < 0.05$ versus control (ethanol-treated) and rhGAL-1-treated mice.

Galectin-1, an endogenous lectin constitutively expressed by several tissues, is involved in generating and maintaining central and peripheral tolerance by inducing apoptosis of unselected or negatively selected thymocytes,[11] and by causing selective apoptosis of activated mature T lymphocytes.[12] The fact that galectin-1 is constitutively expressed in lymphoid organs, such as thymus and lymph nodes,[10,11] further supports a regulatory role in immune homeostasis for this lectin. In the present study we have demonstrated that normal mouse colonic mucosa constitutively expresses high levels of galectin-1, suggesting a potential role for this lectin also in maintaining intestinal immune homeostasis. Of relevance, TNBS administration resulted in a marked decrease in colonic expression of galectin-1, an event that might increase the resistance of hapten-activated LP T cells against apoptosis. Consistent with the view that galectin-1 modulates local immune homeostasis, it has been recently shown that there is a reduced expression of galectin-1 in sinovial tissues of patients affected by juvenile idiopathic arthritis, a chronic inflammatory condition characterized by defective T-cell apoptosis.[32]

To investigate whether rhGAL-1 exerts this therapeutic activity by restoring the susceptibility of hapten-activated T cells to apoptosis, we carried out a series of experiments aimed at exploring the effect of this lectin on TNBS-activated T cells. As shown by cytofluorimetric studies, treatment with rhGAL-1 resulted in an almost complete prevention of TNBS-induced activation of spleen T cells. This effect seems to be related to the selective deletion of TNBS-activated T cells, as demonstrated by the fact that spleen T cells prepared from TNBS-treated mice and cultured with galectin-1 undergo apoptosis, while nonactivated spleen T cells, isolated from control mice, were resistant. Moreover, we found that galectin-1 directly inhibits LP T-cell activity,

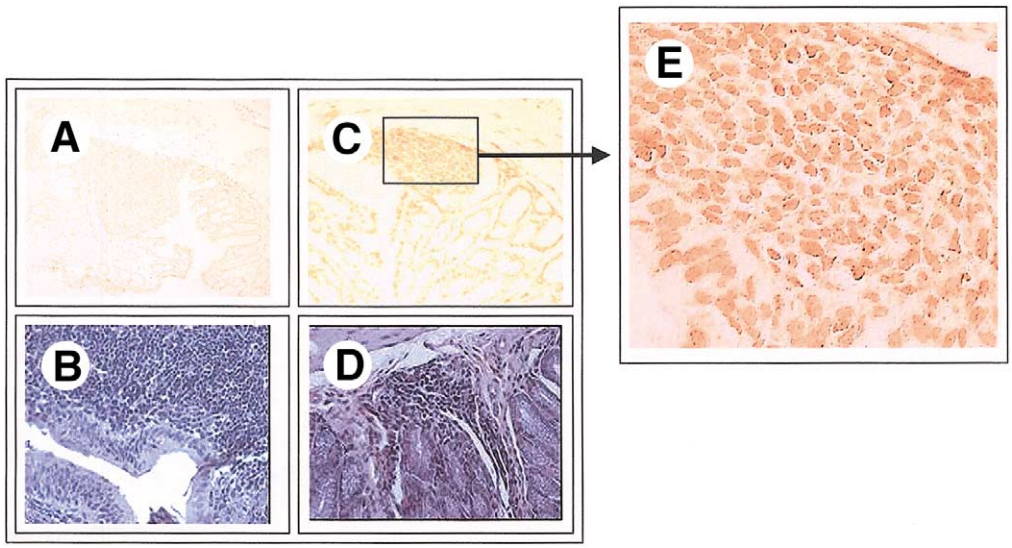

Figure 12. Effect of therapeutic administration of rhGAL-1 on in situ TUNEL staining. (*A*) Colon section from a saline-treated mouse killed 21 days after TNBS administration showing disorganized epithelial architecture and massive mononuclear cell infiltrate (original magnification, 100×). The presence of this important mononuclear cell infiltrate in colon sections of TNBS-colitis was clearly evidenced by H&E staining (*B*, original magnification, 200×). (*C*) Colon section from a mouse treated with rhGAL-1 from day 14 to day 21 and killed 21 days after TNBS administration (original magnification 100×). The epithelial architecture is almost completely recovered, together with a dramatic reduction in the mononuclear cell infiltrate, confirmed by H&E staining (*D*, original magnification 200×). Most of the remaining mononuclear cells were found to be apoptotic (*E*, original magnification 400×).

as demonstrated by the fact that LP T cells prepared from mice pretreated with rhGAL-1 not only released almost undetectable levels of IFNγ spontaneously, but were also refractory to stimulation with anti-CD3/anti-CD28 agonistic antibodies. The reduction in IFNγ secretion from LP T cells isolated from rhGAL-1 treated mice is likely related to the proapoptotic ability of the lectin. Indeed, if galectin-1 acted merely by removing the stimulus to IFNγ production, the subsequently isolated LP T cells would be expected to produce increased amounts of IFNγ when restimulated with CD3/CD28 mAbs in vitro. Therefore, it is conceivable that, administered in vivo, galectin-1 causes deletion of the TNBS-sensitive LP T-cell clone. Support for this view comes from the observation that LP T cells prepared from control mice were not susceptible to the proapoptotic activity of rhGAL-1 in vitro, while LP $CD4^+$ T cells isolated from mice with TNBS-induced colitis undergo apoptosis when cultured with the lectin. In a further, more direct study performed to confirm this scenario, we showed that administration of rhGAL-1 to mice with fully established colitis leads to the appearance of apoptotic mononuclear cells in the colonic tissue together with a significant reduction in inflammatory infiltrate. Taken together, these data indicate that TNBS administration results in the development of a subset of TNBS-sensitive IFNψ-producing LP Th1 clone that is susceptible to the proapoptotic activity of galectin-1.

Galectin-1 binding with the receptor initiates a variety of signal-transduction events, such as ERK-2 phosphorylation, calcium influx, and Bcl-2 downregulation, that influence T-cell physiology and survival.[12] However, the mechanisms underlying the proapoptotic activity of galectin-1 are unknown. In the present study, we have shown that apoptosis of LP T cells induced by galectin-1 was associated with a marked increase in caspase 8 and 9 activity, suggesting that galectin-1–induced T-cell death is mediated by caspase activation. Present data confirm the results of a recent study in which T-cell apoptosis induced by galectin-1 was prevented by ZVAD-fmk, a broad-spectrum caspase inhibitor.[33]

To gain further information on the possible mechanism underlying the protective effect exerted in vivo by galectin-1 administration, we monitored both the plasma and colon levels of Th1 cytokines. Our results demonstrated that galectin-1 administration prevented the release of IL-12, a potent proinflammatory cytokine mainly produced by antigen-presenting cells. This cytokine is necessary for the development of the Th1 response in most experimental systems, leading to the increase in IFNγ production, macrophage activation, and B-cell–dependent production of complement-fixing antibodies.[34,35] IL-12 plays a critical role in the pathogenesis of several models of organ-specific autoimmune disease, including collagen-induced arthritis, insulin-dependent diabetes, and experimental autoimmune encephalomyeli-

tis[36,37]; it is also involved in the pathogenesis of IBD[38,39] and TNBS-induced colitis.[3,4] Our data, showing that galectin-1 administration almost completely abolished IL-12 release induced by TNBS suggests that the immunomodulatory activity of this lectin is mediated not only by the deletion of the TNBS-sensitive T-cell clone, but also by blocking the synthesis and/or the release of this potent proinflammatory cytokine. This hypothesis is supported by previous demonstrations that galectin-1 prevents proinflammatory cytokine release from spleen macrophages stimulated by LPS and from T cells stimulated by IL-2, without affecting cell viability.[20,40] Therefore, inhibition of proinflammatory cytokine synthesis and/or release might represent another mechanism underlying the therapeutic activity of galectin-1 on TNBS-induced colitis in mice.

In summary, we have shown that galectin-1 exerts a multilevel regulation of mucosal immune system in a rodent model of colitis. Because galectin-1 is an endogenous lectin that is neither immunogenic nor cytotoxic, it could be potentially useful in treating human diseases involving dysregulated T-cell activation. In this context, galectin-1 would have distinct advantages over the unselective immunosuppressive agents currently used in the treatment of human IBDs.

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