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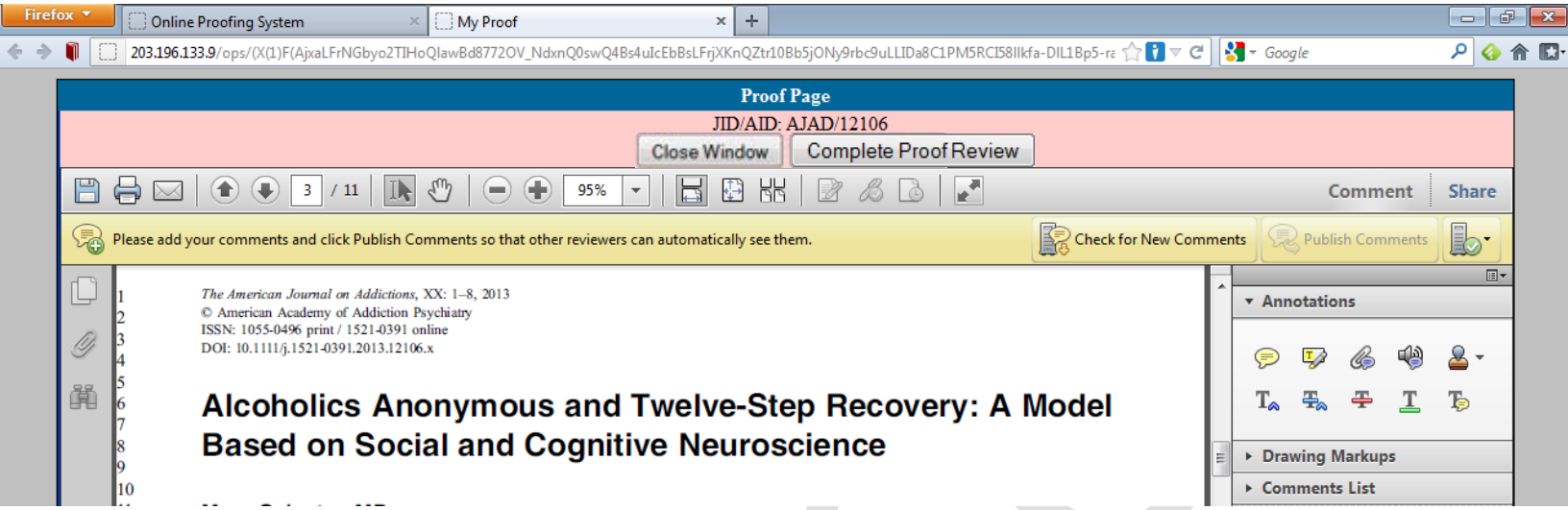

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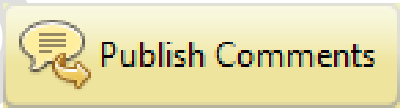

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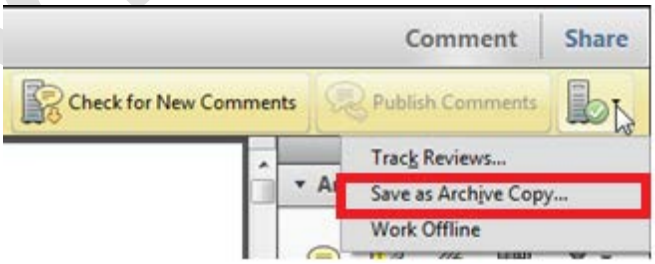

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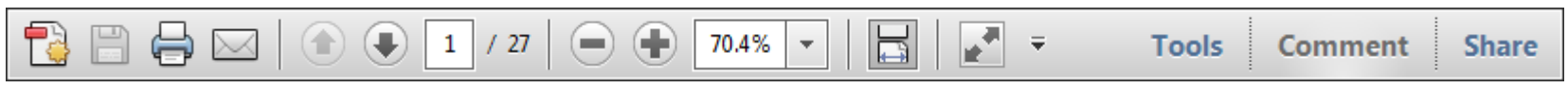

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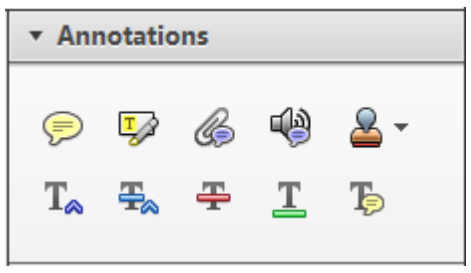

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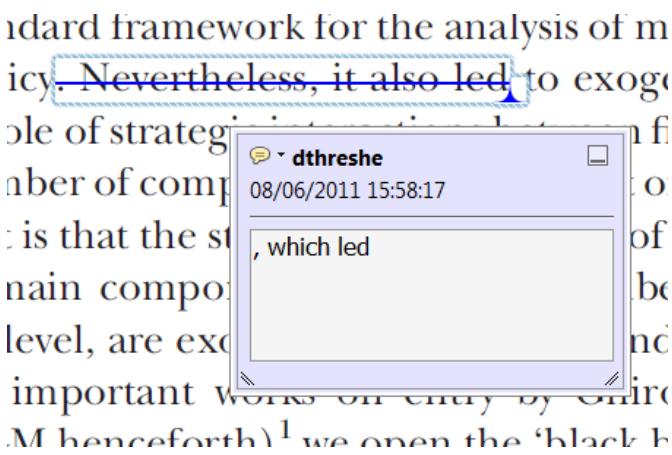

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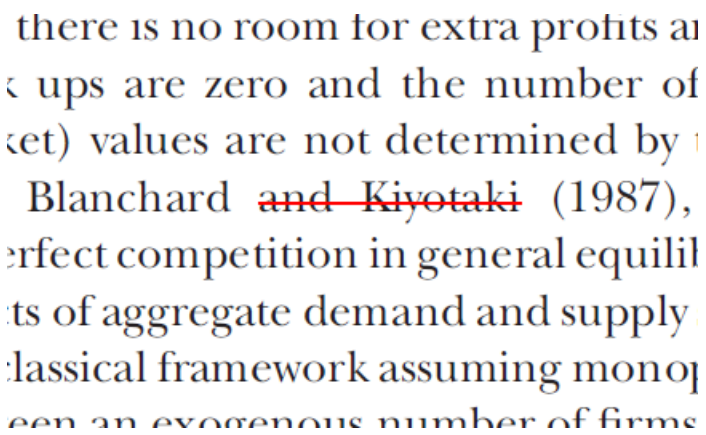

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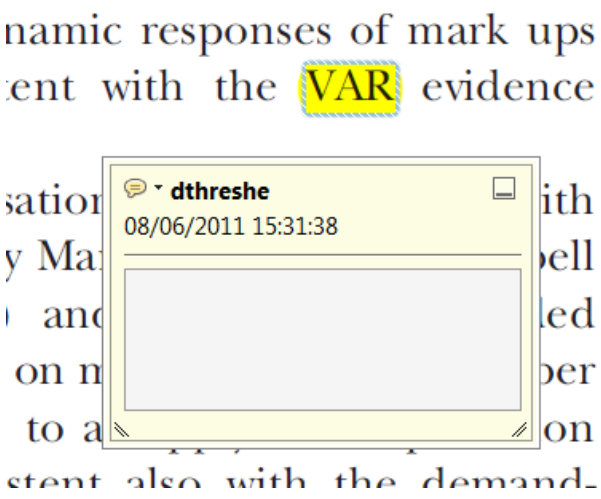

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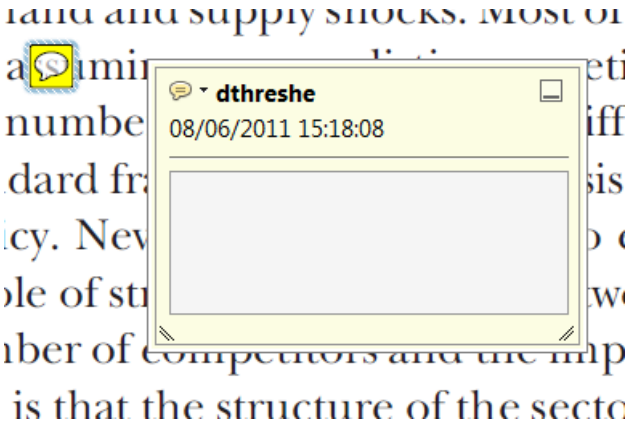

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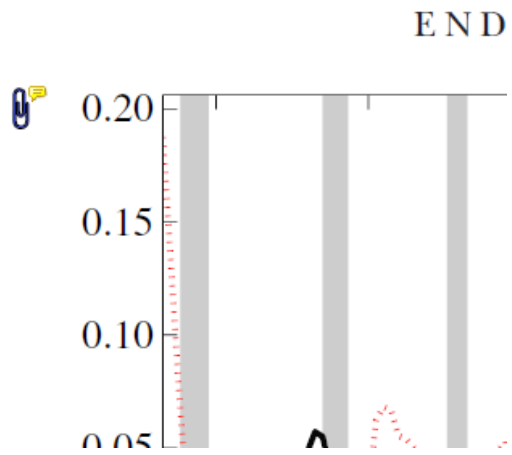

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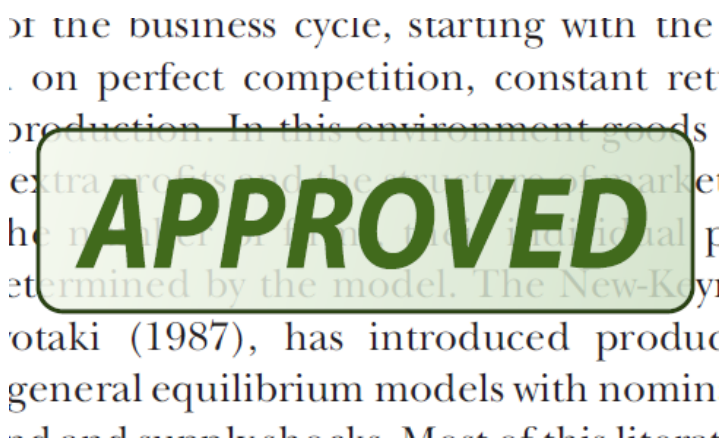

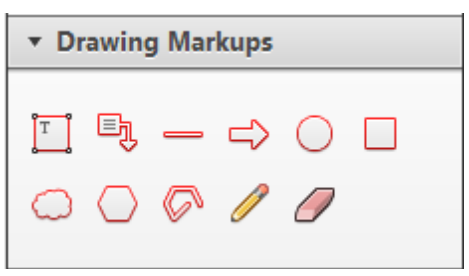

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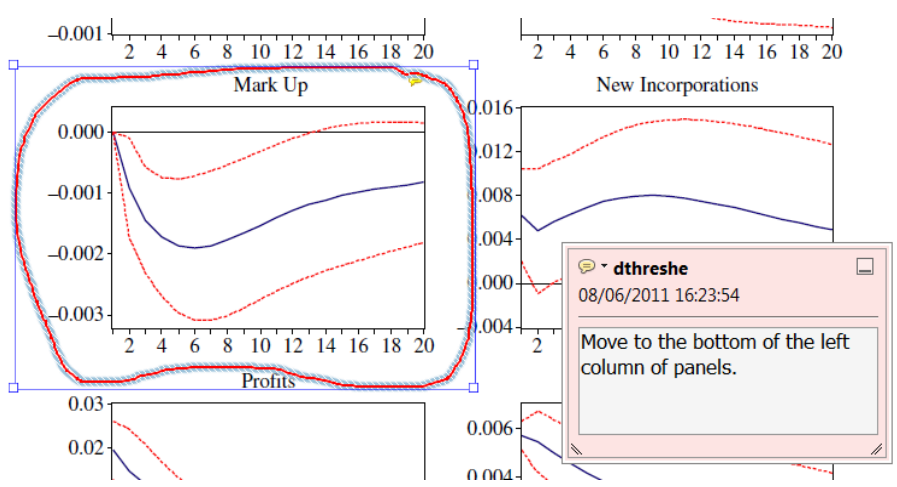

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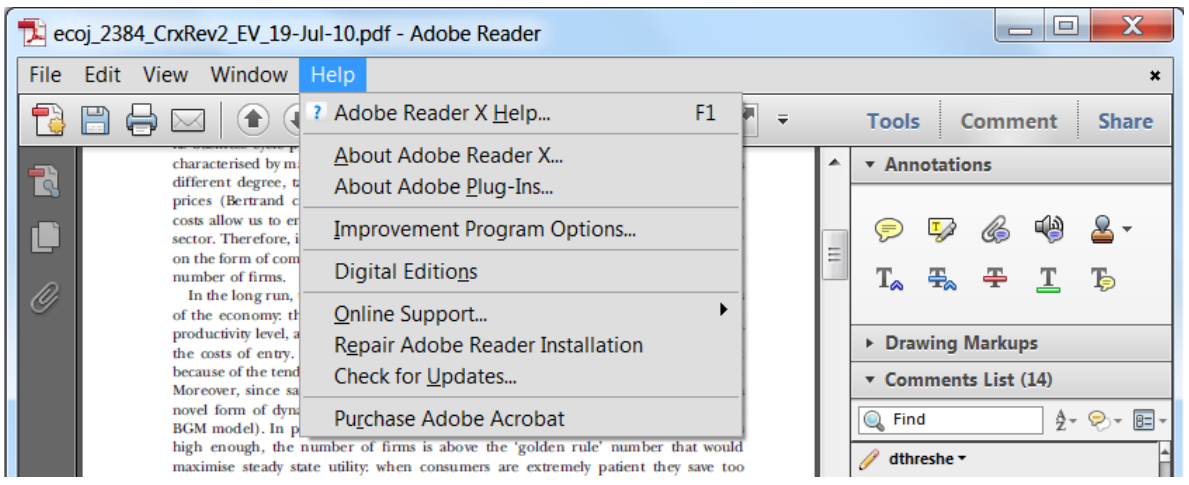

Journal of Cellular Physiology

Inflammation Controls Sensitivity of Human and Mouse Intestinal Epithelial Cells to Galectin-1

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Galectins[Q2] play key roles in the inflammatory cascade. In this study, we aimed to analyze the effect of galectin-1 (Gal-1) on function of intestinal epithelial cells (IECs) isolated from healthy and inflamed mucosa. IECs isolated from mice or patients with inflammatory bowel diseases (IBD) were incubated with different pro-inflammatory cytokines, and Gal-1 binding, secretion of homeostatic factors and viability were assessed. Experimental models of food allergy and colitis were used to evaluate the in vivo influence of inflammation on Gal-1 binding and modulation of IECs. We found an enhanced binding of Gal-1 to: (a) murine IECs exposed to IL-1β, TNF, and IL-13; (b) IECs from inflamed areas in intestinal tissue from IBD patients; (c) small bowel of allergic mice; and (d) colon from mice with experimental colitis. Our results showed that low concentrations of Gal-1 favored a tolerogenic micro-environment, whereas high concentrations of this lectin modulated viability of IECs through mechanisms involving activation of caspase-9 and modulation of Bcl-2 protein family members. Our results showed that, when added in the presence of diverse pro-inflammatory cytokines (TNF, IL-13, and IL-5), Gal-1 differentially promoted the secretion of growth factors including TSLP, EGF, IL-10, IL-25, and TGF-β_1. In conclusion, we found an augmented binding of Gal-1 to IECs when exposed in vitro or in vivo to inflammatory stimuli, showing different effects depending on Gal-1 concentration. These findings highlight the importance of the inflammatory micro-environment of mucosal tissues in modulating IECs susceptibility to the immunoregulatory lectin Gal-1 and its role in epithelial cell homeostasis.

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The intestinal mucosa plays key roles in a diversity of vital functions, including nutrient absorption and orchestration of local and systemic immune responses (Mowat, 1999). The maintenance of an intact epithelium is critical for the integrity of the barrier and permeability, and for intestinal homeostasis (Nusrat et al., 2000; Yamada, 2008). In this regard, intestinal epithelial cells (IECs) constitute relevant players for the generation of tolerance and barrier repair, which are critical to maintain mucosal function and tissue homeostasis (Mowat, 1999; Cario and Podolsky, 2000; Strober et al., 2003; Mowat et al., 2004). To accomplish this function, IECs are equipped with a myriad of receptors that respond to inflammatory mediators and components of the microbiota to orchestrate innate immune responses that are required for the control of luminal pathogens (Cario and Podolsky, 2000; Van De Walle et al., 2010). Under certain circumstances, the delicate balance that leads to homeostasis is broken and inflammatory responses arise (Olson, 2006; Maloy and Powrie, 2011; Nalle and Turner, 2015).

Uncontrolled activation of inflammatory cascades or impaired regulation of the immune system is observed in inflammatory bowel diseases (IBD), which comprise a complex

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group of disorders characterized by chronic and relapsing inflammation of the colon and small intestine. Crohn's disease (CD) and ulcerative colitis (UC) are the major types of IBD (Baumgart et al., 2007), both involving deregulation of the epithelial cell compartment with a compromised intestinal barrier function (Zhou et al., 2004; Heller et al., 2005; Nalle and Turner, 2015). In addition to IBD, other inflammatory processes may condition intestinal homeostasis, such as food allergies. Compelling evidence during the past decade have demonstrated that T cell-mediated mechanisms promote mucosal inflammation in IBD (Neurath et al., 2001; Sturm et al., 2008; Gerner et al., 2013) and dramatically compromise the epithelial cell compartment (Siggers & Hackam, 2011; Planell et al., 2012; Tang et al., 2014; Thagia et al., 2014).

Galectins are an evolutionary conserved family of β-galactoside-binding proteins, being galectin-1 (Gal-1) one of the most studied members. Gal-1 exerts multiple functions in a variety of physiologic and pathologic processes, including regulation of cell cycle progression, vascular signaling, and inflammatory responses (Rabinovich et al., 1999; Toscano et al., 2006; Elola et al., 2007; Starossom et al., 2012; Rabinovich et al.; Bacigalupo et al., 2015). We have previously demonstrated that this lectin selectively promotes apoptosis of Th1 and Th17, but not Th2 cells through glycosylation-dependent mechanisms (Toscano et al., 2007), promotes the differentiation of IL-27-producing tolerogenic dendritic cells (DCs; Ilarregui et al., 2009) and modulates vascular signaling programs (Croci et al., 2014). Interestingly, Gal-1 is ubiquitously expressed in the intestine and is implicated in several intestinal disorders including IBD and colorectal cancer (Santucci et al., 2003; Mizoguchi and Mizoguchi, 2007; Hokama et al., 2008; Paclik, Lohse et al., 2008; Barrow et al., 2011; Ose et al., 2012). Studies performed in mouse models of colitis (Santucci et al., 2003) and human intestinal specimens of patients with IBD or colon cancer (Hittelet et al., 2003) suggested that Gal-1 could be an attractive candidate to control intestinal inflammatory disorders and a target for the treatment of malignancy. We have previously demonstrated that Gal-1 controls the viability of human and mouse enterocytes (Muglia et al., 2011). However, the impact of distinct inflammatory micro-environments on the sensitivity of IECs to Gal-1 and the subsequent regulation of mucosal function have not yet been explored (Paclik, Lohse et al., 2008; Muglia et al., 2011). Here, we studied the in vitro and in vivo influence of different inflammatory contexts in the regulatory activity of Gal-1 in human and mouse enterocytes. Thus, we investigated the biology and function of Gal-1 in the context of: (a) mouse IECs exposed to different pro-inflammatory cytokines to which these cells are known to be sensitive including IL-1β, IFN-γ, TNF, IL-5, and IL-13 (O'Connell et al., 2000; Nikawa et al., 2001a,b; Makins and Ballinger, 2005; Yeruva et al., 2008; Weber et al., 2010); (b) a cholera toxin-driven food allergy mouse model that promotes a mild inflammatory response of the small bowel; (c) a colitis mouse model induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS); and (d) human intestinal specimens from IBD (CD and UC) patients. Overall, our findings indicate that different pro-inflammatory settings fine-tune Gal-1 binding and function, thus highlighting the selective role of healthy or inflamed mucosal tissue in regulating lectin-mediated epithelial cell homeostasis.

Materials and Methods

Isolation of intestinal epithelial cells

Epithelial cells were obtained from human and mouse intestines. Male BALB/c mice (6-week old) were housed and cared at the animal facilities (School of Sciences, University of La Plata), according to institutional guidelines. Experimental protocols were approved by the local Institutional Animal Care and Use Committee (School of Veterinary Sciences, University of La Plata). For analysis of human samples, projects were approved by the Ethics Committee of the Gastroenterology Hospital Dr. Bonorino Udaondo of Buenos Aires and patients signed a written informed consent. Patients were diagnosed considering clinical, endoscopic, and histopathological findings. Intestinal biopsies were obtained by colonoscopy in seven UC (five women and two men, mean age 44 ± 15 years old), two CD (two women, mean age 40 ± 18 years old), and seven non-IBD control patients (four women and three men, age 48 ± 16 years old).

Human biopsies or mouse small bowel segments were handled as previously described for cell isolation (Muglia et al., 2011). Briefly, biological specimens were placed in ice-cold HBSS supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin until use. After washing with HBSS, samples were incubated in HBSS/penicillin/streptomycin plus 0.5 mM EDTA to obtain epithelial cells. Cells were pelleted, re-suspended in RPMI 1640 supplemented with 5% FBS and penicillin/streptomycin and used immediately.

Gal-1 binding assay

Isolated IECs were incubated for 18 h with 5 ng/ml IL-5 or IL-13 or 50 ng/ml of TNF, IFN-γ or IL-1β, respectively, in RPMI 1640 supplemented with 5% FBS, penicillin/streptomycin. Biotinylated rGal-1 (10 ng/ml) was added and incubated for 20 min followed by streptavidin-APC (1:100, 20 min at room temperature [BD Pharmingen, Franklin Lakes, NJ]). As control of specificity, samples were incubated in the presence of rGal-1 and 100 mM lactose. Fluorescence acquisition was performed with a FACScalibur cytometer (Becton[Q3] Dickinson) using QuestProCell software. Data were analyzed with the FlowJo software.

Annexin V/propidium iodide staining

Cells were incubated with different concentrations of rGal-1 (0.3, 1, 3, or 5 μM), or biotinylated rGal-1 for 18 h. Apoptosis was analyzed by flow cytometry using Annexin V-FITC and propidium iodide (PI).

Assessment of caspase activity

Isolated cells were lysed in ice-cold lysis buffer (10 mM EDTA, 50 mM Tris–HCl pH 7.4, 150 mM sodium chloride, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, 10 mg/ml leupeptin, and 2 mg/ml aprotinin) and the protein content was quantified with the bicinchoninic acid kit (Pierce BCA Protein Assay, Pierce, Rockford, IL) using BSA as standard. Caspase activity was analyzed by fluorimetric assays. Briefly, lysates were incubated with the caspase-specific substrates: Ac-Asp-Glu-Val-Asp-AFC (Ac-DEVD-AFC; AFC = 7-Amino-4-trifluoromethylcoumarin), Ac-Ile-Glu-Thr-Asp-AFC (Ac-IETD-AFC), or Ac-Leu-Glu-His-Asp-AFC (Ac-LEHD-AFC; Biomol International, Plymouth Meeting, PA) for caspase-3, -8, and -9, respectively. The fluorogenic AFC released by the activated caspases was spectrofluorometrically detected at 505 nm following excitation at 400 nm. The arbitrary fluorescence units were normalized to the total protein content of the sample. In order to evaluate the specificity of the reaction, specific aldehyde inhibitors were included: Ac-DEVD-CHO, Ac-IETD-CHO, or Ac-LEHD-CHO (Biomol International) for caspase-3, -8, and -9, respectively. Values obtained for cells incubated with saline alone were subtracted from those corresponding to cells incubated with rGal-1.

Evaluation of Bad, Bax, and Bcl-2 protein expression

Pro-apoptotic Bad and Bax, and anti-apoptotic Bcl-2 proteins were evaluated in IEC by flow cytometry after incubation with 5 μM Gal-1 for 18 h. Cells were fixed and permeabilized using the

Intracellular Fixation and Permeabilization buffer set (eBioscience, San Diego, CA). Anti-Bad-FITC (BD Biosciences, San José, CA), anti-Bax, and anti-Bcl-2 from Santa Cruz Biotechnology Inc. (Dallas, TX) were used in a 1:50 dilution. In the two later cases after incubation with the first antibody, cells were washed with FACS buffer and then incubated with anti-rabbit-FITC labeled secondary antibody (1:100; BD Biosciences). In all cases, appropriate isotype controls (BD Biosciences) were included.

Mouse model of food allergy

An IgE-mediated mouse model of milk allergy was developed as described (Smaldini et al., 2012). Briefly, male 6- to 8-week-old BALB/c mice received 6 weekly intra-gastric doses of 20 mg of cow's milk protein (CMP) plus 10 μg of cholera toxin (CT; Sigma Aldrich) in bicarbonate buffer. A control group of age- and sex-matched naïve mice received 6 weekly intra-gastric doses of 20 mg CMP. Ten days after the final boost, mice were intra-gastrically challenged twice (days 45 and 46) with 20 mg CMP and sacrificed the following day. The allergic reaction was detected as follows:

Clinical signs. Mice were evaluated for symptoms immediately after oral challenge in a blinded fashion by two independent investigators. Clinical scores were assigned according to the following range: 0 = no symptoms; 1 = scratching and rubbing around the nose and head; 2 = puffiness around the eyes and mouth, diarrhea, pilo-erection, reduced activity, and/or decreased activity with increase respiratory rate; 3 = wheezing, labored respiration, cyanosis around the mouth and the tail; 4 = no activity after prodding, or tremor and convulsion; and 5 = death.

Skin test. Mice were injected with 20 μg of CMP in 20 μl of sterile saline in one footpad, and saline in the contralateral footpad as negative control. Evans blue dye (Anedra, Buenos Aires, Argentina) was intravenously injected as 100 μl of a 0.1% solution. Blue skin color 30 min after the injection was considered as a positive skin test.

Detection of serum immunoglobulins. IgE and IgG1 specific antibodies were measured as previously described by EAST (Ceska, 1981) and ELISA, respectively (Smaldini et al., 2012).

Determination of secreted cytokines. Spleens were removed and cells were isolated. Splenocytes were plated in RPMI 1640 supplemented with 10% FBS, penicillin/streptomycin and stimulated for 72 h with CMP (0.35 mg/ml). Supernatants were collected and cytokines were quantified by ELISA: IL-5 and IFN-γ (Invitrogen, Carlsbad, CA), IL-13 (R&D Systems, UK), and TNF (BD Biosciences).

Quantification of mucosal cytokine expression. Twenty-four hours after the oral challenge, mice were killed and mRNA was extracted from the jejunum using the illustra RNAspin Mini Isolation kit (GE Healthcare, Germany) according to the manufacturer's specifications. Complementary DNA (cDNA) was obtained (Invitrogen Life Technologies, Carlsbad, CA) and mRNA expression was determined by real-time quantitative PCR using the ABI PRISM Sequence Detection System (BioRad, Berkley, CA). Values were referred to β-actin to standardize the total amount of cDNA. The fold change of mRNA expression was defined as the ratio between the normalized values corresponding to the sensitized mouse and the control, using the $2^{-\Delta\Delta C}$T method (Livak and Schmittgen, 2001).

Mouse model of colitis

Colitis was induced in male BALB/c mice (6- to 8-week old, 18–22 g) by rectal administration, of 0.1 ml 50% ethanol followed with 0.1 ml of 2.5 or 5 mg of 2,4,6-trinitrobenzene sulfonic acid (TNBS; Sigma–Aldrich, Deisenhofen, Germany; Neurath et al., 1995) at day 0 using a plastic catheter at 2.5 cm from the anus. Mice were killed at day 7. Control group received 0.1 ml 50% ethanol.

To analyze the effects of TNBS, we evaluated the body weight, clinical and histological scores, and weight and length of the colon. The Disease Activity Index (DAI) was daily evaluated considering weight, water/food consumption, morbidity, stool consistency, pilo-erection, and the presence of rectal bleeding. The DAI was calculated independently by two blinded investigators.

Histological analysis of the colon

Colon tissue was fixed in 5% neutral-buffered formalin and embedded in paraffin. Samples were cut and mounted onto positive-charged glass slides, heated at 60°C for 30 min and stained with hematoxylin and eosin (H&E). The histologic activity index (HAI) was scored according to cell infiltration (0, no infiltration; 1, increased number of inflammatory cells in the lamina propria; 2, inflammatory cells extending into the submucosa; 3, transmural inflammatory infiltrates) and tissue damage (0, no mucosal damage; 1, discrete epithelial lesions; 2, erosions or focal ulcerations; 3, severe mucosal damage with extensive ulceration extending into the bowel wall; Lee et al., 2005). At least five sections per animal were analyzed.

Measurement of soluble growth factors and cytokines

IECs were incubated with pro-inflammatory cytokines: IL-1β (50 ng/ml), TNF (50 ng/ml), IL-5 (5 ng/ml) or IL-13 (5 ng/ml), and Gal-1 (1 μM) during 16 h. As controls, cells were incubated with medium, Gal-1 or pro-inflammatory cytokines alone. Supernatants were collected and soluble factors were quantified by ELISA: TSLP (Biolegend, San Diego, CA), EGF (R&D Systems, Minneapolis, MN), TGF-β_1 (eBioscience), IL-10 (eBioscience), and IL-25 (eBioscience).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 software. The significance of the difference was determined using an independent sample *t*-test for paired or unpaired data when appropriate. One-way analysis of variance (ANOVA) with a post hoc Bonferroni's test was used for analysis of more than two groups. A *P*-value less than 0.05 was regarded as statistically significant.

Results

An inflammatory micro-environment controls Gal-1 binding to enterocytes

To investigate whether an inflammatory milieu could modify the capacity of IECs to bind Gal-1, we incubated isolated murine enterocytes in the presence of different pro-inflammatory cytokines. As shown in Figure 1A, we found a significantly increased binding of Gal-1 to enterocytes in the presence of IL-1β, TNF, or IL-13. The binding of Gal-1 was also assessed in colonocytes isolated from IBD or control patient biopsies (Fig. 1B). Paired samples taken from inflamed and non-inflamed areas of the same patient were analyzed and 100 mM lactose was used to determine sugar-binding specificity. We observed a higher binding of Gal-1 to cells from inflamed areas compared with that from non-inflamed or non-IBD tissues; paired samples showed a more intense binding to cells from inflamed tissues compared to cells located in non-inflamed areas.

Elevated concentrations of Gal-1 control IEC viability via caspase3- and 9-dependent pathway

As our previous studies demonstrated a regulatory role for Gal-1 in modulating epithelial cell viability (Muglia et al., 2011), we sought to evaluate the impact of inflammation in this

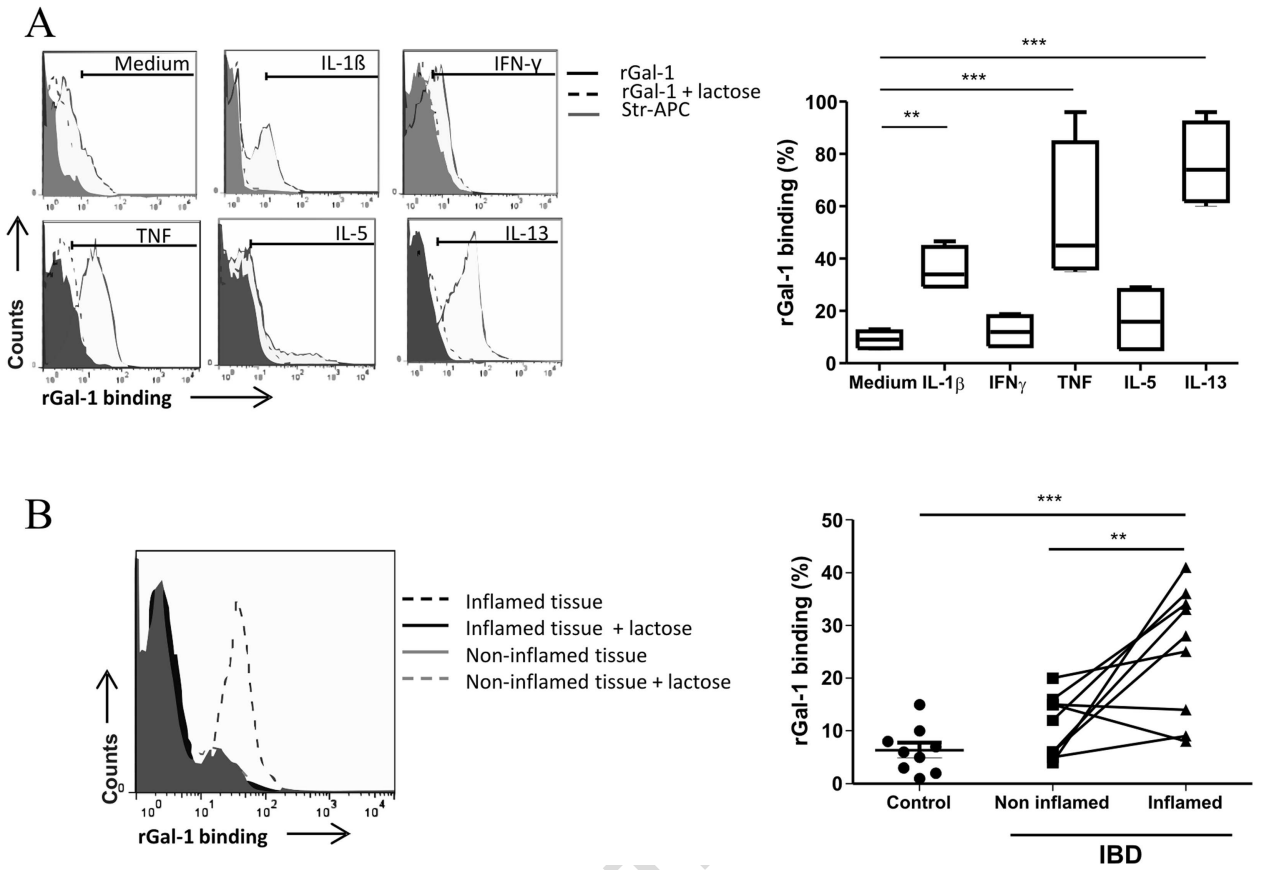

Fig. 1. Gal-1 binding to mouse and human IECs. (A) Flow cytometry of Gal-1binding to enterocytes cultured with either IL-1β (50 ng/ml), IFN-γ (50 ng/ml), TNF (50 ng/ml), IL-5 (5 ng/ml), IL-13 (5 ng/ml) or media alone, and then incubated with biotinylated rGal-1 followed with streptavidin-APC. As control, 100 mM lactose was added to inhibit binding of rGal-1 to specific glycosylated sites (n = 12 animals). (B) Left: flow cytometry of Gal-1 binding to IECs isolated from colonic biopsies of control and IBD patients (macroscopically inflamed and non-inflamed areas); right: frequency of Gal-1$^+$ cells in control and paired samples from the same IBD patient (inflamed and non-inflamed areas). Lactose (100 mM) was added to inhibit the rGal-1 binding as control. Data are expressed as mean values ± SEM ($^{**}P < 0.01$, $^{***}P < 0.001$).

process. We found no changes in IEC survival when exposed to 0.1–3 μM Gal-1, whereas a significant proportion of Annexin V$^+$ IECs were observed in the presence of high concentrations (5 μM) of this lectin (Fig. 2A). We further confirmed that 79.4 ± 5% of Gal-1$^+$ cells were Annexin V$^+$ (Fig. 2B). Interestingly, exposure to Gal-1 in the presence of pro-inflammatory cytokines, such as IL-1β, TNF, and IL-13, led to considerable increase in the frequency of Annexin V$^+$ IECs (Fig. 2C). Analysis of the mechanisms underlying this effect revealed increased activity of caspase-3 and -9 (Fig. 2D), which correlated with increased Bad and Bax expression and diminished Bcl-2 levels (Fig. 2E). The use of different caspase inhibitors confirmed the involvement of specific caspase activation in IECs incubated with Gal-1 (Fig. 2D). Thus, inflammation controls epithelial cell viability by modulating sensitivity to Gal-1.

Intestinal inflammatory micro-environments favor a Gal-1-permissive glycophenotype in enterocytes

To evaluate if a pro-inflammatory milieu could in vivo regulate enterocyte susceptibility to Gal-1 binding, we used two different murine models of intestinal inflammation: (a) a food allergy mouse model and b) a colitis mouse model.

A CT-driven food allergy mouse model was employed to analyze the effect of a T helper 2-dependent inflammatory response in Gal-1 binding to IECs. The experimental protocol is illustrated in Figure 3A. To characterize the immune response elicited in mice we firstly scored the hypersensitivity symptoms elicited immediately after oral challenge. Sensitized mice exhibited higher scores compared with control mice (Fig. 3B). To further investigate the immune mechanism underlying the allergic reaction, we monitored the serum CMP-specific immunoglobulins and cytokine secretion by splenocytes. As shown in Figure 3C, serum CMP-specific IgE and IgG1 antibodies were induced in sensitized mice, which was functionally reflected by positive skin tests (Fig. 3D). Consistent with these findings, we found an increased production of IL-5, IL-13, and TNF in CMP-stimulated splenocytes from allergic mice, while IFN-γ remained low and unchanged (Fig. 3E). To confirm if systemic Th2-biased immune activation was reflected at the mucosal site, the mRNA expression of cytokines was evaluated in duodenum and we found increased levels of *il13* and *il5* mRNA in sensitized mice (Fig. 3F). In addition, we observed low *ifng* mRNA levels in the gut of sensitized mice. Thus, an inflammatory Th2 context was locally and systemically induced in sensitized mice. This effect was accompanied by edematized villi, with infiltration of lamina propria mononuclear cells in intestines of sensitized compared to control mice (Fig. 3G). Interestingly, we

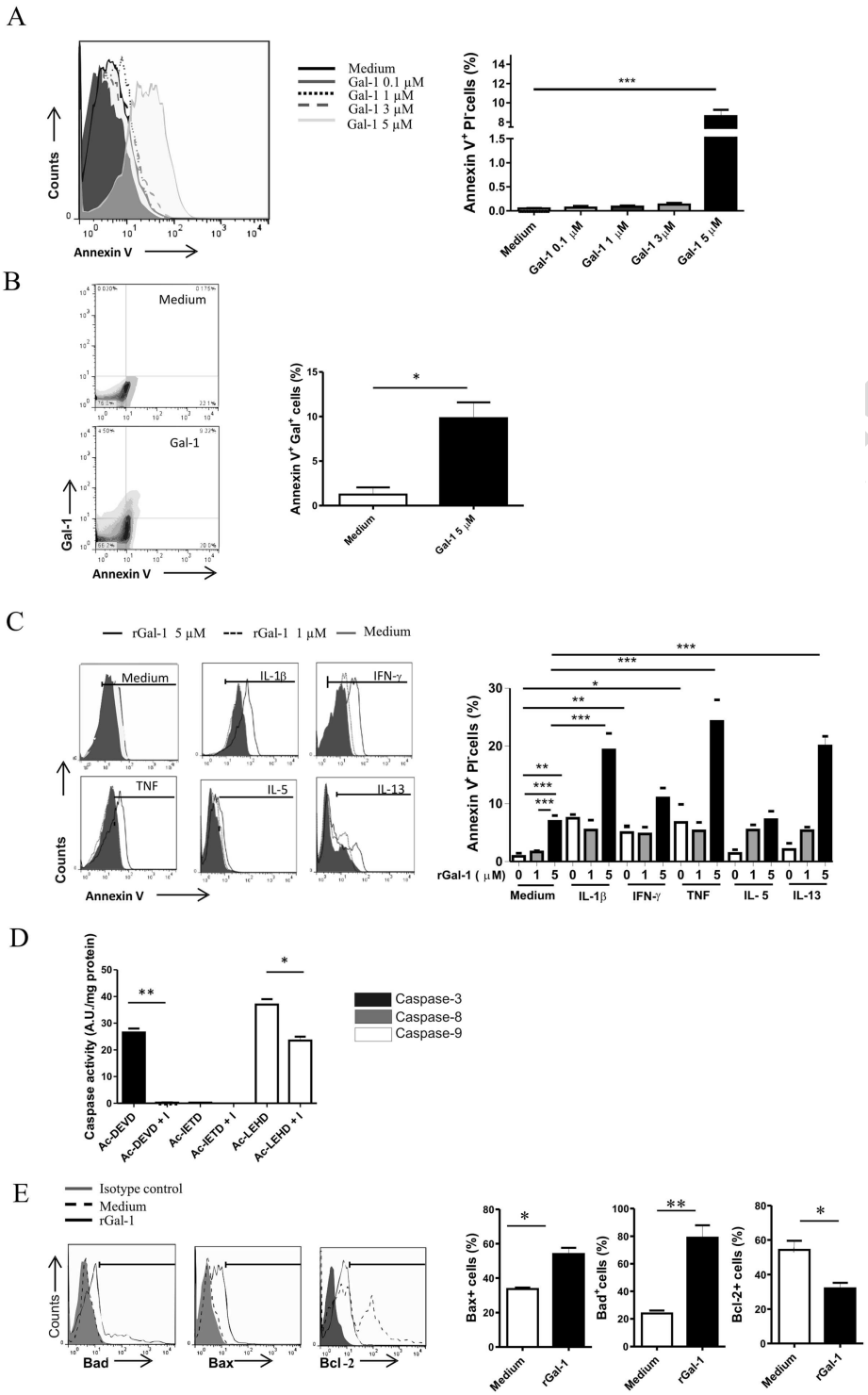

Fig. 2. Gal-1 controls death of murine IECs. Representative histograms and statistical analysis are shown for (A) Annexin V/PI analyzed by flow cytometry in murine enterocytes incubated with different concentrations of rGal-1 or medium alone (n = 5 mice); (B) IECS were incubated with 5 μM biotinylated rGal-1 for 18 h and then stained with Annexin V and streptavidin-APC. Left: representative histograms; right: frequency of Gal-1$^+$Annexin V$^+$ cells (C) Annexin V/PI analyzed by flow cytometry in enterocytes incubated with rGal-1 (1 and 5 μM) and pro-inflammatory cytokines (50 ng/ml IL-1β, 50 ng/ml TNF, 50 ng/ml IFN-γ, 5 ng/ml IL-5, and 5 ng/ml IL-13) or medium alone; (D) caspase activity by fluorometry in enterocytes incubated with rGal-1 (5 μM) or medium. Enterocytes were harvested and caspase-3, -8, and -9 activities were assessed using specific substrates, with or without specific caspase-inhibitors (I). Values of caspase activity corresponding to medium were subtracted; (E) frequency of cells intracellularly stained for Bad, Bax, and Bcl-2 by flow cytometry (n = 6). Data are expressed as mean value ± SEM ($^*P < 0.05$, $^{**}P < 0.01$).

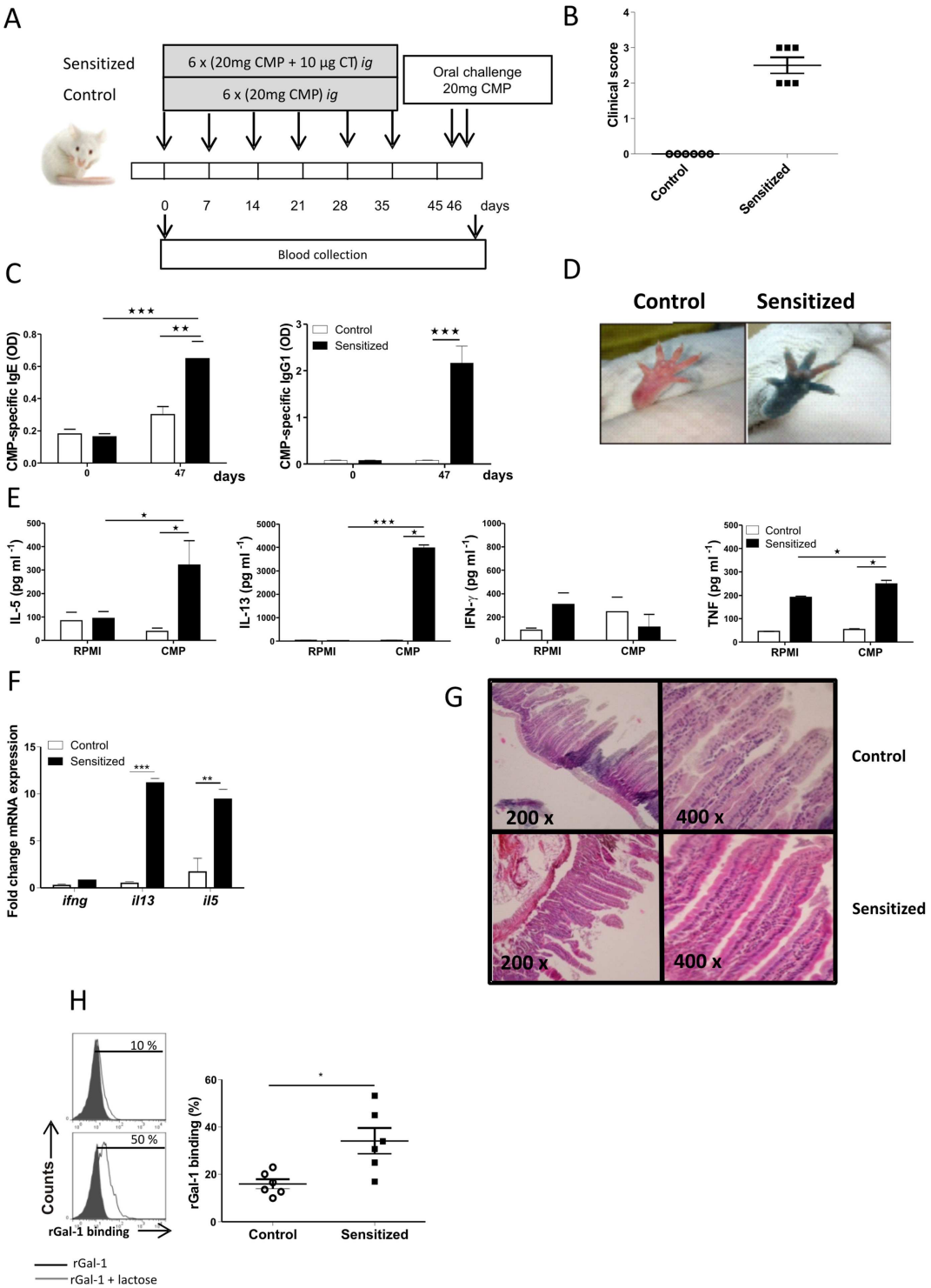

Fig. 3. Gal-1 binding to enterocytes in a mouse model of food allergy with a Th2 dominant micro-environment. An IgE-mediated food allergy model was induced in BALB/c mice (n = 6/group) (A) Outline of the experimental design for the food allergy mouse model. (B) Clinical scores corresponding to hypersensitivity symptoms following the oral challenge with CMP. (C) Serum CMP-specific IgE and IgG1 antibodies by EAST and ELISA, respectively. (D) Skin test performed with CMP in sensitized and control mice (a representative result is depicted). (E) Cytokines secreted by spleen cells stimulated with medium or CMP (350 µg/ml). Supernatants were collected and analyzed by ELISA. (F) Gene expression corresponding to *ifng*, *il13*, and *il5* mRNA was quantified by qPCR in jejunum segments 24 h following the oral challenge. β-Actin expression was used to standardize the total amount of cDNA and fold change of mRNA expression was defined as the ratio between the normalized values corresponding to sensitized and control mice. (G) Histopathological analysis of small bowel in control and sensitized animals (hematoxylin and eosin staining; magnifications are indicated). (H) Gal-1 binding to enterocytes isolated from jejunum of sensitized and control animals by flow cytometry using biotinylated rGal-1. Histograms and statistical analysis of the frequency of Gal-1$^+$ cells are shown. These results are representative of two independent experiments with similar results. Data are expressed as mean values ± SEM ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). ig, intra-gastric administration.

found increased binding of rGal-1 to IEC isolated from inflamed intestinal tissue from sensitized mice compared to their control counterpart (Fig. 3H).

To further evaluate the effect of inflammation on Gal-1 binding, we generated a colitis mouse model by intrarectal administration of the hapten TNBS, which induces Th1 and Th17 responses in the BALB/c susceptible strain. Mice receiving 5 mg of TNBS showed a rapid colitis onset marked by weight loss (Fig. 4A), diarrhea, loose feces, and a significant DAI elevation (Fig. 4B). Macroscopical examination of intestines showed a significant reduction of length and increase in weight of colon in 5 mg TNBS colitis compared to 2.5 mg TNBS and control mice (Fig. 4C). This result correlated with the histopathological analysis that revealed edema, hyperproliferative crypts, and cell infiltration of lamina propria (Fig. 4D), with a significant higher HAI (Fig. 4E) in the acute colitis model provoked with 5 mg versus 2.5 mg, and control mice.

Furthermore, study of mRNA expression for cytokines and/or transcription factors involved in innate (IL-1β, IL-6, and TNF) and adaptive (T-bet and IFN-γ) Th1-dependent immunity revealed a significant induction of *tnf*, *t-bet*, and *ifng* mRNA in TNBS-treated mice (5 mg) versus mice treated with 2.5 mg TNBS or vehicle (ethanol)-treated mice (Fig. 4F and G), suggesting a mucosal Th1 dominant pro-inflammatory response. Of note, IECs isolated from mice with TNBS-induced colitis (5 mg) showed a significant increased Gal-1 binding compared to IECs from mice receiving 2.5 mg TNBS and IECs isolated from vehicle-treated animals (Fig. 4H). Thus, Gal-1 binds with greater affinity to IECs exposed in vitro or in vivo to inflammatory stimuli.

Inflammatory stimuli control Gal-1-induced secretion of growth factors and cytokines

We finally investigated how pro-inflammatory stimuli influence Gal-1-induced secretion of growth factors and cytokines by mouse IECs (Fig. 5). Freshly isolated IECs were exposed to 1 μM Gal-1 in the presence of pro-inflammatory (Th1- or Th2-polarizing) cytokines (IL-1β, TNF, IL-5, and IL-13). We found that TNF augmented the secretion of epidermal growth factor (EGF), thymic stromal lymphopoietin (TSLP), IL-10, and TGF-β_1 induced by Gal-1 (1 μM) on IECs. Furthermore, Gal-1 co-incubated with IL-13 induced an up-regulation of IL-10, TGF-β_1, and IL-25 secretion, whereas IL-5 rendered an augmented production of IL-25 in Gal-1-exposed IECs. Altogether these findings suggest that Gal-1 controls the release of anti-inflammatory cytokines and growth factors and may contribute to create a tolerogenic micro-environment, thus protecting the intestinal epithelium in response to an inflammatory challenge.

Discussion

To deal with the continuous antigenic and microbial challenges, the mucosal surface of the intestine is equipped with a complex network of cells and soluble mediators that maintain a permanent balance and tissue homeostasis (Williams, 2006). In this complex scenario, the epithelial compartment is a key functional component of the mucosa that expresses several glycosylated receptors that exhibit galectin-specific saccharides. These glycans displayed on the epithelial surface may have key roles in immune homeostasis and microbial recognition to penetrate the mucosa[Q7] (Ciarlet et al., 2001; Mahdavi et al., 2013; Nita-Lazar et al., 2015). It has been documented that lectins are expressed in normal as well as in pathological situations in the digestive tract (Demetter et al., 2008; Hokama et al., 2008). Specifically, Gal-1 has been implicated in IBD with a protective role in a TNBS-induced colitis in mice through a selective pro-apoptotic effect on activated Th1 cells (Santucci et al., 2003). Moreover, this lectin instructs the differentiation of IL-27-producing tolerogenic dendritic cells which promotes T-cell tolerance through IL-10-dependent mechanisms (Ilarregui et al., 2009), although this mechanism has not yet been confirmed in the gut. Importantly, although galectins have been linked to IBD (Santucci et al., 2003; Hokama et al., 2008; Lippert et al., 2008; Paclik, Berndt et al., 2008; Paclik, Danese et al., 2008; Paclik, Lohse et al., 2008; Frol'ová et al., 2009), there is still no study investigating their role in the homeostasis of IECs, which are critical components in mucosal homeostasis and permeability. Increased leakage of the epithelial layer with an uncontrolled permeability has been implicated in the development of chronic inflammatory diseases (Nusrat et al., 2000; Randall-Demllo et al., 2013). Collectively, these evidences prompted us to investigate, using in vitro and in vivo strategies, the function of Gal-1 within the intestinal epithelial cell compartment under different inflammatory settings.

We have previously shown that Gal-1 induces mouse and human enterocyte apoptosis through a mitochondrial- and caspase-3-dependent pathway (Muglia et al., 2011). In spite of the fact that human intestinal Caco-2 cell line underwent apoptosis upon incubation with recombinant Gal-1 through a calpain-dependent, caspase-independent mechanism (Paclik, Lohse et al., 2008), here we confirmed that the caspase-dependent intrinsic pathway of apoptosis was induced with 5 μM of Gal-1. We showed that the cell death programs induced in IECs involve acaspase-9-dependent pathway accompanied by increased Bax and Bad, and decreased Bcl-2 expression levels.

Several reports have postulated that apoptosis of IECs may be linked to the pathogenesis of mucosal inflammation. Di Sabatino et al. (2003) demonstrated that increased enterocyte apoptosis is associated with CD with a higher rate of apoptosis in inflamed as compared to non-inflamed areas. Consistent with this finding, Heller et al. (2005) described IL-13 as an effector pro-inflammatory cytokine that affected the epithelial barrier function by inducing epithelial cell apoptosis in patients with ulcerative colitis. Accordingly, Trautmann et al. (2002) reported that apoptosis is a key pathogenic event leading to bronchial epithelial cell shedding in asthma. In this study, we showed that intestinal inflammation induced by Th1/Th17-polarized (colitis model and IBD patients) and Th2-polarized (food allergy mouse model) responses promoted a permissive micro-environment that positively regulated binding of Gal-1 to IECs. Although several reports showed that asialo-core 1-O-glycans and core-2O-glycans are differentially exposed in epithelial cells under inflammatory or neoplastic conditions (Fu et al., 2011; An et al., 2007; Campbell et al.), there is still no study investigating the impact of Gal-1 binding on epithelial cells of inflamed mucosa and its associated biological function. Our results show that enhanced binding of Gal-1 in the presence of pro-inflammatory cytokines may lead to a compensatory effect on IECs to protect the epithelial cell compartment and restore intestinal homeostasis. In this regard, different soluble factors have been described to be involved in gut homeostasis (Clark et al., 2006; Li and Flavell, 2008; Zaph et al., 2008; Zeuthen et al., 2008; Hardy et al., 2013; Suzuki, 2013) and their up-regulation has been documented in epithelial cells exposed to a variety of inflammatory stimuli (Mahdavi et al., 2013; Munari et al., 2014). Further studies in IBD and food allergy models are mandatory to explore the differential expression of glycosyltransferases and glycosidases on immune or endothelial target cells exposed to pro-inflammatory or tolerogenic micro-environments. Interestingly, we have recently identified changes in the glycosylation signature of endothelial cells upon exposure to immunosuppressive (IL-10 or TGF-β_1) versus pro-inflammatory (IFN-γ or IL-17) cytokines, thus compromising Gal-1 binding and pro-angiogenic activity (Croci et al., 2014). In

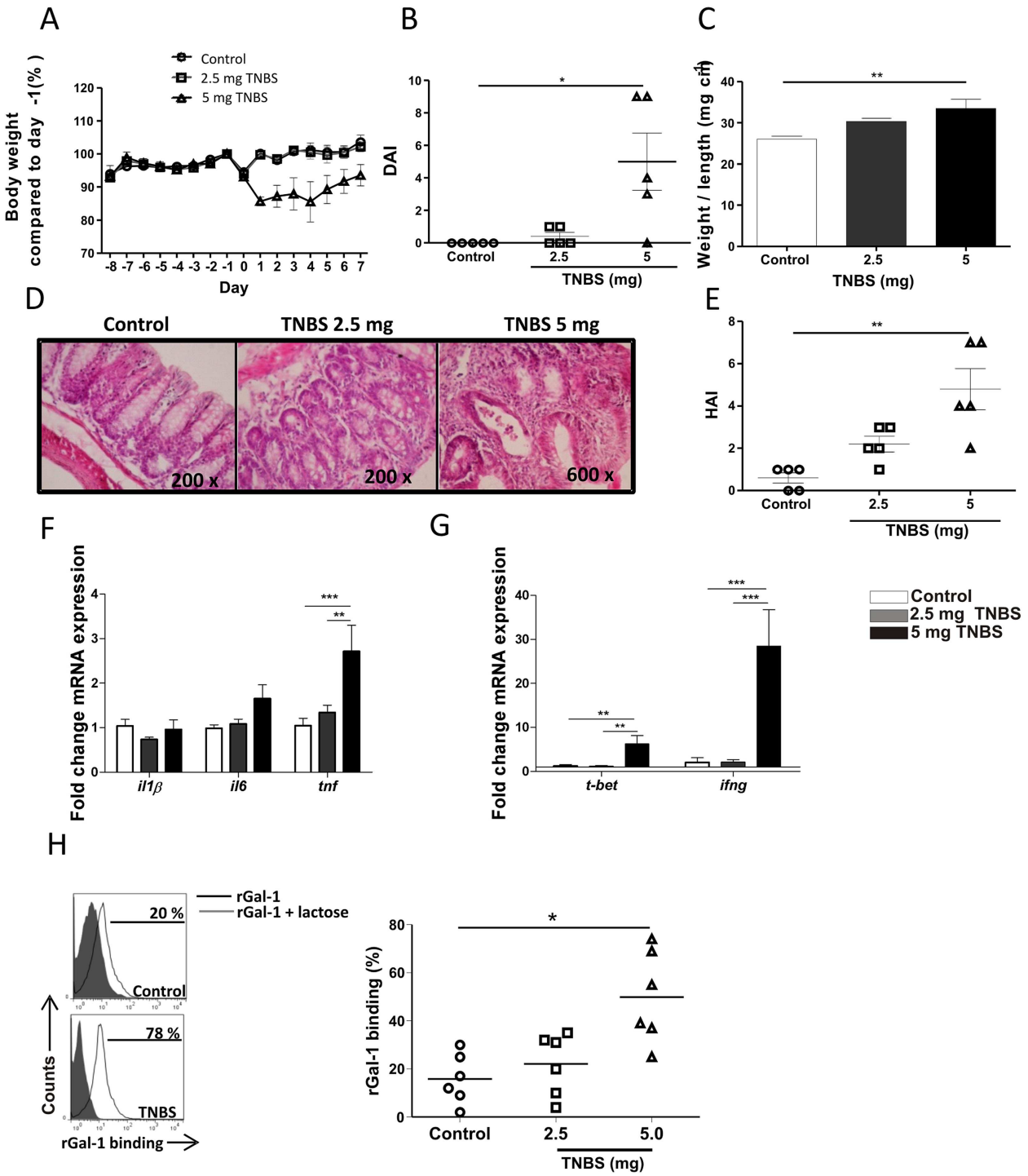

Fig. 4. Gal-1 binding to enterocytes in a colitis model with a Th1/Th17 dominant micro-environment. The colitis model was performed in BALB/c mice through the intrarectal administration of 2.5 and 5 mg of TNBS in ethanol. Mice were killed at day 7 (n = 6/group). (A) Body weight was monitored throughout the experiment. (B) Disease activity index (DAI) at day 7. (C) Weight/length ratio of the whole large bowel. (D) Histopathological analysis of colon from control and colitis mice (hematoxylin and eosin staining; magnifications are indicated). (E) Histological Activity Index (HAI). (F) Gene expression corresponding to *il1b*, *il6*, and *tnf* mRNA was determined by qRT-PCR in colon segments. β-Actin expression was used to standardize the total amount of cDNA and the fold change of mRNA expression was defined as the ratio between the normalized values corresponding to TNBS-treated and control mice. (H) Gal-1 binding in colonocytes isolated from colonic segments of TNBS-treated and control mice by flow cytometry. Histograms and statistical analysis of the frequency of Gal-1$^+$ cells are shown. Results are representative of two independent experiments. Data are expressed as mean values ± SEM ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

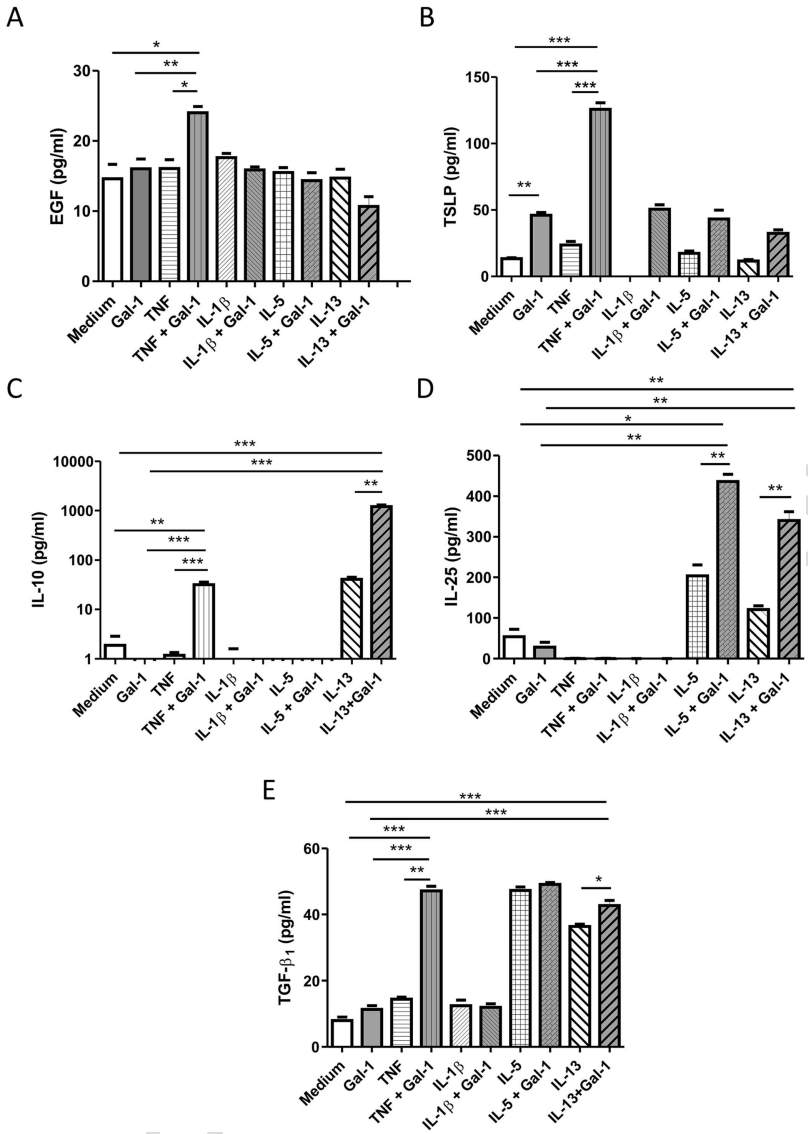

Fig. 5. Soluble factors released by mouse enterocytes upon incubation with Gal-1 in the presence of pro-inflammatory cytokines. IECs were exposed to rGal-1 (1 μM) for 16 h in the presence of pro-inflammatory cytokines, including IL-1β (50 ng/ml), TNF (50 ng/ml), IL-5 (5 ng/ml), IL-13 (5 ng/ml), or medium alone. Soluble factors were analyzed in culture supernatants by ELISA; (A) TSLP; (B) EGF; (C) IL-10; (D) IL-25; and (E) TGF-β_1. Results shown are representative of two independent experiments. Data are expressed as mean values ± SEM ($^{*}P < 0.05$, $^{**}P < 0.01$).

addition, we previously demonstrated the differential ability of activated Th1 and Th17 cells to bind Gal-1, an effect which was associated to the differential glycosylation of cell surface glycoconjugates (Toscano et al., 2007). Here, we show that under different inflammatory intestinal conditions, IECs become more sensitive to Gal-1 binding which contributes to the release of a variety of tolerogenic and anti-inflammatory cytokines. This effect could contribute to barrier recovery and to counteract the effects that pro-inflammatory cytokines exert on expression of tight junction proteins that regulate paracellular permeability (Prasad et al., 2005). Importantly, we showed that at low physiologic concentrations Gal-1 favors the release of anti-inflammatory and tolerogenic cytokines from IECs, whereas higher concentrations of this lectin (5 μM) influence IEC viability. In addition, the inflammatory intestinal environment may delineate a differential glycosylation of the epithelial cell surface, thereby modulating microbe recognition and susceptibility to infections. It has been described that in inflammation some commensal bacteria may become pathogenic and some pathogenic bacteria may become more virulent. The recognition of glycans has been implicated in the pathological sequelae associated to host-microbe interaction at different levels in the mucosa (Mahdavi et al., 2013; Nita-Lazar et al., 2015). It should be interesting to investigate if the inflammatory conditions here studied may influence the mucosal susceptibility to components of the intestinal microbiota and promote the reprogramming of bacterial behavior.

In conclusion, we show here that a pro-inflammatory (either Th2 or Th1/Th17) micro-environment can regulate the

response of IECs to a regulatory factor such as Gal-1. These findings suggest that dysregulation of the cellular glycome in intestinal inflammatory disorders could alter endogenous mechanisms of tolerance and homeostasis. Further analysis is warranted in order to evaluate the complex changes in the expression of glycosyltransferases and glycosidases in IECs responding to pro-inflammatory insults. Thus, Gal-1 may serve as a backup mechanism to restore intestinal homeostasis and to preserve the epithelial cell compartment against insults induced by pro-inflammatory stimuli.

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