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# Galactomannan as a Potential Modulator of Intestinal Ischemia–Reperfusion Injury

*Pablo Stringa, PhD,*[a,b,1] *Victor Toledano, MS,*[b,c,1]
*Rodrigo Papa-Gobbi, PhD,*[a] *Miguel Arreola, PhD,*[a] *Carlota Largo, PhD,*[a]
*Mariana Machuca, MD,*[d] *Luis A. Aguirre,*[b,c] *Martin Rumbo, PhD,*[e]
*Eduardo López-Collazo, MD,*[b,c,*]
*and Francisco Hernández Oliveros, MD, PhD*[a,**]

[a] *Transplant Group, Experimental Surgery, IdiPAZ, La Paz University Hospital, Madrid, Spain*
[b] *Tumor Immunology Laboratory, IdiPAZ, La Paz University Hospital, Madrid, Spain*
[c] *Innate Immunity Group, IdiPAZ, La Paz University Hospital, Madrid, Spain*
[d] *Special Pathology Laboratory, Faculty of Veterinary Sciences, National University of La Plata, La Plata, Buenos Aires, Argentina*
[e] *Institute for Immunological and Physiopathological Studies (IIFP-CONICET-UNLP), National University of La Plata, La Plata, Buenos Aires, Argentina*

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ABSTRACT

*Background:* Galactomannan (GAL), a polysaccharide present on the cell wall of several fungi, has shown an ability to modulate inflammatory responses through the dectin-1 receptor in human macrophages. However, studies evaluating the modulatory properties of this polysaccharide in *in vivo* inflammatory scenarios are scarce. We hypothesized that GAL pretreatment would modulate local and remote damage related to intestinal reperfusion after an ischemic insult.
*Materials and methods:* Adult male Balb/c mice were subjected to intestinal ischemia–reperfusion injury by reversible occlusion of the superior mesenteric artery, consisting of 45 min of ischemia followed by 3 or 24 h of reperfusion. Intragastric GAL (70 mg/kg) was administered 12 h before ischemia, and saline solution was used in the control animals. Jejunum, lung, and blood samples were taken for the analysis of histology, gene expression, plasma cytokine levels, and nitrosative stress.
*Results:* Intestinal and lung histologic alterations were attenuated by GAL pretreatment, showing significant differences compared with nontreated animals. Interleukin 1β, monocyte chemoattractant protein 1, and IL-6 messenger RNA expression were considerably downregulated in the small intestine of the GAL group. In addition, GAL treatment significantly prevented plasma interleukin 6 and monocyte chemoattractant protein 1 upregulation and diminished nitrate and nitrite levels after 3 h of intestinal reperfusion.

* *Corresponding author.* IdiPAZ, Instituto de Investigacion del Hospital La Paz, Pedro Rico 6, Madrid, Spain. Tel.: +34-912071029.
** *Corresponding author.* IdiPAZ, Instituto de Investigacion del Hospital La Paz, Pedro Rico 6, Madrid, Spain. Tel.: +34-912072483; fax: +34-91-7277576.
E-mail addresses: elopezc@salud.madrid.org (E. López-Collazo), fhernandezo@salud.madrid.org (F. Hernández Oliveros).
[1] These authors contributed equally to this work.
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*Conclusions:* GAL pretreatment constitutes a novel and promising therapy to reduce local and remote damage triggered by intestinal ischemia–reperfusion injury. Further *in vivo* and *in vitro* studies to understand GAL's modulatory effects are warranted.

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## Introduction

Intestinal ischemia–reperfusion injury (IRI) is a phenomenon that occurs in many intestinal diseases, such as strangulated hernia, volvulus, necrotizing enterocolitis, and small bowel and multivisceral transplantation, among others.[1]

This dynamic process involves an ischemic period characterized by reduction in cellular mitochondrial adenosine triphosphate generation, hydrolase activation, alteration of cell membrane permeability, and increased calcium influx into ischemic cells. At the same time, the ischemic insult is exacerbated during reperfusion because of a marked proinflammatory cytokine release, mainly interleukin (IL)-6 and tumor necrosis factor (TNF)-$\alpha$, increased production of reactive nitrogen and oxygen species, upregulation of toll-like receptor signaling, and activation of proinflammatory transcription factors.[2-4]

Nevertheless, IRI in the intestine not only affects local tissue but this damage also triggers a systemic inflammatory response leading to morphologic and functional alterations in remote organs, particularly the kidneys, liver, and lungs, which can lead to multiple organ dysfunction syndrome.[5,6]

In this sense, any strategy capable of mitigating IRI will improve organ recovery and patient survival. In fact, this issue has become one of the main aims for basic and translational research in the field of intestinal surgery; novel strategies, including antioxidant administration, cellular therapies, ischemic preconditioning, and various drug treatments, have already been tested.[7-11]

On the other hand, immune system modulation therapies have created new opportunities for intervention. Among them, the possibility of reprogramming the innate activation of immune cells by various means could contribute to new alternatives in this field.[12,13] Our group has previously reported that galactomannan (GAL), a component of the cell walls of several fungi that is released during growth, is capable of reprogramming macrophages' inflammatory response in humans. Although the entire mechanism is not fully understood, when macrophages are pretreated *in vitro* with this polysaccharide, they express less class-II transactivator and less human leukocyte antigen-DR. Moreover, GAL inhibits the nuclear factor kappa-light-chain-enhancer of activated B cells 2 (NFκB2) pathway, which, in turn, diminishes TNF-$\alpha$ and IL-6 expression after lipopolysaccharide stimuli. All these data suggest a potential role for GAL as a controller of the inflammatory response in some clinical situations.[14,15]

Considering that GAL's anti-inflammatory properties have never been assessed in an *in vivo* model, our main goal was to evaluate GAL administration as a strategy to attenuate local and remote intestinal IRI in mice.

## Materials and methods

### *Animal use and care*

Adult male Balb/c mice (average weight, 27 ± 0.54 g) were housed in a climate-controlled room (21°C ± 2°C and relative humidity of 45% ± 15%) on a 12-h light–dark cycle at our institution's animal facilities. This study was performed in strict accordance with the European Union Criteria for Animal Use in Scientific Experimentation recommendations (63/2010 EU) and related Spanish legislation (RD 53/2013). The protocol was approved by the Animal Welfare Ethics Committee of La Paz University Hospital (PROEX 139/17).

### *Surgical procedure*

Intestinal IRI by reversible occlusion of the superior mesenteric artery (SMA) was performed as we had previously described.[16] Briefly, mice were anesthetized with isoflurane (5% induction and 1.5%-2% maintenance), and tramadol hydrochloride (subcutaneous, 20 mg/kg) was used for pain control. After celiotomy, SMA was isolated and occluded with a vascular clamp using microsurgical instruments and a microscope (10×) to magnify the surgical field. After the stipulated time, the clamp was removed, and the small bowel returned immediately to its normal color (Supplementary Fig. 1-A). After surgery, mice recovered on a heating unit at 38°C to prevent hypothermia and were placed in a housing cage until sampling.

### *Experimental design*

Three groups of 10 animals each were established. Except for the sham group, 40 min of SMA occlusion was performed.

*Sham groups*

Intragastric saline (0.2 mL, $n = 5$) or GAL (70 mg/kg in 0.2 mL, $n = 5$) was administered 12 h before surgery (celiotomy and SMA dissection without occlusion). The animals were euthanized, and samples were taken 3 h after surgical procedure. The GAL concentration was chosen because of previous results of *in vitro* and *in vivo* rat experiments in our group (data not shown).

*Control group*

This group received intragastric saline 12 h before SMA occlusion. Animals were euthanized and sampled 3 h ($n = 5$) or 24 h ($n = 5$) after reperfusion.

*GAL group*

A single dose of intragastric GAL was administered as described for the sham group. SMA occlusion was performed 12 h later. Animals were euthanized 3 h ($n = 5$) or 24 h ($n = 5$) after reperfusion.

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A representative diagram of the experimental design is shown in (Supplementary Fig. 1-B).

*Sampling*

The animals were euthanized by exsanguination under general anesthesia after the corresponding reperfusion period (3 or 24 h). In all the animals, the distal jejunum, lungs, and blood samples from the abdominal vena cava were taken for local and remote assessment of intestinal IRI.

*Intestinal and lung histologic evaluation*

Sections of the animals' small bowel and lungs were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin for histologic evaluation performed blindly by two pathologists.

Intestinal IRI histologic damage was scored according to Park's classification as previously described[16] (score 0, normal mucosa; 1, subepithelial space at villus tip; 2, more extended subepithelial space; 3, epithelial lifting along villus side; 4, denuded villi; 5, loss of villus tissue; 6, crypt layer infarction; 7, mucosal infarction; and 8, transmural infarction). Intestinal villus length was assessed using Image J software (LOCI, University of Wisconsin, Madison). At least 20 individual villi were measured in each mouse to determine this parameter.

To evaluate lung histologic damage, we assessed neutrophil infiltration, interstitial edema, airway epithelial cell damage, hyaline membrane formation, and hemorrhage. Each parameter was scored (0, normal; 1, mild change; 2, moderate change; or 3, severe change). Samples received a general score resulting from adding each evaluated parameter.

*Cytometric bead array and flow cytometer analysis*

IL-6, IL-10, monocyte chemoattractant protein (MCP)-1, interferon (IFN)-γ, and TNF-α plasma levels were analyzed by flow cytometry using a Cytometric Bead Array Mouse Inflammation Kit (552364; BD Bioscience), following the manufacturer's instructions. This Cytometric Bead Array employs a series of particles with discrete fluorescence intensities to simultaneously detect multiple soluble analytes, which, combined with flow cytometry, creates a multiplexed assay. Flow cytometry was performed using a FACSCalibur cytometer (BD Biosciences), and the data were analyzed with FCAP Array Software v3.0 (BD Biosciences).

*Measurement of plasma nitrate/nitrite levels*

Nitrate/nitrite plasma concentrations were performed with a Nitrite/Nitrate Assay Kit (23479-1KT-F; Sigma–Aldrich) in blood samples taken from the abdominal vena cava following the manufacturer's instructions. In particular, the commercial kit focuses on determining nitric oxide metabolite levels using a Griess assay, in which nitrate is converted to nitrite by the enzyme nitrate reductase. The Griess assay's mechanism is summarized as the azo coupling between diazonium species, which are produced from sulfanilamide with nitrite and naphthylethylenediamine. This product, proportional to the nitric oxide metabolite present, was measured spectrophotometrically at a wavelength of 540 nm.

*RNA isolation and quantification*

The total RNA was isolated from the distal jejunum and right lung. These samples were washed once with phosphate-buffered saline and were kept with RNAlater Stabilization Solution (Invitrogen) at 4°C and stored at −80°C until processing. The RNA was isolated using TRI Reagent (Sigma–Aldrich; St. Louis, MO) and further purified with DNAse type 1 from Amersham Biosciences (Piscataway, NJ). The complementary DNA (cDNA) was obtained by reverse transcription of 1 μg of RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression levels were analyzed by real-time quantitative polymerase chain reaction, using the CFX96 Real-Time PCR Detection System (Bio-Rad) and cDNA obtained as described above. The quantitative polymerase chain reactions were performed using the QuantiMix Easy kit from Biotools (Madrid, Spain) and specific primers for MCP-1, TNF-α, IFN-γ, IL-1β, IL-6, and IL-10. The relative gene expression level was determined using mouse GAPDH as a normalizer. The primer sequences were as follows: MCP-1 forward: 5′- TTA AAA ACC TGG ATC GGA ACC AA -3′; reverse: 5′- GCA TTA GCT TCA GAT TTA CGG GT -3′, TNF-α forward: 5′- TAG CCA GGA GGG AGA ACA GAA AC-3′; reverse: 5′- CAG TGA GTG AAA GGG ACA GAA CC -3′, IFN-γ forward: 5′- CGG CAC AGT CAT TGA AAG CCT A-3′; reverse: 5′- GTT GCT GAT GGC CTG ATT GTC-3′, IL-1β forward: 5′-GCA ACT GTT CCT GAA CTC AAC T-3′; reverse: 5′-ATC TTT TGG GGT CCG TCA ACT-3′, IL-6 forward: 5′- AAA CCG CTA TGA AGT TCC TCT CTG -3′; reverse: 5′-ATC CTC TGT GAA GTC TCC TCT CC-3′, IL-10 forward: 5′-GGT TGC CAA GCC TTA TCG GAA ATG -3′; reverse: 5′- CAC TCT TCA CCT GCT CCA CTG C -3′; and GAPDH forward: 5′- AAC TTT GGC ATT GTG GAA GG -3′; reverse: 5′- ACA CAT TGG GGG TAG GAA CA-3′. All primers were synthesized, desalted, and purified by Bonsai Biotech.

*Statistical analysis*

Comparisons between groups were performed using an analysis of variance followed by Tukey's test for multiple comparisons or Student's *t*-test followed by a Mann–Whitney test for unpaired or paired data, as appropriate. Results were expressed as mean ± standard error of the mean. Differences between means were considered statistically significant when $P < 0.05$.

A linear discriminant analysis was used to separate predefined groups, using a linear combination of variables (gene expression).[17] Data were plotted in a multidimensional graph in which similar objects lie close to one another and are clustered.

## Results

*GAL preserves intestinal architecture against IRI*

We first assessed local IRI in tissue architecture; therefore, we performed an exhaustive histopathologic analysis of intestinal samples and scored the damage according to Park's classification. As depicted in Figure 1A, after 3 h of reperfusion, the control (CT) group showed denuded villi and crypt layer infarction, with epithelial lifting among villi the most representative injury in this group. The histologic score average of

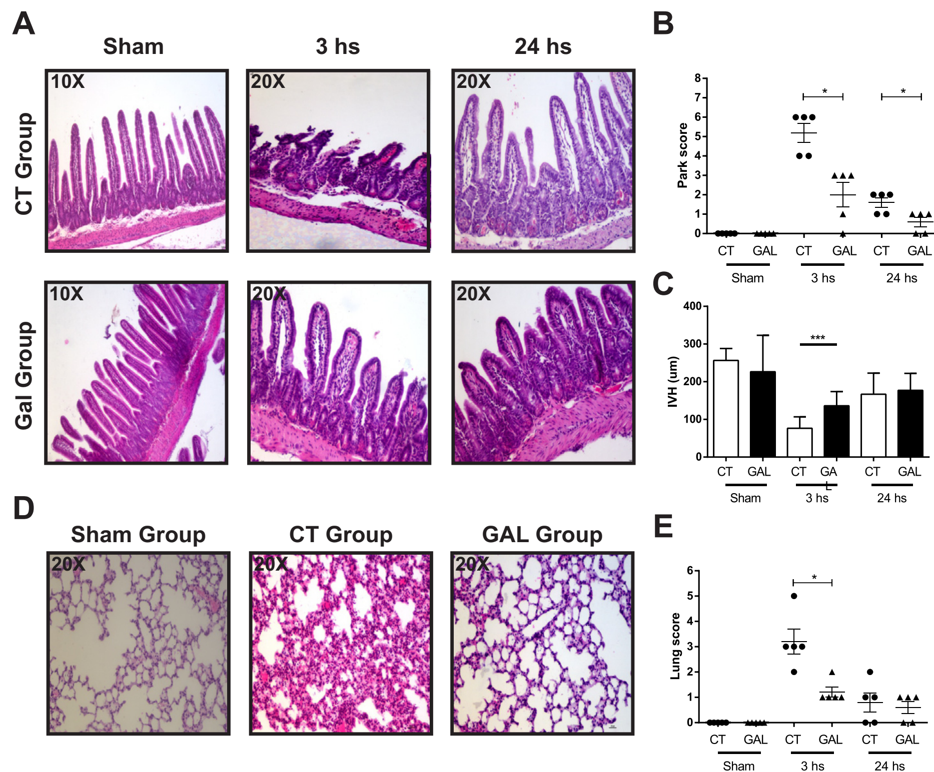


**Fig. 1 – Representative light microscopy (hematoxylin and eosin staining) images of treated and nontreated intestine of mice at 3 and 24 h after intestinal reperfusion (A). Histopathologic intestinal damage induced by IR was studied using Park score. Each points depict a simple sample evaluation ($n = 5$ per group). Also, mean and standard deviation represented by horizontal lines in each group and observation time are shown (B). Bar graph (mean and standard deviation) showing intestinal villus length at different times of reperfusion in sham, CT, and GAL groups (C). GAL pretreatment modulates remote damage cause by intestinal IRI. The highest damage was observed in CT group compared with GAL and sham animals (D). Vertical scatter plot showing lung histologic evaluation was done (each point represents an individual mouse). A significant difference between GAL and CT group was observed at 3 h of jejunum–ileum reperfusion (E). (Color version of figure is available online.)**

the CT group 3 h after IR was 5.2 ± 0.9, with a median of 6 (Fig. 1B). At this reperfusion time, less damage was observed in the GAL-pretreated intestinal samples, with an average of 2 ± 1.2 and a median of 3 ($P < 0.05$; Fig. 2B). After 24 h of reperfusion, the CT and GAL groups showed a tendency to recover intestinal villi integrity, with an average of 1.6 ± 0.4 and 0.6 ± 0.4 in the CT and GAL groups, respectively; significant differences between groups were still observed ($P < 0.05$; Fig. 1A and B). Samples from both sham groups showed normal small bowel morphology (grade 0 in the Park classification).

Intestinal IRI markedly impacted intestinal villus length in all the animals after blood restoration. Interestingly, as shown in Figure 1C, GAL treatment reduces this effect 3 h after reperfusion (CT: 85.11 ± 29; GAL: 133.25 ± 40 μm; $P < 0.05$). However, at 24 h of reperfusion animals, a tendency to recover villi height was observed in both the CT and GAL groups (183.55 ± 47.23 and 183.12 ± 47.06 μm, respectively). No statistically significant differences were observed between groups at this evaluation time (Fig. 1C).

*GAL reduces remote damage triggered by intestinal IRI*

As previously mentioned, IRI affects not only local tissues but also remote organs. In fact, lungs from the CT group showed alterations, characterized by the presence of moderate neutrophil infiltration and mild to moderate edema (three of five samples for each parameter; Fig. 1D) 3 h after reperfusion. Interestingly, significantly less damage was observed at that time in the GAL-treated group in comparison with the CT group ($P < 0.05$; Fig. 1E). Mild neutrophil infiltration was the most frequently observed alteration in the lungs of the GAL-treated animals; no edema was reported in any sample.

The histologic evaluation performed 24 h after reperfusion showed a tendency of lung tissue to recover, with mild neutrophil infiltration as the most characteristic damage. No statistical differences between the CT and GAL groups were observed at the time of study. Microscopic evaluation of the lungs showed normal parenchyma in the GAL and CT sham groups (Fig. 1E).

*GAL modulates the systemic response of cytokines after IRI*

To evaluate the systemic effect of IRI and GAL treatment, we analyzed the plasma levels of several pro- and anti-inflammatory mediators. As depicted in Figure 2A-D, IR induced a significant increase in TNF-α, IFN-γ, IL-6, and MCP-1 levels in the CT group compared with the sham group ($P < 0.05$ for TNF-α and IFN-γ; $P < 0.01$ for IL-6 and MCP-1). Interestingly,

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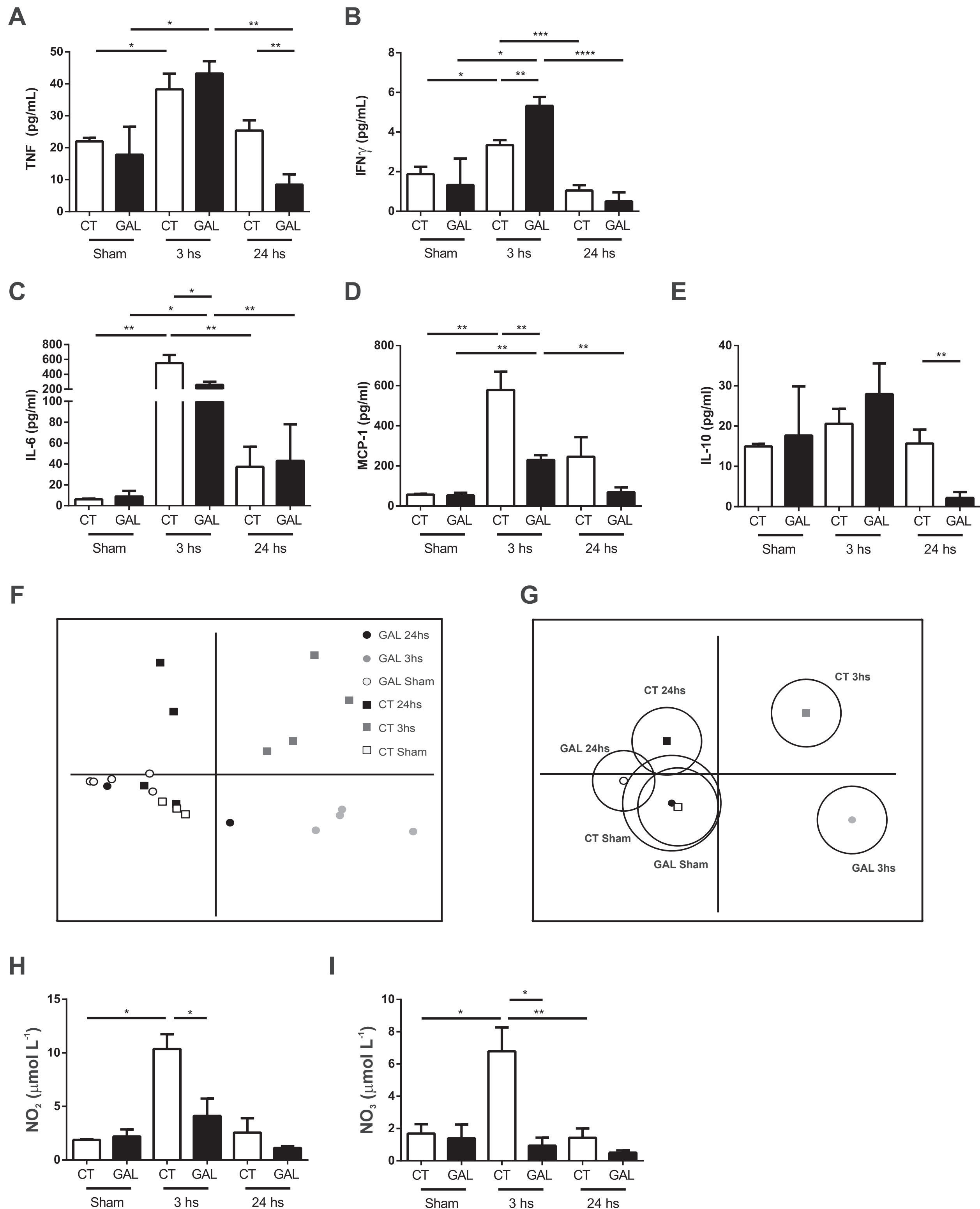


**Fig. 2 – Plasma levels of inflammatory mediators in experimental groups during different reperfusion times. Significant differences in terms of TNF-α, IFN-g, IL-6, MCP-1 and IL-10 were observed between CT and GAL groups (A-E). Principal component analysis of plasma inflammatory mediators in CT, GAL, and sham groups (F and G). Nitrosative stress was studied by nitrate (H) and nitrite (I) plasma level determination after intestinal reperfusion. Significant differences between treated and nontreated animals at 3 h of reperfusion were observed.**

GAL treatment significantly prevented IL-6 and MCP-1 upregulation after 3 h of IR compared with the CT group (IL-6: $P < 0.05$, Fig. 2C; MCP-1: $P < 0.01$, Fig. 2D. Furthermore, TNF-α levels were significantly lower in the GAL-treated animals than in the CT animals 24 h after IR (Fig. 2A). Figure 2F and G shows the linear discriminant analysis of all these immune mediators. This joint analysis revealed that, although after 3 h of IR, both the CT and GAL-treated animals were clustered separately from the sham groups; they were also clearly separated from each other. These data suggest that GAL pretreatment has the ability to modulate cytokine release to the systemic compartment on IRI.

Considering that IRI is also associated with increased production of nitric and oxygen reactive species, we decided to evaluate nitrite and nitrate plasma levels in both the CT and the GAL-treated animals. As depicted in Figure 2H and I, 3 h postreperfusion, the CT group showed a significant increase in nitrite and nitrate compared with the sham group (in both cases $P < 0.05$). In concordance with the histology results, GAL pretreatment prevented the accumulation of nitrites and nitrates in the plasma at that time (in both cases $P < 0.05$). All these data suggest that GAL acts not by modulating a single mechanism but by impacting on a wide variety of immune modulators.

### *GAL modulates the intestinal and lung inflammatory profile*

To elucidate the protective mechanism of GAL administration in the IR process, we analyzed the intestinal and pulmonary expression profile of various pro- and anti-inflammatory mediators. Based on previous reports, we focused on MCP-1, TNF-α, IFN-γ, IL-1β, IL-6, and IL-10 messenger RNA (mRNA)

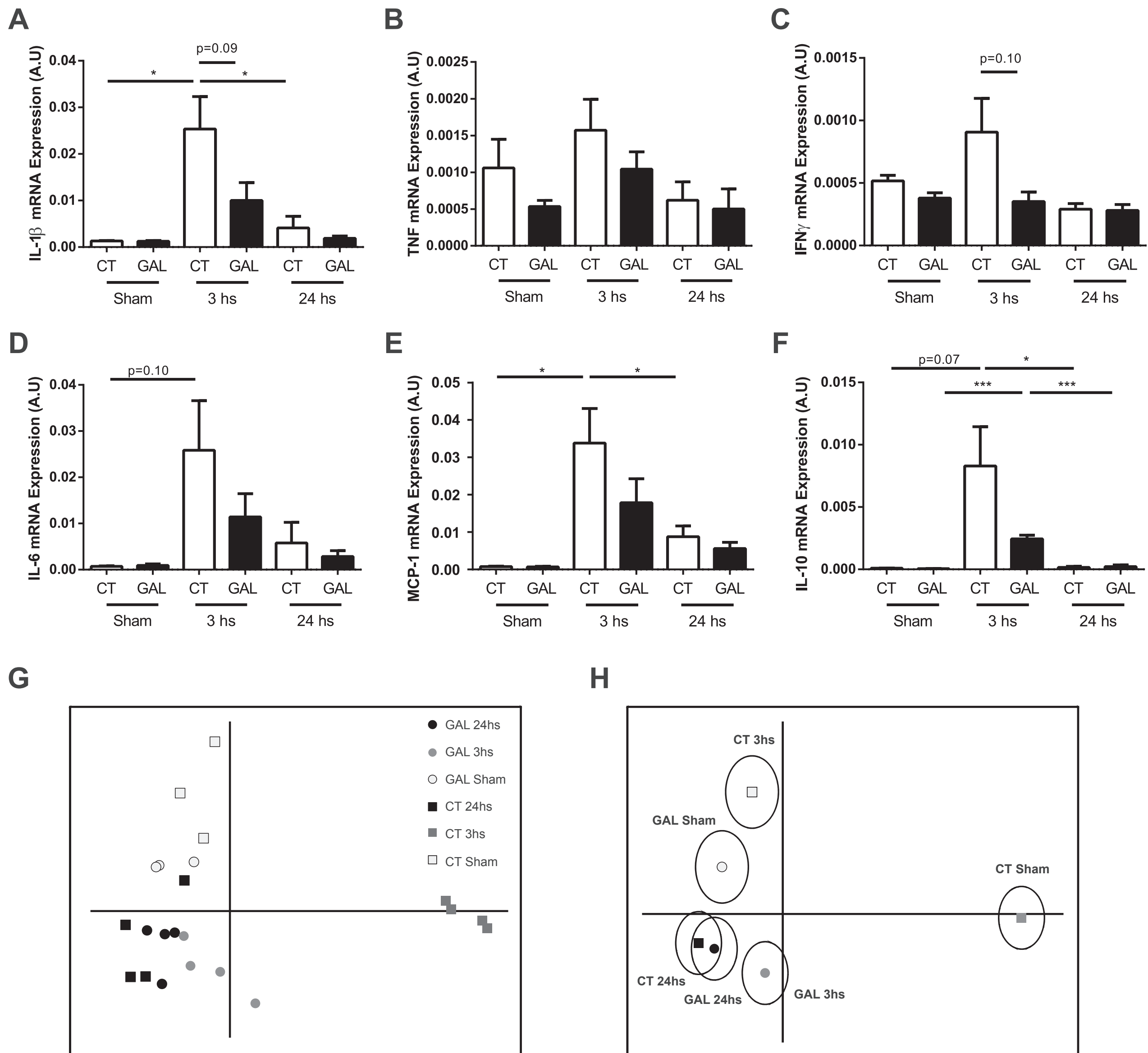


**Fig. 3 – Intestinal mRNA gene expression of IL-1b, TNF-α, IFN, IL-6, MCP-1, and IL-10 by quantitative polymerase chain reaction did not show differences between treated and nontreated animals (A-F). However, when a multivariate analysis was done, a considerable difference between CT intestines after 3 h of reperfusion and the remaining experimental groups was observed (G and H).**

levels and found that IR induces a significant upregulation of MCP-1 and IL-1β in the intestine after 3 h compared with the sham group (in both cases $P < 0.05$). Consistent with the histologic analysis, the mRNA levels of the proinflammatory genes returned to baseline levels after 24 h of reperfusion. Interestingly, the GAL-pretreated animals did not show a significant increase in any of those proinflammatory genes in comparison with the sham group. Nevertheless, a single gene analysis did not show statistical differences between the pretreated and nonpretreated GAL groups (MCP-1, $P = 0.2$; TNF-α, $P = 0.31$; IFN-γ, $P = 0.09$; IL-1β, $P = 0.10$; and IL-6, $P = 0.26$). On the other hand, IL-10 mRNA levels were upregulated in both the GAL and CT groups ($P = 0.07$ and $P < 0.001$, respectively). In the GAL nontreated animals, the differences of IL-10 mRNA expression levels with the sham group were clear but not statistically significant, probably because of data dispersion. Considering that a single gene analysis could not clearly explain the protective GAL effect observed in the histology, we performed an integrated analysis of all these pro- and anti-inflammatory mediators. As depicted in Figure 3G, after 3 h of IR, the animals without GAL treatment were separately clustered from all the other groups. The fact that both the sham and CT groups after 24 h were clustered close to each other shows that acute proinflammatory response after IR is physiologically overcome in 24 h. Interestingly, all the GAL-treated animals were also clustered close to the sham groups, indicating that the administration of this polysaccharide prevents the "cytokine storm" that occurs in the intestine during IRI. Altogether, these data suggest that GAL treatment acts not by a single mechanism but by modulating a complex network of proinflammatory mediators.

Given that GAL pretreatment also prevents IRI in the lung, we analyzed the expression profile of these pro- and anti-inflammatory mediators in lung samples. As shown in Figure 4A, IR significantly upregulated IL-1β levels compared with the sham group ($P < 0.05$). Interestingly, GAL treatment

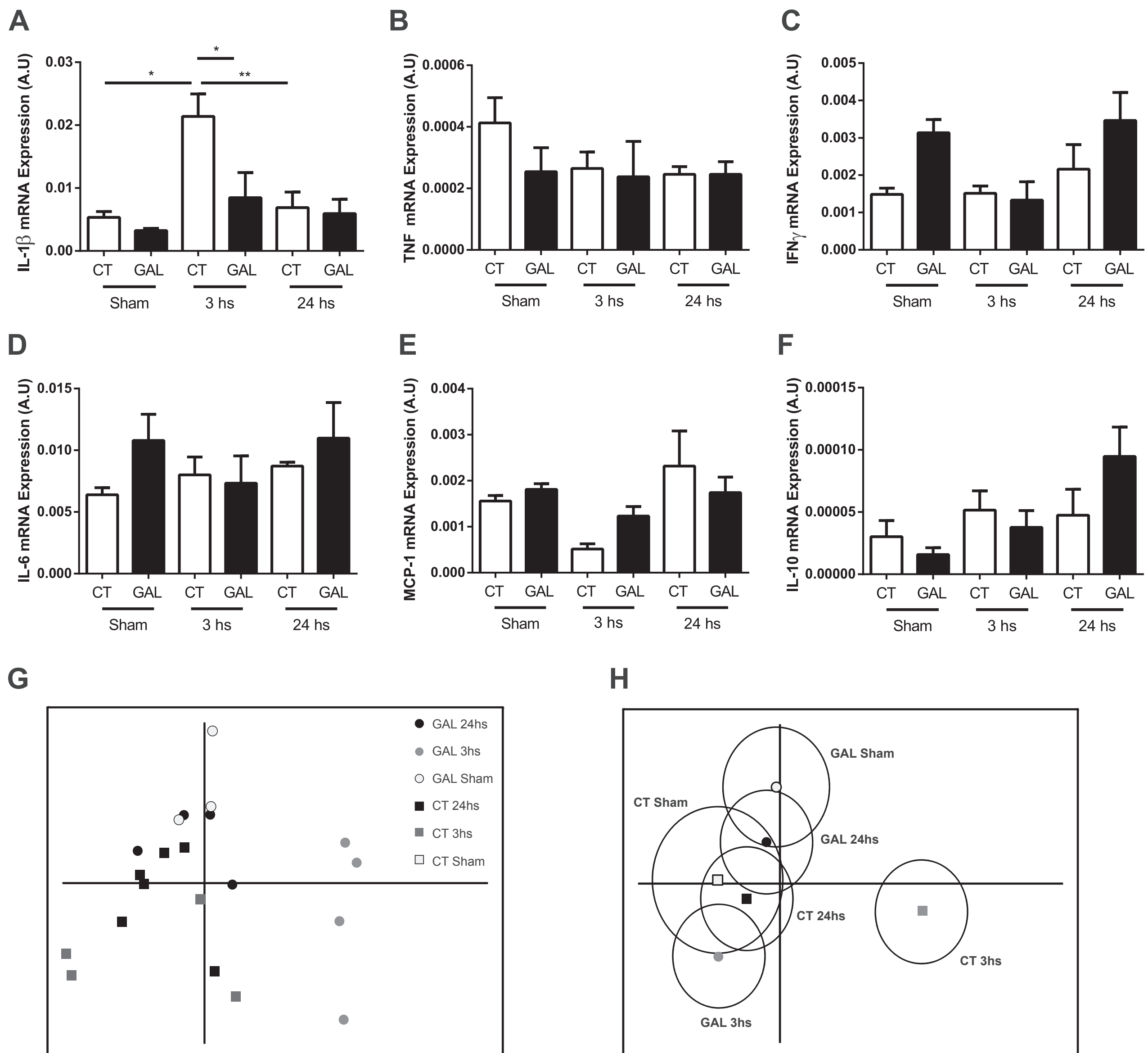


**Fig. 4 – Relative mRNA lung proinflammatory gene expression at 3 and 24 h after intestinal IRI (A-F). Analysis of principal components (G and H).**

prevented IL-1β upregulation ($P < 0.05$) and showed no significant differences with the sham group. No changes in any other inflammatory mediators were observed (Fig. 4).

## Discussion

GAL, a component in the wall of several fungi, has previously been documented as capable of reprogramming human monocytes, downmodulating their inflammatory profile in a subsequent lipopolysaccharide exposure, with certain similarity to endotoxin tolerance. In contrast to endotoxins, however, GAL does not induce an inflammatory response, constituting an attractive candidate to be used as a modulator agent.[14]

Reversible clamping of vessels in rodents for the experimental study of IRI in several organs, including the intestine, is widely used by the scientific community. These models allow us to study IR pathogenesis and develop corresponding strategies to diminish this inflammatory phenomenon, with the ultimate goal of transferring knowledge from basic research to the clinical scenario of gastrointestinal surgery.[18,19]

Considering this, we used an *in vivo* model of intestinal IRI in mice to evaluate, for the first time, the role of intragastric GAL administration as a pretreatment to attenuate local and remote damage caused by mesenteric ischemia followed by reperfusion.

Our findings show that GAL pretreatment significantly diminished histologic IR damage in the small intestine according to the Park score. In accord with our previous reports, GAL induced a reprogramming of the inflammatory response in human macrophages.[14] Although the entire mechanism is not fully understood, when macrophages/monocytes are pretreated with this polysaccharide, they express less class-II transactivator and human leukocyte antigen-DR.[20,21] Moreover, GAL inhibits the NFκB2 pathway, which, in turn, reduces TNF-α and IL-6 expression after lipopolysaccharide stimuli.[14] Our *in vivo* model showed that GAL downmodulates MCP-1, TNF-α, and IL-6 plasma levels, as well as IL-1β, MCP-1, and IL-6 mRNA expression in the small intestine. Considering that NFκB is a master regulator of MCP-1, TNF, and IL-6 expression, we hypothesized that the lower level of those proinflammatory mediators observed in plasma would be, at last in part, the result of GAL-induced inhibition of the NFκB pathway.[22]

These results are in concordance with Badia *et al.*, who had shown that GAL inhibits a *Salmonella*-induced proinflammatory profile in intestinal epithelial cells, downmodulating levels of MCP-1, TNF, IL-6, and IL-1α, among others, *in vitro*.[23]

We observed that the administration of GAL through the gastrointestinal tract is able to modulate IRI. Although it is not clear which mechanism is responsible for this *in vivo* activity, there is evidence that various polysaccharides can modulate inflammatory pathways through their activity on intestinal epithelial response.[24-26]

The inflammatory process of intestinal IRI is known not only to affect local tissues but also remote tissues and organs such as the lungs, kidneys, and liver. This feature is primarily because of the passage of bacteria and endotoxins from the intraluminal compartment to the systemic circulation, along with other proinflammatory mechanisms, such as cytokine release, which cause lesions in the whole organism. Our group and others have previously reported that intestinal IRI in rodents impacted negatively on the lungs, with neutrophil infiltration, edema, and airway epithelial cell damage as the main characteristic alterations during the reperfusion phase.[5,9] In this study, we have observed that histologic remote lung damage occurs at 3 h after reperfusion, and that GAL pretreatment was able to reduce morphologic alterations compared with the CT group. We suggest that the protective effect of GAL against IRI in the intestine is because of the reduction of circulating inflammatory mediators, resulting in less pulmonary inflammation.

In conclusion, the data presented in this study indicate that GAL pretreatment reduces IRI, probably because of the downmodulation of several proinflammatory mediators and its antioxidant properties. To our knowledge, this is the first *in vivo* study of GAL's protective capacity. The main limitation of our study is related to the limited conditions used to evaluate GAL's protection that should be extended to other conditions and timing. Although further studies need to be performed to corroborate this polysaccharide's ability to reprogram the immune response in clinical situations, our results show that GAL might be used pre-emptively in those surgeries in which blood supply to any specific organ would be compromised.

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Authors' contributions: E.L-C, P.S., R.P.-G., and V.T. conceived and designed the study and collected, analyzed and interpreted the data. M.N.A., L.C., and M.M. collected the data. M.R. and F.H.O. wrote the article and provided critical revisions that are important for the intellectual content. L.A.A. and E.L.-C. approved the final version of the article.

## Disclosure

The authors reported no proprietary or commercial interest in any product mentioned or concept discussed in this article.

## Appendix ASupplementary data

Supplementary data to this article can be found at https://doi.org/10.1016/j.jss.2019.10.027.

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