Protective Effects of Lactococci Strains Delivering Either IL-10 Protein or cDNA in a TNBS-induced Chronic Colitis Model

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Background: Oral treatment with *Lactococcus lactis* strains secreting the anti-inflammatory cytokine interleukin (IL)-10 has previously shown success as a therapy for inflammatory bowel diseases (IBD).

Goals: Our aim was to compare the protective effects of IL-10, delivered by recombinant lactoccoci using 2 novel expression systems, in a murine colitis model mimicking the relapsing nature of IBD. The first system is based on a Stress-Inducible Controlled Expression system for the production and delivery of heterologous proteins at mucosal surfaces and the second allows the delivery to the host cells of an *il-10* cDNA cassette, harbored in a eukaryotic DNA expression vector (pValac).

Study: Colitis was induced in female BALB/c mice by intrarectal injection of 2,4,6-trinitrobenzenesulphonic acid (TNBS). Mice that recovered received one of the bacteria treatments or saline solution orally during 14 days. Colitis was reactivated 25 days after the first TNBS injection with a second TNBS challenge. Three days after colitis reactivation, cytokine profiles and inflammation in colon samples were evaluated.

Results: Animals (N = 9) receiving *L. lactis* strains secreting IL-10 using Stress-Inducible Controlled Expression system or delivering pValac:*il-10* plasmid showed lower weight loss ($P < 0.005$), lower damage scores ($P < 0.005$), and immune activation in their large intestines compared with inflamed nontreated mice.

Conclusions: Our results confirm the protective effect of IL-10 delivered either as a protein or as a cDNA in a colitis model mimicking the relapsing nature of IBD and provides a step further in the "proof-of-concept" of genetically engineered bacteria as a valid system to deliver therapeutic molecules at mucosal level.

Key Words: *Lactococcus lactis*, interleukin-10, DNA vaccine, inflammatory bowel diseases (IBD)

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The authors declare that they have nothing to disclose.

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Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis, is a group of chronic inflammatory disorders of the gut characterized by an uncontrolled and relapsing inflammatory response. Patients with IBD suffer bloody diarrhea, abdominal pain, and rectal bleeding. Although its etiology is still unknown, it appears to be multifactorial. As there is no cure for IBD, the short-term aim of current medical treatments is to bring the symptoms under control. Therefore, therapies currently used to treat IBD are based on the combination of anti-inflammatory and immunosuppressive drugs.[1] Positive results obtained in animal models and human clinical trials have led to a growing interest in the use of probiotics, especially lactic acid bacteria (LAB), to modulate IBD-related dysbiosis.[2] Probiotics have been defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host."[3] Furthermore, the use of LAB as live delivery systems has been widely described, allowing improved mucosal targeting with a large variety of therapeutic molecules.[4–6] It was previously shown that *L. lactis*, the model LAB, was able to produce the interleukin (IL)-10,[7] a cytokine with anti-inflammatory properties and clinical benefits[8] that plays a key role in the treatment of gastrointestinal inflammatory diseases.[9] Recombinant *L. lactis* secreting biologically active IL-10 was effective in preventing damages in induced colitis using different mouse models.[10] Moreover, a biological *L. lactis* containment system was constructed for human IL-10 production[11] and evaluated in CD patients without adverse effects.[12] However, the clinical results did not reveal a statistically significant difference in mucosal healing versus the placebo group. Altogether, these facts, as well as the requirement of more efficient delivery strategies, prompted us to search for new delivery systems to better target these molecules to the mucosa, avoiding any possible undesirable systemic side effects. From this perspective, several new controlled-expression systems, such as inducible promoters, have recently been developed.[13–16] Also, another promising strategy to deliver molecules in vivo and in vitro is the DNA delivery which allows the eukaryotic cell to produce the molecule of interest itself.[17–19]

In this study, we used 2 different approaches to deliver IL-10 cytokine: an inducible protein delivery system and a cDNA delivery system. The former is a Stress-Inducible Controlled Expression system for the expression of IL-10 in *L. lactis*.[15] This system is based on a stress-inducible promoter (pGroESL) that allows the production of the heterologous protein of interest in situ (ie, colon) by *L. lactis*.

Interestingly, this strain was previously found to be protective in different murine models of IBD[20] and irritable bowel disease (IBS) (Martín et al, unpublished data). The latter is a system based on a new vector (ie, plasmid), for DNA delivery using lactococci, named pValac (vaccination using LAB).[21] This plasmid harbors an eukaryotic region containing the cytomegalovirus promoter, the open reading frame of *Mus musculus il-10* gene and the polyadenylation signal of bovine growth hormone (BGH polyA), required for gene expression by eukaryotic host cells, as well as a prokaryotic region containing the RepA/RepC replication origins for both *Escherichia coli* and *L. lactis,* and a chloramphenicol resistance gene (*Cm*) for bacteria selection. Recently, an invasive *L. lactis* strain producing Fibronectin Binding Protein A (FnBPA +) from *Staphylococcus aureus* and containing pValac:*il*10 plasmid was found to be effective in the prevention of inflammation in an acute 2,4,6-trinitrobenzenesulphonic acid (TNBS) murine model of colitis.[22] The same strain without FnBPA was also shown to be effective in the prevention of colitis in a dextran sulfate sodium-induced murine model.[23] The aim of this work is to compare the effect of both lactococci strains in a chronic murine model of IBD mimicking the relapsing nature of this disease as a method to discern the efficacy of both delivery systems.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

L. lactis MG1363 wild-type strain (LL) and *L. lactis* MG1363 strain harboring either pValac:*il-10* or pGroeESL:*il-10* plasmids (LL-pValac:IL-10 and LL-pGroESL:IL-10, respectively) were grown for 16 hours at 30°C without agitation in 10 mL of LAPTg (1% glucose, 1.5% peptone, 1% tryptone, 1% yeast extract, and 0.1% Tween 80) medium containing 10 μg/mL chloramphenicol. These cultures were washed, and resuspended in 1 mL of saline solution (0.9% NaCl) to obtain a final concentration of 1×10^{10} CFU/mL for animal feeding.

Induction of Colitis and Bacteria Administration

Chronic colitis was induced following the protocol described previously[24] (Fig. 1). BALB/c mice (n = 90, female, 5 wk old) were fully anesthetized by an intraperitoneal injection of ketamine hydrocholoride (100 μg/g body weight; Holliday-Scott S.A., Buenos Aires, Argentina) mixed with xylazine hydrochloride (5 μg/g body weight, Rompun; Bayer, División Sanidad Animal, Buenos Aires, Argentina). Then, colitis was induced by an intrarectal injection of a TNBS (Sigma, St. Louis, MO) solution (2 mg/mouse) dissolved in 50% ethanol (EtOH) and phosphate-buffered saline (PBS) 0.01 M, pH 7.4, using a 4 cm long catheter. The TNBS solution was slowly instilled into the colon after which the mice were held in a vertical position for 30 seconds. Control mice received only EtOH and PBS. Body weight and mortality ratio were monitored daily. After 2 weeks (recovery period), mice that survived the first TNBS challenge and fully recovered their initial body weight received once daily during 14 days (remission period) either 100 μL of each bacterial suspension (containing 1×10^9 CFU) or 100 μL of saline solution (control groups) using a gavage syringe. Control mice received 100 μL of saline solution. Animals were challenged with a second injection of TNBS 25 days after the first TNBS injection to reactivate colitis. TNBS-TNBS mice were subdivided into 4 experimental groups: TNBS-TNBS group (chronic inflammation control group), TNBS-TNBS-LL group, TNBS-TNBS-LL-pVALAC:IL-10 group, or TNBS-TNBS-LL-pGroESL:IL-10 group. Control mice received a second inoculation with EtOH PBS instead of TNBS (mock group). On day 3 after the second TNBS injection, 10 mice per group were killed and samples were collected.

During the whole experiment, all groups were fed ad libitum with balanced rodent diet and maintained in a room with a 12-hour light/dark cycle at 18 ± 2°C.

Macroscopic and Histologic Damage Scores

Colon and cecum samples were removed, visually inspected for macroscopic inflammation, and then fixed in formaldehyde solution (10% in PBS) for histologic analysis using standard methods. Serial paraffin sections of 4 μm were made and stained with hematoxylin and eosin (H&E) for light microscopy examination. Macroscopic lesions and extent of colonic damage and inflammation were assessed using previously described grading systems.[22] The analyses were performed by 2 different scientists. High macroscopic or histologic damage scores indicate increased damage in the colon.

Cytokine Profile on Intestinal Samples

Intestinal contents were collected from the colons of mice with 500 μL of PBS containing Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche Molecular Biochemicals), centrifuged (8000*g*, 10 min, 4°C) and supernatants were stored at 20°C until further analysis. These samples were then essayed with the BD Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit (BD Bioscience, San Diego, CA) to measure IL-6, interferon (IFN)-γ, TNF, IL-17A, and IL-10 cytokine levels.

A small section of the colon, approximately 5 mm in length, was mechanically disrupted in 200 μL PBS with Complete Mini EDTA-free Protease Inhibitor Cocktail, and homogenized in a Bead Beater apparatus (Biospec Products

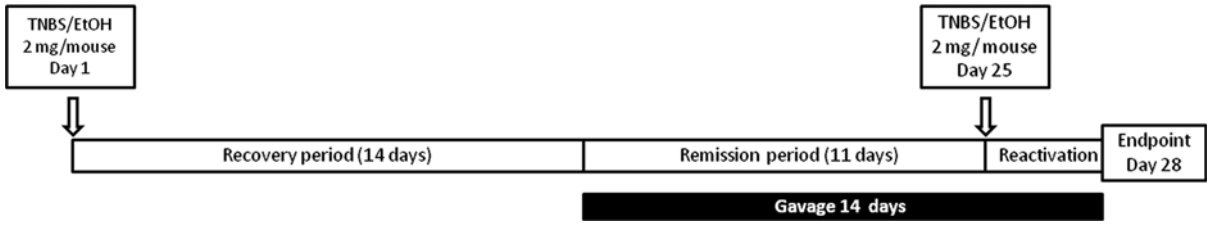

FIGURE 1. TNBS chronic colitis experimental protocol used in this work. Colitis was induced by administration of 2 mg/mouse of TNBS solution. Fourteen days following TNBS period, either different bacteria or saline were intragastrically administrated to mice during 14 days (gavage period). Colitis was reactivated 25 days after the first TNBS injection (recovery period) with a second administration of 2 mg/mouse of TNBS solution. Three days after colitis reactivation mice were killed. TNBS indicates 2,4,6-trinitrobenzenesulphonic acid.

Inc., Bartlesville, OK) with 0.1 mm zirconia/silica beads (Catalogue #110791012; Biospec Products Inc.). After 4 cycles of disruption (2 min) alternated with 2 minutes of incubation on ice, samples were centrifuged (8000*g*, 10 min, 4°C) and the supernatants were immediately used to determine cytokine levels as described above. The results were expressed as cytokine ratios for each mouse to show the balance between the anti-inflammatory IL-10 and the proinflammatory cytokines in the animals and not only the individual cytokine concentrations in the intestinal fluids or tissues.

Statistical Analysis

Statistical analyses were performed using MINITAB 16 Statistical Software (Minitab, State College, PA). Comparisons were performed by an ANOVA general linear model followed by Tukey's post hoc test for body weight or damage scores analysis or by Dunnett's post hoc test for cytokine analysis.

RESULTS

Lactococci Strains Delivering Either IL-10 Protein or cDNA Reduce the Severity of TNBS-induced Chronic Colitis

To determine the potential protective effect of LL-pValac:IL-10 and LL-pGroESL:IL-10 strains in a chronic TNBS-induced murine colitis model, different parameters were determined. First, we measured the weight change after a second TNBS injection as a read-out of mice status after colitis reactivation (Fig. 2A). The percentage of loss of body weight was significantly higher in the inflamed control group (TNBS-TNBS) compared with the noninflamed control group (mock group) ($P < 0.05$) (Fig. 2A). Strikingly, both LL-pValac:IL-10 and LL-pGroESL:IL-10 administrations led to significant improvements in body weight compared with TNBS-TNBS mice ($P < 0.005$) maintaining the weight percentages similar to the mock group 3 days after chronic colitis reactivation (Fig. 2A). The specific effect of IL-10 delivery by recombinant lactococci was confirmed by the lack of a protective effect in the group receiving the wild-type *L. lactis* strain (LL). This group showed similar weight loss than the inflamed control mice (TNBS-TNBS, Fig. 2A).

Macroscopic damages of the colon were then asserted by means of a scoring system based on the presence of erythema, hemorrhage, edema, stricture formation, ulceration, fecal blood, presence of mucus, diarrhea, and adhesions. Total macroscopic scores were significantly higher ($P < 0.005$) for the TNBS-TNBS group than for the mock group (Fig. 2B). Mice treated with LL showed similar macroscopic scores than inflamed control group (TNBS-TNBS), whereas both LL-pValac:IL-10 and LL-pGroESL:IL-10 decreased the macroscopic damage score compared with TNBS-TNBS mice ($P < 0.005$) (Fig. 2B). Furthermore, TNBS-TNBS-LL-pGroESL:IL-10 mice showed no statistical significant differences with the control mock group and a slight higher effect compared with the TNBS-TNBS-LL-pValac:IL-10 (Fig. 2B). The mean histologic scores showed similarly significant differences in

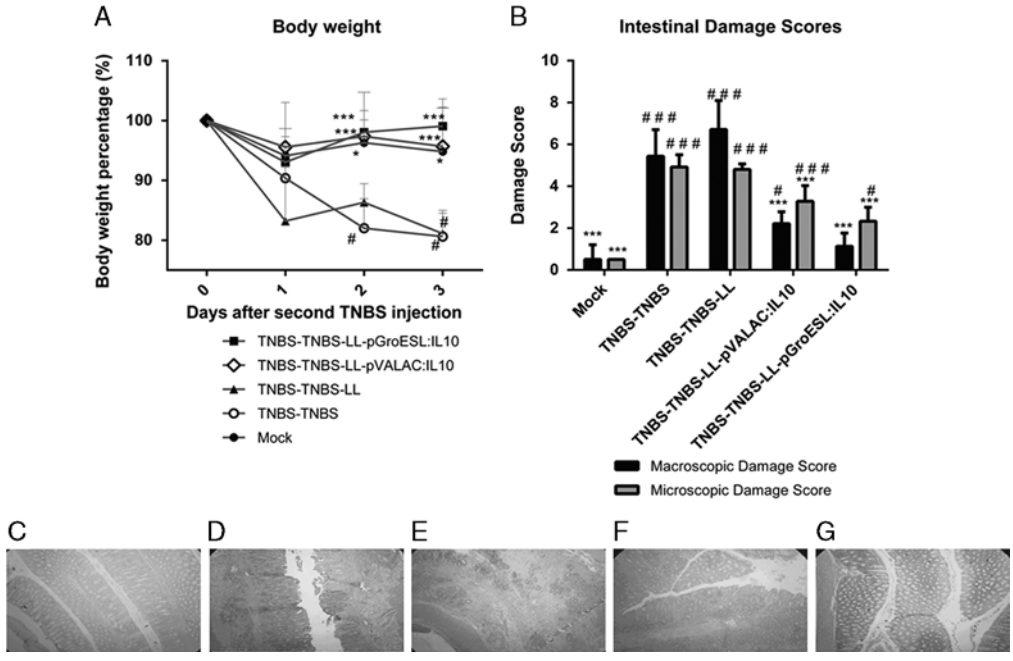

FIGURE 2. Body weight percentage (A) and colon damage scores (B) in mice from mock, TNBS-TNBS (chronic colitis control group), TNBS-TNBS-LL (receiving treatment with *L. lactis* MG1363 wild-type strain), TNBS-TNBS-LL-pValac:IL-10 (receiving genetically engineered *L. lactis* MG1363 for IL-10 DNA delivery), or TNBS-TNBS-LL-pGroESL:IL-10 (receiving genetically engineered *L. lactis* MG1363 for IL-10 protein delivery) groups. Body weight is represented as a percentage of the initial body weight on the day of the second induction of colitis. Macroscopic (black bars) and microscopic (gray bars) damage scores correspond to samples taken 3 days after the second injection with TNBS. Each value represents the mean of n = 10 ± SD. Means with $*P<0.05$ or $***P<0.005$ differ significantly from TNBS-TNBS group, whereas means with $\#P<0.05$ or $\#\#\#P<0.005$ differ significantly from control noninflamed group (mock). Representative microphotographs (as observed at ×100 magnification) of H&E-stained colon sections of mice belonging to: (C) mock, (D) TNBS-TNBS, (E) TNBS-TNBS-LL, (F) TNBS-TNBS-LL-pValac:IL-10, and (G) TNBS-TNBS-LL-pGroESL:IL-10 experimental groups. H&E indicates hematoxylin and eosin; TNBS, 2,4,6-trinitrobenzenesulphonic acid.

inflammation between both the TNBS-TNBS group and TNBS-TNBS-LL treated group ($P < 0.005$) compared with the mock group, and improvements in mice treated with both LL-pValac:IL-10 and LL-pGroESL:IL-10 ($P < 0.005$), compared with TNBS-TNBS group (Fig. 2B). H&E staining of colon samples from mice of TNBS-TNBS (Fig. 2D) and TNBS-TNBS-LL groups (Fig. 2E) revealed large areas of coagulative necrosis with severe neutrophil infiltration and distortion of the crypt architecture. In contrast, H&E staining of colon samples from mice of TNBS-TNBS-LL-pValac:IL-10 (Fig. 2F) and TNBS-TNBS-LL-pGroESL:IL-10 (Fig. 2G) showed a significantly lower damage in the colon; however, they did not reach the appearance of the mock group (Fig. 2C).

Mucosal Restoration of IL-10 Concentrations After Administration of *L. lactis* Delivering IL-10 Either as a Protein or as cDNA

Analysis of IL-10 concentrations in both colonic tissue (Fig. 3A) and colon content (Fig. 3B) revealed a significant restoration of IL-10 production in TNBS-treated mice after administration of either LL-pValac:IL-10 ($P < 0.05$) or LL-pGroESL:IL-10 ($P = 0.0695$ in colonic tissue and $P < 0.05$ in colonic tissue) compared with both TNBS-TNBS and TNBS-TNBS-LL groups where IL-10 production was significantly decreased after TNBS treatment (Figs. 3A, 3B). No significant differences were observed in IL-10 levels between mice receiving LL and the recurrent colitis control group.

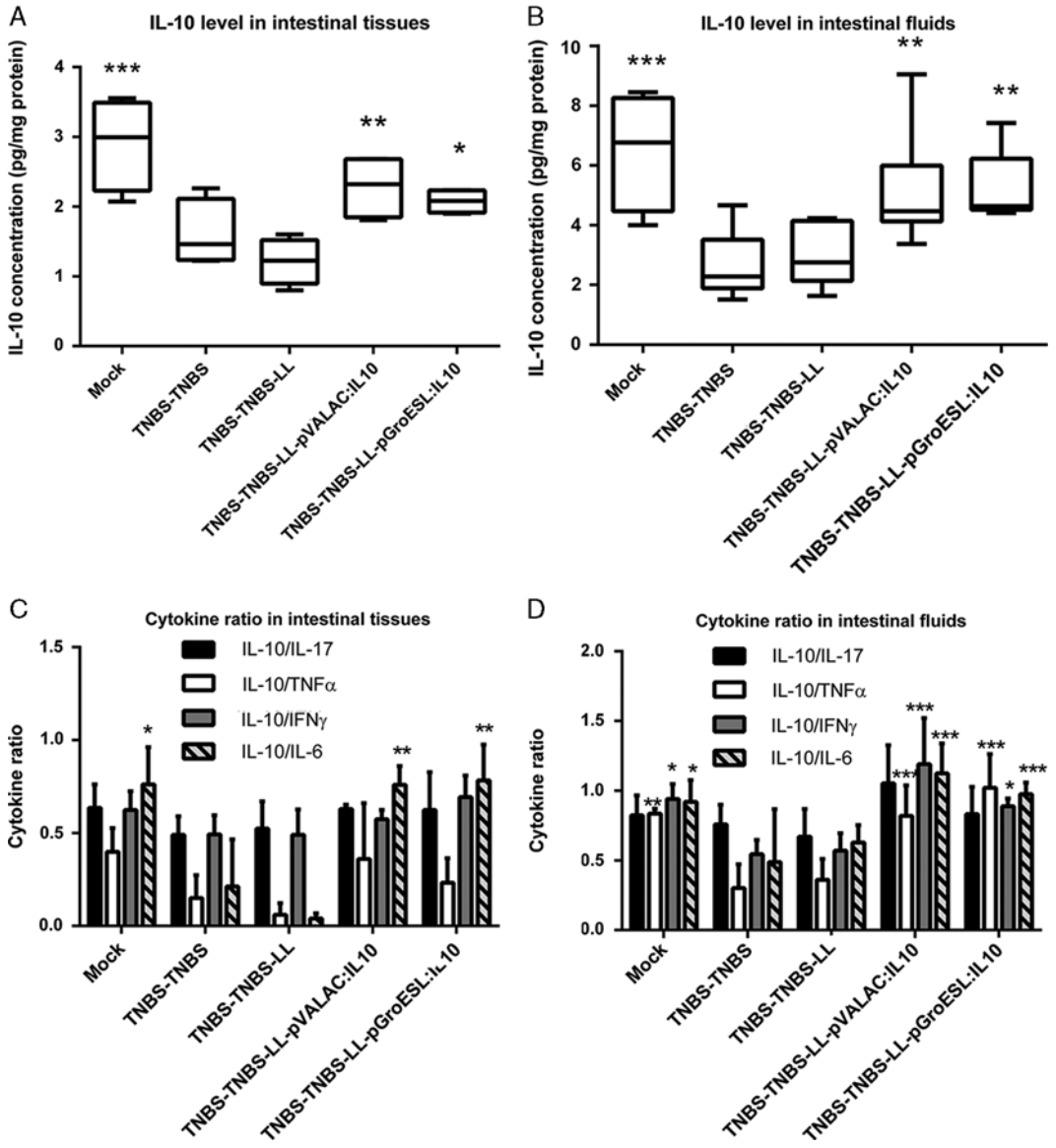

FIGURE 3. Concentration of interleukin (IL)-10 in intestinal tissues (A) or intestinal fluids (B) of mice from mock, TNBS-TNBS (chronic colitis control group), TNBS-TNBS-LL, TNBS-TNBS-LL-pValac:IL-10, or TNBS-TNBS-LL-pGroESL:IL-10 groups. Ratio between anti-inflammatory IL-10 and proinflammatory cytokine concentration is also represented for each experimental group in intestinal tissues (C) or fluids (D): IL-10/IL-17 (black bars), IL-10/TNF-α (white bars), IL-10/IFN-γ (gray bars), and IL-10/IL-6 (dashed bars). Each value represents the mean of n = 10 ± SD. Means with $*P < 0.1$, $**P < 0.05$, or $***P < 0.005$ differ significantly from TNBS-TNBS group. IFN indicates interferon; TNBS, 2,4,6-trinitrobenzenesulphonic acid.

An Anti-Inflammatory Profile of Cytokine Production Correlates With the Protective Effects Observed by Recombinant Lactococci

The analysis of cytokines present in the intestinal tissue showed significant differences in IL-10/IL-6 cytokine ratios between the mock and TNBS-TNBS groups ($P < 0.05$) (Fig. 3C). No significant differences were observed for IL-10/TNF-α, IL-10/IFN-γ, and IL-10/IL-17 (Fig. 3C). Treatment with LL-pValac:IL-10 and LL-pGroESL:IL-10 restored IL-10/IL-6 ratios to the values of mock group, showing significant differences ($P < 0.05$) compared with TNBS-TNBS mice (Fig. 3C).

The results from the analysis of the cytokines secreted to the intestinal lumen showed statistical significant differences in IL-10/TNF-α ($P < 0.05$), IL-10/IFN-γ ($P = 0.061$), and IL-10/IL-6 ($P = 0.054$) ratios between TNBS-TNBS and mock groups (Fig. 3D). LL-pValac:IL-10 treatment restored the levels of all these ratios to similar values to those of the mock group, maintaining significant differences ($P < 0.005$) with the TNBS-TNBS group (Fig. 3D). LL-pGroESL:IL-10 treatment also maintained significant differences in the IL-10/TNF-α ($P < 0.005$), IL-10/IFN-γ ($P = 0.056$), and IL-10/IL-6 ($P < 0.005$) cytokine ratios of their intestinal contents compared with those of the TNBS-TNBS group.

No significant differences were observed in the group of mice treated with LL strain compared with TNBS-TNBS control neither in colonic tissue nor in colon contents.

DISCUSSION

IL-10 is one of the main molecules of interest analyzed in IBD-related models. Indeed, it has been proven to be an important immunoregulatory cytokine that successfully suppress the exacerbated mucosal immune response associated with IBD.[25] Also, a defect in IL-10 production may be involved in the pathogenesis of IBD due to the importance of IL-10-mediated immune responses in maintaining intestinal homeostasis and commensal microbiota tolerance.[26] However, parenteral IL-10 treatment resulted in side effect pharmacokinetics and tissue distribution and has only limited success in leading to clinical remission.[27,28] Although clinical trials up to date have shown disappointing results, it has been recently postulated that with the new technologies in protein delivery, a mucosal delivery and the correct choice of patients, IL-10 supplementation could become a viable treatment option.[26] For instance, this cytokine has been delivered to the gastrointestinal tract (GIT) by means of different approaches varying from nanoparticles-in-microsphere systems to different LAB-related delivery systems.[15,22,29]

The use of genetically modified microorganisms to deliver active molecules at mucosal surfaces has been widely studied due to the increasing interest in future novel therapeutic approaches for IBD.[2] Consequently, the aim of this study was to investigate the anti-inflammatory and protective effects of 2 strains of *L. lactis* delivering either IL-10 protein or cDNA in a TNBS-induced chronic colitis model. TNBS-induced colitis share many histopathologic features observed in CD patients.[30] As CD is a relapsing/chronic IBD mainly affecting the GIT,[8] here colitis induction was made at 2 different time points to mimic flare episodes. We observed that treatment with LL-pValac:IL-10 or LL-pGroESL:IL-10 strains during the remission period decreased the severity of relapse after the second TNBS inoculation. These beneficial effects are an evidence of the improved symptoms of chronic colitis without loss of body weight and lower histologic and macroscopic damage scores than TNBS-TNBS control (Fig. 2).

Changes in the intestinal cytokine profile were also analyzed to determine the role of IL-10 in the modulation of the production and secretion of other proinflammatory cytokines involved in the immune response associated to IBD. We observed that both delivery systems were effective in restoring the decrease of IL-10 in the intestine of mice treated with TNBS (Figs. 3A, 3B). This restoration was also associated to the modulation of other proinflammatory cytokines at intestinal level and luminal content. A proinflammatory status was confirmed in nontreated mice with lower IL-10/TNF-α, IL-10/IL-6, and IL-10/IFN-γ ratios, compared with the mock group. This proinflammatory balance switched into an anti-inflammatory profile in mice orally treated with both LL-pValac:IL-10 and LL-pGroESL:IL-10, restoring the ratio to mock levels. Decreases on intestinal tissue and fluid IL-10 levels in inflamed control group compared with mock group correlated with the presence of intestinal damages. Indeed, as the LL strain did now shown any protective effect in terms of macroscopic, microscopic scores, health status and cytokine profiles, the effect of both LL-pValac:IL-10 and LL-pGroESL:IL-10 might be specifically because of a correct IL-10 protein and cDNA delivery at the mucosal level, which appears in higher concentrations not only in intestinal fluids but also in intestinal tissues compared with the LL strain. This is in agreement with previous results that confirmed active delivery of pValac:*il-10* plasmid and correct expression of the cytokine by eukaryotic cells in vitro and in vivo by an invasive strain of *L. lactis*[22] and by the noninvasive strain.[23] In this study, we decided to use a noninvasive *L. lactis* strain as vehicle as it has been recently reported that plasmid transfer also occurs in the noninvasive strain allowing heterologous protein production in vivo.[31] This would represent an additional benefit considering that *L. lactis* has been widely used as a safe protein delivery vector because of its noninvasive and noncolonizing status having a reduced time of action as its permanence in the GIT is never higher than 24 hours.[4–6] This bacterium has thus less potential to trigger immunotolerance or side effects upon prolonged use.[32]

Here LL-pGroESL:IL-10 also allowed a regular production of IL-10 in the intestinal lumen (intestinal fluids) due to the continuous administration and the local induction of IL-10 expression by the stress conditions (temperature, pH, bile salts) found at the intestinal level.[15] This represents an advantage compared with systems of expression used in other studies where addition of inductors was needed before oral administration of the strain and where no significant changes were detected in IL-10 fluid levels.[33] A higher IL-10 production was also found in the intestinal tissues of animals treated with LL-pGroESL:IL-10 consistent with previous observations.[20] Because of the normal intestinal epithelial cell turnover[22] and the noncolonization ability of *L. lactis*, this regular IL-10 production is due to bacterial daily administration and causes an increase in the intestinal IL-10/proinflammatory cytokine ratios analyzed in this work. Besides, the use of a controlled system for local delivery of therapeutic molecules and the local delivery of cDNA were validated as no systemic side effects were observed in mice after administration of both recombinant strains.

In conclusion, in this paper we compared the beneficial effects induced in mice through DNA or protein

delivery by administration of noninvasive recombinant *L. lactis* strain harboring pValac:*il-10* or pGroeESL:IL-10 plasmids. Our results showed a similar anti-inflammatory effect with both strains in a chronic TNBS-induced colitis model, highlighting the relevance of IL-10 as a therapeutic molecule to be delivered by live nonpathogenic carriers such as *L. lactis* to treat inflammation-related diseases such as IBD.

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