Immunomodulation of *Lactobacillus reuteri* CRL1324 on Group B *Streptococcus* Vaginal Colonization in a Murine Experimental Model

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Keywords
Group B Streptococci, immune cells, *Lactobacillus*, mice, preventive effect, vaginal tract

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Submission August 4, 2015;
accepted October 6, 2015.

Citation
De Gregorio PR, Juárez Tomás MS, Nader-Macías MEF. Immunomodulation of *Lactobacillus reuteri* CRL1324 on group B *Streptococcus* vaginal colonization in a murine experimental model. Am J Reprod Immunol 2015

doi:10.1111/aji.12445

Problem
Maternal Group B *Streptococcus* (GBS) colonization is a risk factor for infectious disease in newborns. One promising strategy is the modulation of vaginal defense to increase the host's ability to combat infection.

Method of Study
The effect of intravaginal (i.va.) *Lactobacillus reuteri* CRL1324 inoculation on different immune cell populations, cytokines, and immunoglobulin isotypes in a murine model of GBS vaginal colonization was evaluated.

Results
Seven i.va. inoculations of *L. reuteri* CRL1324 previous to GBS challenge showed an immunomodulatory effect on the cells and mediators of innate immunity, decreasing the number of neutrophils induced by the pathogen and increasing the activated macrophage population. Moreover, increases in B lymphocytes and IgA and IgG subclasses were observed in mice inoculated with *L. reuteri* CRL1324 and then challenged with GBS.

Conclusion
Lactobacillus reuteri CRL1324 shows a protective effect against GBS colonization that could be mediated by the modulation of the immune response.

Introduction

Streptococcus agalactiae (Group B *Streptococcus*, GBS) is a Gram-positive microorganism that can asymptomatically colonize the vaginal tract in approximately 25% of healthy women.[1,2] GBS can be vertically transmitted to neonates during peripartum through contact with vaginal fluids, causing severe diseases. This pathogen is known as a principal cause of pneumonia, bacteraemia, sepsis, and meningitis in newborns.[2] Other pathologies, such as urinary tract infections, vaginitis, and fine superficial fissures and erythema of vulvar skin in women, have also been attributed to GBS.[3–6]

For the prophylaxis and treatment of GBS disease, penicillin is the most frequently recommended. Alternatives include ampicillin, cefazolin, vancomycin, or clindamycin for penicillin-allergic patients. However, resistance to penicillin and alternative antibiotics has been reported lately.[7–9] Therefore, there is a growing concern referred to possibly inadequate prophylaxis or therapies with these antibiotics, as GBS continues to be one of the main causes of early-onset neonatal sepsis.[10,11]

At present, there is a pressing request for the development of novel preventive strategies or treatments to control GBS vaginal colonization and transmission to the susceptible newborn, due to the lack of a successful vaccine against GBS and to the emerging antibiotic resistance. A promising strategy is the modulation of the vaginal defense mechanisms that increase the host's ability to fight against

microbial infection. A novel and valid alternative includes the use of beneficial vaginal lactobacilli (BVL) with antibacterial and immune modulatory properties. Lactobacilli are the predominant microorganisms in the equilibrated vaginal ecosystem.[12] They can protect against urogenital tract infections by competition for epithelial cell attachment sites or nutrients, production of antimicrobial substances (lactic acid, H_2O_2, bacteriocins), and/or modulation of the immune response.[13,14]

Our research group has previously reported that *Lactobacillus reuteri* CRL1324, a BVL strain, is a potential candidate to control GBS vaginal colonization.[15] *L. reuteri* CRL1324 was able to co-aggregate with GBS strains and antagonize their growth by organic acid production in *in vitro* experiments.[16] Also, a dose-dependent preventive effect of *L. reuteri* CRL1324 on GBS vaginal colonization in an experimental mouse model was previously demonstrated.[15] Seven doses of *L. reuteri* CRL1324 were able to significantly reduce the numbers of viable GBS cells, while four doses showed no preventive effect.

The aim of this work was to evaluate the effect of *L. reuteri* CRL1324 on different immune cell populations, cytokines, and immunoglobulin isotypes present in the murine vaginal tract in order to determine whether the decrease in the number of viable GBS cells evidenced after seven intravaginal (i.va.) inoculations of *L. reuteri* CRL1324 could be due to a modulation of the immune system.

Material and methods

Microorganisms and Culture Conditions

Lactobacillus reuteri CRL (Centro de Referencia para Lactobacilos Culture Collection) 1324 was previously isolated from human vagina in Tucumán, Argentina.[17] *Streptococcus agalactiae* (GBS) NH17 (capsular serotype IB) was previously isolated from a patient with urogenital infections at the Nuevo Hospital 'El Milagro' (Salta, Argentina). Microorganisms were stored and cultured as previously described by De Gregorio et al.[15]

Animals and Experimental Groups

Female BALB/c mice of 2 month old, weighing 25–30 g, from the inbred colony of CERELA (Centro de Referencia para Lactobacilos) were used. All the mice were housed, fed, and induced in a pseudo-estrous condition (to promote lactic acid bacteria colonization),[15–18] as previously described by De Gregorio et al.[15] The experiments were independently performed three times employing three animals (minimum) per experimental group and sampling day. The Institutional Laboratory Animal Care and Use Committee of Centro de Referencia para Lactobacilos authorized the experimental CRL-BIOT-LMP-2011/2A protocol employed in this work.

The animals were randomly assigned to five experimental groups as previously described by De Gregorio et al.[15]: (i) *Lactobacillus* (Lb)-pathogen (Pt)-treated mice [i.va. inoculated with 10^8 colony-forming units (CFU) of *L. reuteri* CRL1324 twice a day (with 10 hr in between) for 4 days (a total of seven doses) and later i.va. challenged with a single dose of around 1×10^7 CFU of GBS NH17], (ii) Pt-challenged mice (i.va. inoculated seven times with saline and later i.va. challenged with 1×10^7 CFU of GBS NH17), (iii) Lb-treated mice (i.va. inoculated seven times with 10^8 CFU of *L. reuteri* CRL1324 and later with saline), (iv) HC mice (inoculated only with saline), and (v) ApC mice (inoculated only with agarized peptone, which was the support used for lactobacilli inoculation).

The *Lactobacillus* and GBS inocula and the inoculation procedure were performed as previously described by De Gregorio et al.[15]

Sampling and Analytical Procedures

Every sampling day, vaginal washings (v.w.) were obtained under sterile conditions, using automatic pipettes with tips loaded with 50 μL of phosphate-buffered saline (PBS) (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, pH 7.2). Seven v.w. were pooled from each mouse for the different determinations. An aliquot of pooled v.w. was centrifuged at $200 \times g$ for 10 min at 4°C. The supernatants were stored at −70°C for cytokine and antibody determination. Subsequently, mice were killed by cervical dislocation and dissected to aseptically remove the vaginal tissue.

Evaluation of the Vaginal Cell Populations Involved in Innate and Adaptive Immune Response in Animals Inoculated with *L. reuteri* CRL1324 and Challenged with GBS

Mice vaginal tissues were removed and later subjected to flow cytometry to identify the specific

immune cell populations. The vaginas (three mice per experimental group and sampling day) were pooled, cut into small pieces (~2 mm diameter), and placed in 3 mL of RPMI media (Sigma-Aldrich, St. Quentin Fallavier, France) with 0.05% (v/v) ethylenediaminetetraacetic acid and 5% (v/v) of fetal bovine serum (FBS, from NATOCOR, Córdoba, Argentina) on ice. Then, the tissues were agitated at room temperature for 15 min and later for 1 hr at 37°C in 3 mL RMPI with 0.5 mg/mL of collagenase A (Sigma-Aldrich, Milan, Italy), 10% (v/v) FBS, and 1% (v/v) penicillin–streptomycin (Gibco, Burlington, Canada). The collagenase step was applied twice, and the remaining tissue pieces were disaggregated using the plunger from a 5-mL syringe through a 70-μM filter screen into RPMI with 5% (v/v) FBS. The resulting cell suspension was filtered through a 40-μM filter screen once more. Then, the erythrocytes were lysed by adding 2 mL of 10% (v/v) lysing solution (Lysing Solution; Becton Dickinson, Franklin Lakes, NJ, USA). The cells were later washed with RPMI with 5% (v/v) FBS, and the number of viable leukocytes was determined by the trypan blue (Sigma Chemical Co, St. Louis, MO, USA) exclusion technique using a Neubauer counting chamber.

To determine the expression of the cell surface markers on the vaginal cells isolated, 1×10^6 cells were pre-incubated with anti-mouse CD32/CD16 monoclonal antibody (Fc block) for 15 min at 4°C. The cells were washed with 2% (v/v) FBS-PBS and incubated with specific fluorochrome-conjugated monoclonal antibodies for 30 min at 4°C in the dark. The following antibodies were used: fluorescein isothiocyanate (FITC)-labeled anti-mouse CD3, phycoerythrin (PE)-labeled anti-mouse Gr-1, PE-labeled anti-mouse histocompatibility complex class II (MHC-II), biotinylated anti-mouse B220, biotinylated anti-mouse CD11b, allophycocyanin (APC)-labeled anti-mouse CD45, and APC-labeled anti-mouse F4/80. After incubation, the cells were washed with 2% (v/v) FBS-PBS. Samples treated with biotinylated antibodies were incubated with streptavidin–peridinin–chlorophyll proteins (PerCP) for 15 min at 4°C. All the antibodies, except APC-labeled anti-mouse F4/80 (eBioscience, Frankfurt, Germany), were obtained from BD PharMingen, Franklin Lakes, NJ, USA.

The data were acquired on a BD FACSCalibur cytometer (BD PharMingen) and analyzed using FSC Express V3 software (Glendale, CA, USA). The leukocyte gate was selected from the plots of forward scatter (FSC) versus side scatter (SSC) according to cell size (FSC), complexity (SSC), and CD45 expression. Later, the expression of the different cell markers on the vaginal population was evaluated. The cell populations studied were as follows: neutrophils (using Gr-1 marker), macrophages (CD11b and F4/80), and T and B lymphocytes (CD3 and B220, respectively). In addition, the levels of MHC-II expression allowed the determination of the activation state of macrophages.

The results were expressed as percentage of cells in each population, considering the total cell count from each sample as 100%. A minimum number of 100,000 gate events were recorded for each sample.

Evaluation of Cytokines in v.w. of Animals Inoculated with *L. reuteri* CRL1324 and Challenged with GBS

The analysis of the cytokines (TNF-α, IFN-γ, IL-6, IL-10, IL-2, IL-17, and IL-4) released in v.w. were carried out with a Mouse Cytokine Cytometric Beadarray Kit (BD PharMingen). Data were acquired on a BD FACSCalibur cytometer and analyzed using FCAP Array™ version 3.0 CBA analysis software (BD Biosciences, San Jose, CA, USA). Standard curves were prepared for each cytokine from a range of 0–5000 pg/mL. According to the manufacturer's instruction, the detection threshold for these assays ranges from 0.03 to 16.80 pg/mL, depending on the substance analyzed.

Evaluation of Antibodies in v.w. of Animals Inoculated with *L. reuteri* CRL1324 and Challenged with GBS

The evaluation of the antibodies (IgM, IgA, IgE, and IgG subclasses: IgG_1, IgG_{2a}, IgG_{2b} IgG_3) released in the v.w. was carried out with a Mouse Immunoglobulin Isotyping Cytometric Bead Array Kit (BD PharMingen). The data were acquired on a BD FACSCalibur cytometer and analyzed using FCAP Array™ version 3.0 CBA analysis software (BD Biosciences). The results were expressed as the MFI of each experimental group on the different sampling days. A higher MFI was associated with a higher amount of antibodies in each sample. However, MFI did not follow a linear behavior where proportional relationships (e.g., a twofold increase in MFI registers representing a twofold increase in immunoglob-

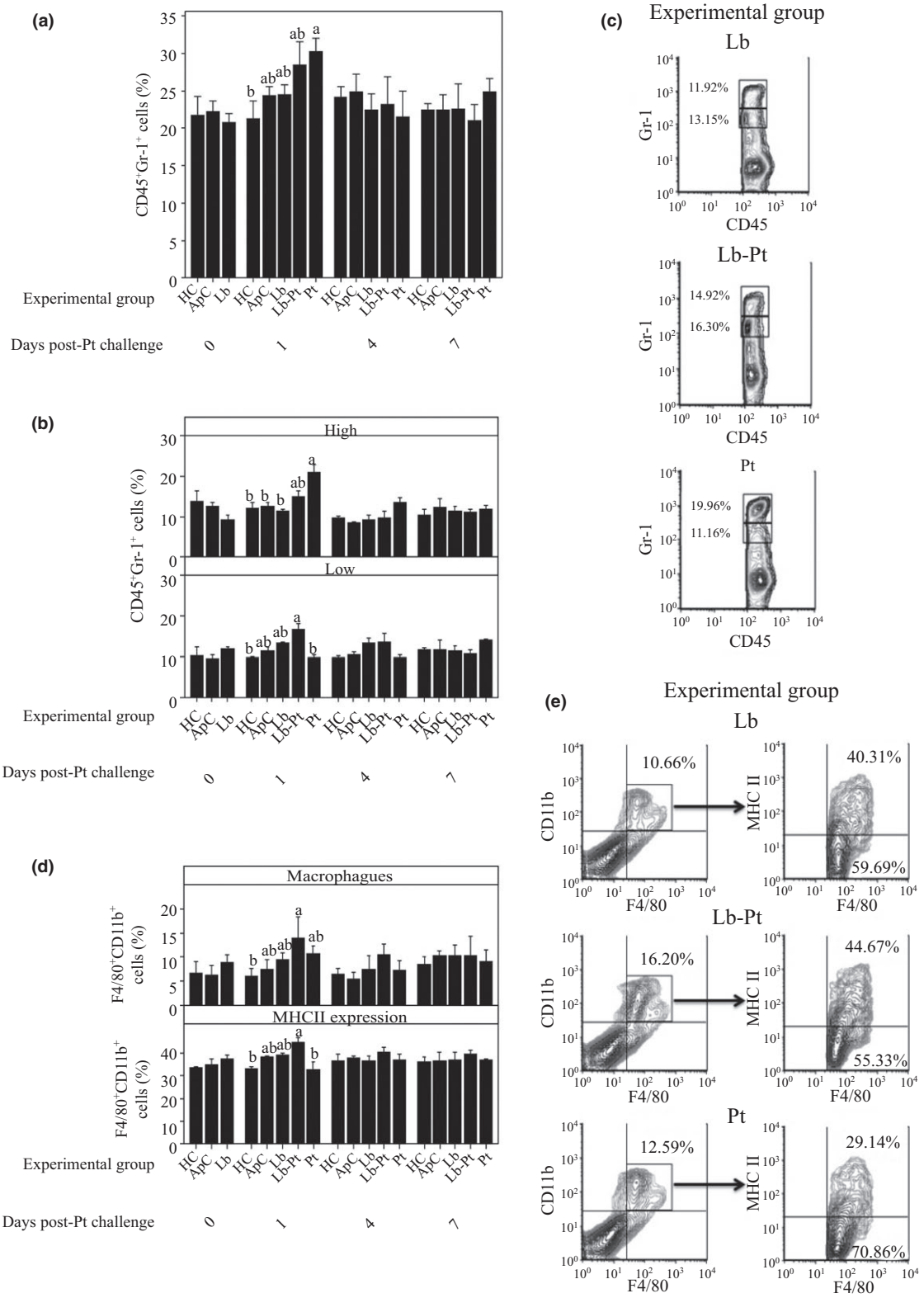

Fig. 1 Granulocyte and macrophage populations in vaginal cells of hormone control (HC), agarized peptone control (ApC), Lb [inoculated with *L. reuteri* CRL1324 (CRL1324)], Lb-Pt [inoculated with CRL1324 and then challenged with *S. agalactiae* NH17 (NH17)], and Pt (challenged with NH17) mice. Percentage (mean value ± standard error) of (a) $CD45^+Gr\text{-}1^+$, (b) $CD45^+Gr\text{-}1^{high}$ and $CD45^+Gr\text{-}1^{low}$, and (d) $F4/80^+CD11b^+$ cells and MHC-II expression in $F4/80^+CD11b^+$ cells. Different letters indicate differences between groups on the same sampling day ($P < 0.05$). Contour plots of (c) $CD45^+Gr\text{-}1^{high}$ and $CD45^+Gr\text{-}1^{low}$ cells and (e) $F4/80^+CD11b^+$ cells and their MHC-II expression in different experimental groups on day 1 post-Pt challenge. Results are representative of three independent experiments ($n \geq 9$ mice per experimental group in each sampling day).

ulin values) could be established. According to the manufacturer's instruction, the detection threshold for these assays was 10 ng/mL.

Microbiological Studies

Viable bacteria numbers in v.w. were determined by the serial dilution method by plating in selective media: MRS agar pH 5.5 supplemented with 15 μg/mL vancomycin (Sigma Chemical Co) to selectively quantify *L. reuteri* CRL1324 and chromIDStrepto B agar to quantify GBS NH17.[15] The plates were incubated under aerobic conditions at 37°C for 24 h (for quantification of GBS NH17) or 48 hr (for *L. reuteri* CRL1324).

Statistical Analysis

Analysis of variance (ANOVA) using a general linear model was applied to determine the main and interaction effects of factors (experimental group and day post-inoculation) on the percentage of immune cells, concentrations of cytokines, MFI of antibodies in vagina, and CFU of GBS NH17 or *L. reuteri* CRL 1324. Significant differences (P value <0.05) between mean values were determined by Tukey's test. All statistical analyses were performed using Minitab 16 statistical software (Colonia, Germany).

Results

Evaluation of the Cell Populations Involved in the Innate and Adaptive Immune Responses in Mice Inoculated with *L. reuteri* CRL1324 and Challenged with GBS

Different cell populations involved in the innate and adaptive immune responses were determined in mice vagina enzymatically disaggregated. When evaluating the $Gr\text{-}1^+CD45^+$ cell populations (markers expressed in granulocytes, monocytes, and macrophages),[19] an increase in the percentage of these cells was observed at day 1 post-GBS challenge in the Lb-Pt and Pt experimental groups. However, this increase was significant only in the Pt-challenged mice compared with hormone control (HC) mice ($P = 0.042$) (Fig. 1a). The higher percentage of $Gr\text{-}1^+\text{-}CD45^+$ cells at day 1 post-Pt challenge in the Pt experimental group was associated with an increased expression of $CD45^+Gr\text{-}1^{high}$ cells (neutrophil population), while in the Lb-Pt experimental group it was associated with a higher expression of $CD45^+Gr\text{-}1^{low}$ cells (possible macrophage population) (Fig. 1b,c). The increase in the percentage of $CD45^+Gr\text{-}1^{high}$ cells observed in Pt-challenged mice was significant ($P < 0.05$) compared with Lb, HC, and ApC mice, but not referred to Lb-Pt-treated mice. However, i.va. treatment with Lb previous to GBS challenge reduced the $CD45^+Gr\text{-}1^{high}$ cell percentage to levels similar to those evidenced in control animals (Fig. 1b). The higher $CD45^+Gr\text{-}1^{low}$ cell percentage in the Lb-Pt group was significant only with respect to HC and Pt mice (Fig. 1b). No significant differences were obtained in the percentage of $Gr\text{-}1^+CD45^+$ cells in either control or Lb groups or between all the experimental groups during the remaining sampling days (Fig. 1a).

When using markers of higher specificity than Gr-1 to evaluate the macrophage population (F4/80 and CD11b), an increase in the percentage of $F4/80^+CD11b^+$ cells was observed at day 1 post-GBS challenge in Lb-Pt-treated mice compared with all the experimental groups (Fig. 1d,e). This difference was significant ($P < 0.05$) only between the Lb-Pt and HC groups. The increase in macrophages is coincident with the highest percentage of $Gr\text{-}1^{low}$ cells described previously (Fig. 1b,c). On day 4 post-Pt challenge, a non-significant increase ($P > 0.05$) in the percentage of $F4/80^+CD11b^+$ cells in Lb-Pt-treated mice was also obtained.

On the other hand, the expression of the MHC-II was evaluated in the $F4/80^+CD11b^+$ population as a feature of macrophage activation. The strategy applied for data analysis and the cytometric plots obtained on day 1 post-Pt challenge in the different experimental groups are shown in Fig. 1e. The highest expression of MHC-II marker (44.38% ± 1.30) was obtained in Lb-Pt-treated mice on day 1 post-Pt

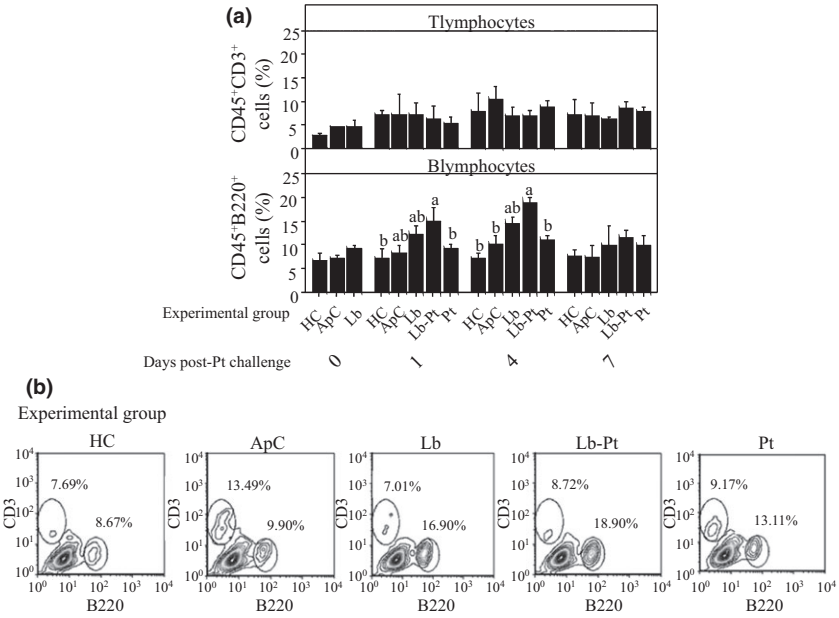

Fig. 2 T and B lymphocytes in vaginal cells of hormone control (HC), agarized peptone control (ApC), Lb [inoculated with *L. reuteri* (CRL1324)], Lb-Pt [inoculated with CRL1324 and then challenged with *S. agalactiae* (NH17)], and Pt (challenged with NH17) mice. (a) Percentage of $CD45^+CD3^+$ and $CD45^+B220^+$ cells. Data are plotted as the mean values of cell percentage ± standard error. Different letters indicate differences between experimental groups on the same sampling day ($P < 0.05$). (b) Contour plots of $CD45^+CD3^+$ and $CD45^+B220^+$ cells in the HC, ApC, Lb, Lb-Pt, and Pt groups on day 4 post-challenge with NH17. Results are representative of three independent experiments ($n \geq 9$ mice per experimental group in each sampling day).

challenge compared with the other experimental groups (HC: 32.67% ± 3.50; ApC: 39.37% ± 2.82, Lb: 42.90% ± 0.97; Pt: 33.30% ± 4.96) (Fig. 1d,e). On the other sampling days, no significant differences in the expression of MHC-II marker were evidenced (Fig. 1d).

The cell populations involved in the mice adaptive immune responses were also evaluated. The expression of CD3 (T lymphocytes) and B220 (B lymphocytes) in $CD45^+$ cells (Fig. 2) was studied. When evaluating T lymphocytes population, no significant differences ($P > 0.05$) were detected in the percentage of $CD45^+CD3^+$ cells between experimental groups, or between sampling periods (Fig. 2a). However, the B lymphocyte population showed a significant increase ($P < 0.05$) in the percentage of $CD45^+B220^+$ cells in Lb-Pt-treated mice on day 1 post-Pt challenge compared with the Pt and HC experimental groups. This increase was also evidenced in Lb-Pt-treated mice on day 4 post-Pt challenge compared with the HC, ApC, and Pt groups (Fig. 2a,b).

Evaluation of Cytokines in v.w. of Animals Inoculated with *L. reuteri* CRL1324 and Challenged with GBS

Different cytokines were determined in the mice v.w. of the experimental groups on all the sampling days. The quantification of cytokines involved in both innate and adaptive immunity (TNF-α, IFN-γ, IL-10, and IL-6) evidenced a significant increase ($P < 0.05$) in the levels of TNF-α (152.56 pg/mL ± 14.95) at day 1 post-Pt challenge in the Pt-challenged mice compared with the HC (44.65 pg/mL ± 17.54), ApC (51.17 pg/mL ± 15.49), and Lb (50.91 pg/mL ± 21.80) groups. On the subsequent sampling days, a decrease in this cytokine was observed in Pt-challenged mice. Treatment with Lb prior to GBS challenge (Lb-Pt-treated mice) reduced the levels of TNF-α (94.29 pg/mL ± 27.65) to values similar to those of control and Lb-treated mice (Fig. 3a).

Different results were obtained with IL-10. Significantly lower levels (1.46 pg/mL ± 0.51) were detected in Pt-challenged mice at day 1 post-Pt challenge compared with the HC (8.06 pg/mL ± 1.42), ApC (7.52 pg/mL ± 0.97), and Lb (7.76 pg/mL ± 1.19) experimental groups. Treatment with Lb prior to challenge with GBS increased the levels of IL-10 (5.34 pg/mL ± 1.39) to values similar to those of control mice at day 1 post-Pt challenge. Also, higher levels of IL-10 were observed in the Pt-challenged mice as days went by. Highest IL-10 values were associated with lowest TNF-α levels (Fig. 3a).

When evaluating IFN-γ and IL-6 levels, the highest concentrations were obtained in the Lb-Pt group compared with all the experimental groups on days 1 and 4 post-Pt challenge. In the case of IFN-γ, the values obtained in the Lb-Pt group were significantly higher ($P < 0.05$) on day 1 post-GBS challenge compared with the control (HC and ApC) and Pt groups.

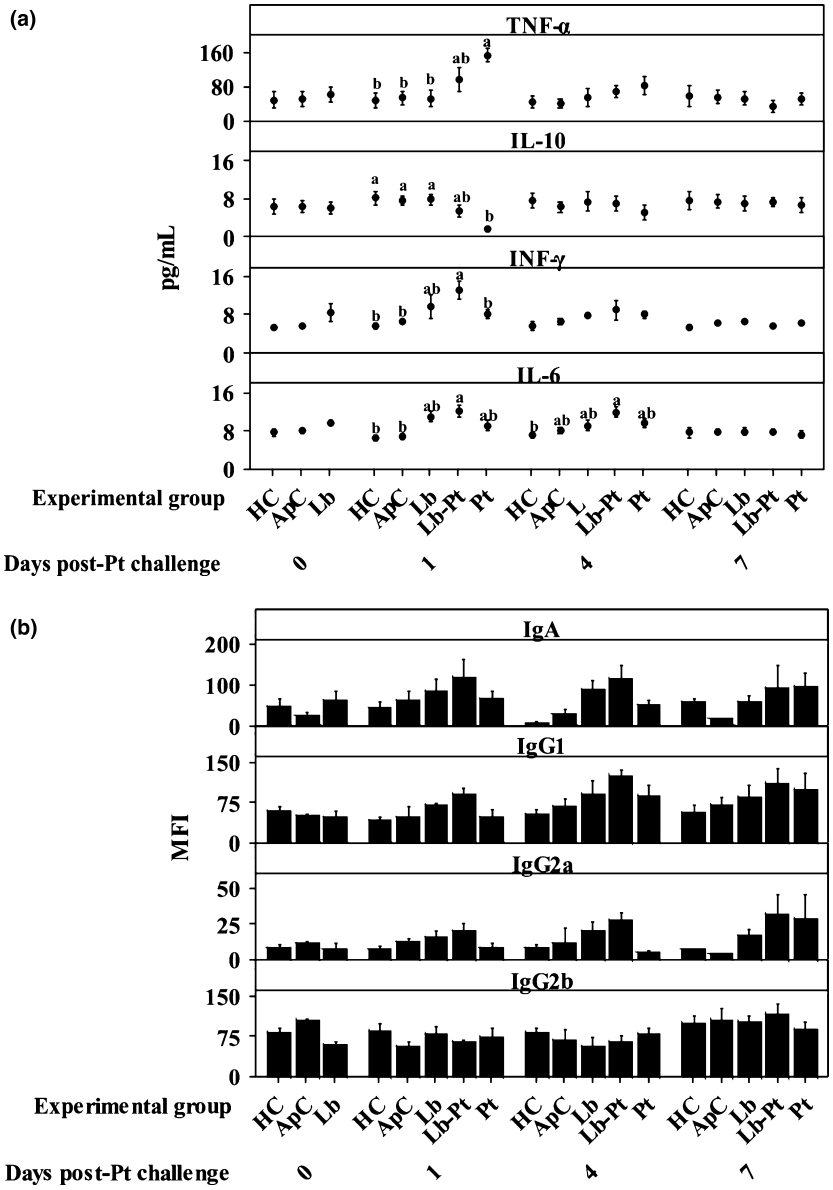

Fig. 3 Cytokine levels and immunoglobulin isotypes in vaginal washings of hormone control (HC), agarized peptone control (ApC), Lb [(inoculated with *L. reuteri* (CRL1324)], Lb-Pt [inoculated with CRL1324 and then challenged with *S. agalactiae* (NH17)], and Pt (challenged with NH17) mice. (a) Quantification (pg/mL) of TNF-α, IL-10, IFN-γ, and IL-6. (b) Mean fluorescence intensity (MFI) of IgA, IgG_1, IgG_{2a}, and IgG_{2b}. Data are plotted as mean values of cytokine levels or MFI ± standard error. Different letters indicate significant differences between experimental groups on the same sampling day ($P < 0.05$). Results are representative of three independent experiments ($n \geq 9$ mice per experimental group in each sampling day).

IL-6 values observed in Lb-Pt-treated mice on day 1 post-Pt challenge were significantly higher ($P < 0.05$) compared with HC and ApC mice, but on day 4 post-Pt challenge they were higher only referred to HC mice. The increased levels of the two cytokines in the Lb-Pt group returned to values close to those of controls on day 7 post-Pt challenge (Fig. 3a).

The evaluation of the cytokines associated with adaptive immunity (IL-2, IL-4, and IL-17) did not show significant differences ($P > 0.05$) between experimental groups and samplings days (data not shown).

Evaluation of Immunoglobulin Isotypes in v.w. from Mice Inoculated with *L. reuteri* CRL1324 and Challenged with GBS

Different immunoglobulin isotypes were determined in the v.w. of mice from the experimental groups at all sampling days. The results showed that only IgA and IgG subclasses (IgG_1, IgG_{2a}, and IgG_{2b}) were detected in the v.w. of mice from all the experimental groups and sampling days, while IgM, IgE, and IgG_3 were not detected (Fig. 3b). A non-significant increase ($P > 0.05$) was evidenced in the mean

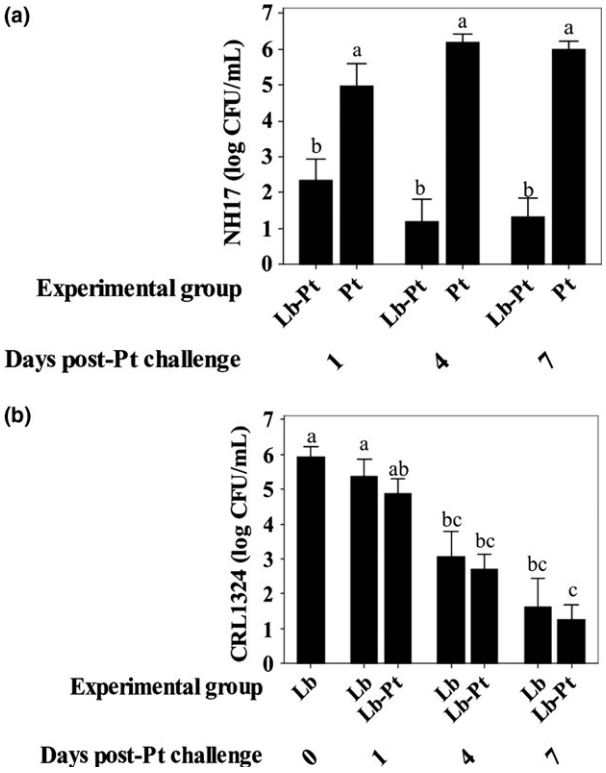

Fig. 4 Persistence of *S. agalactiae* NH17 (NH17) and *L. reuteri* CRL1324 (CRL1324) in murine vaginal washings. (a) NH17 viable cells in Lb-Pt (inoculated with CRL1324 and then challenged with NH17) and Pt (challenged with NH17) mice. (b) CRL1324 viable cells in Lb (inoculated with CRL1324) and Lb-Pt mice. Data are plotted as mean values of the NH17 or CRL1324 viable cell numbers ± standard error. Different letters indicate significant differences between experimental groups on the same sampling day ($P < 0.05$). Results are representative of three independent experiments ($n \geq 9$ mice per experimental group in each sampling day).

fluorescence intensity (MFI) obtained for IgA, IgG_1, and IgG_{2a} in the Lb and Lb-Pt mice at days 1 and 4 post-Pt challenge compared with the other experimental groups. The Lb-Pt-treated mice showed non-significant different levels of IgA, IgG_1, and IgG_{2a} in most of the sampling days compared with the other groups. IgG_{2b} levels obtained in all the experimental groups and sampling days were not different (Fig. 3b).

Persistence of GBS NH17 and *L. reuteri* CRL1324 in v.w. from Mice

Seven doses of *L. reuteri* CRL1324 were able to significantly reduce the numbers of viable GBS cells, as previously demonstrated.[15] When mice were i.va. challenged with GBS NH17, the pathogen was recovered from v.w. up to the last sampling day (day 7 post-Pt challenge). The number of viable GBS NH17 was around 4.97 ± 1.1 log CFU/mL at day 1 post-Pt challenge. No significant increases in the number of viable streptococci were observed in v.w. on day 4 (6.18 ± 0.36 log CFU/mL) and on day 7 (6.01 ± 0.21 log CFU/mL) after pathogen challenge (Fig. 4a). However, when mice were inoculated with seven doses of *L. reuteri* CRL1324 prior to the challenge with GBS NH17, a significantly lower number of GBS viable cells was recovered in the v.w. of Lb-Pt-treated mice compared with Pt-challenged mice in all sampling days.

Viable *L. reuteri* cells in v.w. from Lb-treated mice and Lb-Pt-treated mice were similar, indicating that the i.va. challenge with GBS did not affect the number of viable lactobacilli recovered. A significant decrease in the number of viable lactobacilli was observed throughout the sampling period (Fig. 4b).

Discussion

In a previous work, *L. reuteri* CRL1324 i.va. inoculated in 7 doses before i.va. challenge with GBS NH17 in mice was able to reduce vaginal colonization of the pathogenic microorganism, which was not effective with 4 doses.[15] In addition, the degree of *L. reuteri* CRL1324 vaginal colonization and the murine vaginal pH were not significantly different between mice treated with either 4 or 7 doses, suggesting that these factors are not responsible for the GBS inhibitory effect observed in this experimental model. However, the leukocyte influx induced in the vaginal lumen by the pathogenic microorganism showed a higher reduction in mice treated with seven doses of *L. reuteri* CRL1324 than in mice treated only with four doses.[15]

Several studies have demonstrated that lactobacilli can modulate the host's immune system and produce responses highly related to the dose administered[20–22] and that they are able to exert anti-inflammatory effects on vaginal infection models[23–26] and activate the immune system of the host against urogenital pathogens.[25,27] Therefore, we hypothesized that a higher number of doses of *L. reuteri* CRL1324 might modulate the immune system, improving the host's ability to eradicate GBS while limiting inflammation. In this way, different components involved in the innate and adaptive immune responses were evaluated in the preventive

murine model of *L. reuteri* CRL1324 against GBS NH17 previously set up.[15]

In vitro and *in vivo* studies of GBS vaginal infection have shown that this microorganism during early stages of infection can activate numerous immune pathways, including the secretion of pro-inflammatory cytokines involved in leukocyte recruitment.[28] However, GBS is a microorganism that can limit the expression of virulence factors during vaginal colonization through a regulatory system (CovR/S), reducing the host innate immune response and cytokine production to promote its vaginal colonization.[28] In this work, GBS intravaginally inoculated into mice induced an increase in the population of neutrophils ($CD45^+Gr\text{-}1^{high}$) and in the levels of TNF-α secreted into the vaginal lumen on day 1 post-GBS challenge. On that day, a decrease in the levels of IL-10 was observed in Pt-challenged mice compared with control animals. However, on days 4 and 7 post-Pt challenge, the levels of IL-10 and TNF-α and the neutrophil population returned to values similar to those of control mice. In a similar way, Patras et al.[28] showed that GBS vaginal infection activates numerous immune pathways, including the secretion of pro-inflammatory cytokines and chemokines involved in leukocyte recruitment in the early days post-GBS challenge in *in vitro* and *in vivo* experiments.

The innate immune response is the first barrier responsible for host defense against pathogen entry including non-specific immune protection mediated by neutrophils, monocytes, and macrophages. The innate immune system further regulates the functions of the antigen-specific adaptive immune system, such as the functional balance of the immune response associated with cytokine and chemokine receptor profiles.[29] TNF-α is a pro-inflammatory cytokine that induces activation and recruitment of neutrophils, which can affect epithelial barrier, contributing to pathogen colonization.[30–32] IL-10 is a cytokine that controls the degree and duration of an inflammatory response by preventing the expression of pro-inflammatory cytokines while promoting the expression of anti-inflammatory molecules.[33]

Based on the results obtained in the murine experimental model, it could be suggested that i.va. inoculation of GBS initially triggers pro-inflammatory signals induced by TNF-α but then suppresses them by IL-10 induction. This hypothesis is consistent with earlier findings that show GBS-specific upregulation of IL-10 in infection models.[4,34,35] In this way, Kline et al.[36] have demonstrated that GBS can subvert the immune responses mounted by the host, creating problems for the host's elimination of urogenital pathogens.

On the other hand, strong evidence of the role of probiotics, mainly lactic acid bacteria, in health maintenance or disease prevention by modulating the host immune response was described.[37] When evaluating the effect of *L. reuteri* CRL1324 inoculated seven times into mice prior to GBS challenge, the treatment with lactobacilli reduced the neutrophil numbers ($CD45^+Gr\text{-}1^{high}$ cells) induced by GBS at day 1 post-Pt challenge. These results were correlated with the lower number of leukocytes previously observed in the cytological and histological evaluations of the murine vagina.[15] However, i.va. inoculation of *L. reuteri* CRL1324 increased IL-10 levels and decreased TNF-α levels in vagina (day 1 post-Pt challenge) compared to mice challenged with the pathogen.

An increase in the population of macrophages ($F4/80^+CD11b^+$ and $CD45^+Gr\text{-}1^{low}$ cells) and in the levels of IFN-γ on day 1 post-Pt challenge was also observed in Lb-Pt-treated mice compared with Pt-challenged and control mice (HC and ApC). In addition, macrophage population showed the highest expression of MHC-II in the Lb-Pt experimental group. Macrophages are cells that play a critical role in the initiation of the innate immune response as specialized phagocytes that participate in cellular and molecular clearance as well as in the defense against infection. They also participate in antigen processing, presentation, and activation of the antigen-specific immune response.[29,38] In this way, several studies have demonstrated that *Lactobacillus* strains can activate monocytes and macrophages and induce the upregulation of some maturation markers such as MHC-II on antigen-presenting cells, which suggests that lactobacilli could participate in the antigen presentation process.[39–41]

IFN-γ is a cytokine that contributes to the activation of macrophages, promoting the effective killing of pathogens that are able to survive within them. In addition, an inhibitory effect of IFN-γ on neutrophil traffic and pro-inflammatory Th17 cell differentiation was evidenced.[42–44] In the same way, the results obtained in this work suggest that the higher macrophage numbers and the lower neutrophil quantities found in Lb-Pt-treated mice could be related to the higher levels of IFN-γ detected in the v.w. of these mice compared with the other

experimental groups. Thus, treatment with *L. reuteri* CRL1324 prior to challenge with GBS NH17 could induce an innate immune response mediated by macrophages, which is different in Pt-challenged mice (not treated with Lb) where a predominance of neutrophils was observed. Furthermore, considering the increased expression of MHC-II on F4/80^+CD11b^+ cells in Lb-Pt-treated mice compared with Pt-challenged mice, it could be suggested that treatment with *L. reuteri* CRL1324 could play an important role in the maturation of the macrophage population and in antigen presentation in this specific tract.

Many experimental models and studies in patients have shown that the immunomodulation of some probiotic lactobacilli strains involve the activation of cellular and humoral immunity, increasing the number of T and B lymphocytes and their proliferative activity, regulatory cytokines, and immunoglobulin secretion.[45–49] Therefore, it was important to determine whether the i.va. inoculation of *L. reuteri* CRL1324 and the subsequent GBS challenge produced changes in the T or B lymphocyte population in mice vagina, in the cytokines involved in the adaptive immune response and/or in the immunoglobulins released in murine v.w. Evaluation of the percentage of T cells ($CD3^+CD45^+$ cells) by flow cytometry showed no significant differences in the different experimental groups and sampling days. However, an increase in the percentage of B cells ($B220^+CD45^+$ cells) was induced after i.va. inoculation of *L. reuteri* CRL1324, the largest percentage of these cells being found in the vagina of Lb-Pt-treated mice compared with the other experimental groups. Similarly, Maldonado Galdeano and Perdigón[50] have shown that oral administration of *Lactobacillus casei* CRL431 to mice did not produce changes in intestinal T-cell population, but induced an increase in IgA^+ B cells since day 7 post-Lb inoculation.

The results of this work show that i.va. inoculation of *L. reuteri* CRL1324 induces an increase in IL-6 levels on days 1 and 4 post-Pt challenge. Highest levels of these cytokines were observed in Lb-Pt-treated mice compared with the other experimental groups. IL-6 is a cytokine secreted by epithelial cells, macrophages, dendritic cells, and T cells. This cytokine promotes the maturation of B-cell population, inducing their final development to immunoglobulin-expressing plasma cells.[51,52] The increased levels of IL-6 could be associated with the highest percentage of B lymphocytes observed in Lb-Pt-treated mice and with the highest fluorescence intensities of IgA, IgG_1, and IgG_{2a} recorded in this experimental group.

IgE, IgM, IgD, and IgG_3 isotypes were not detected in v.w. of mice in the different experimental groups with the methodology applied in this work. In contrast, IgA, IgG_1, and IgG_{2a} were evidenced in the v.w. of the different experimental groups, mainly in Lb-Pt-treated mice at day 1 post-Pt challenge. These results agree with previous research showing that in cervico-vaginal fluid, the major immunoglobulins are IgG and IgA, IgG being the predominant immunoglobulin in the vaginal tract.[53–55] Mucosal IgA can be classified into two groups according to its affinity. High-affinity IgA plays an essential role in the neutralization of pathogens and microbial proteins such as toxins and is believed to originate from B cells in a T-cell-dependent manner. Low-affinity IgA, also called natural IgA, is produced mainly by B cells in a T-cell-independent manner. This immunoglobulin is responsible for the immune exclusion of commensal microorganisms and participates in the innate immune response against some pathogens.[56,57] Furthermore, IgG consists of four subclasses which contribute in different ways to humoral immunity against pathogens. Individual subclasses are stimulated by different types of antigens. Antibody response to bacterial antigens and viral proteins is restricted mostly to IgG_1 and IgG_3, whereas IgG_2 is usually produced in response to antigen-presenting carbohydrates such as encapsulated pathogens.[58] Furthermore, Th1 cells stimulate the production of IgG_{2a} antibodies, while IgG_1 antibodies are induced under the control of Th2 cells.[59]

Considering the increase in the percentage of B lymphocytes and immunoglobulin levels in animals i.va. inoculated with lactobacilli, those cytokines characteristic of the adaptive immunity were evaluated: IL-2 (a typical cytokine in the Th1 response), IL-4 (Th2 cytokine), and IL-17 (a cytokine associated with the Th17 response). The results obtained did not show modifications in the three cytokines assayed in any of the experimental groups. These results suggest that the increase in B lymphocyte population and in IgG and IgA levels after i.va. administration of *L. reuteri* CRL1324 could be the result of a T-cell-independent pathway as no changes were observed in T population or in the cytokines produced by this specific population. Furthermore, the modifications in the cytokines secreted by the innate and adaptive immunity

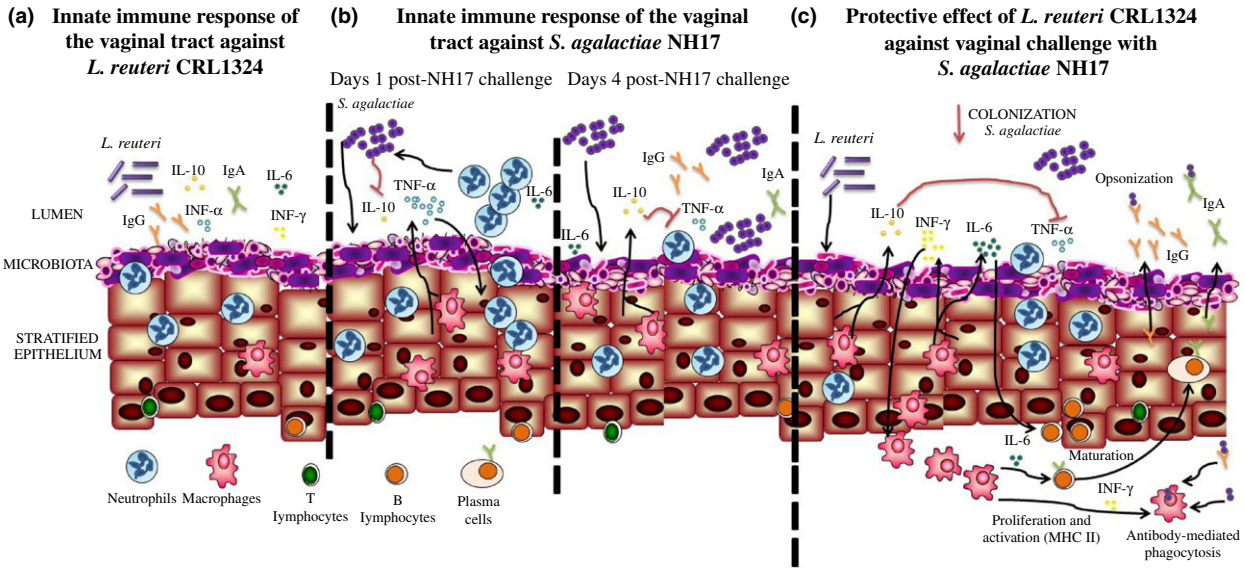

Fig. 5 Mechanisms suggested indicating the different immunological targets modified by *S. agalactiae* NH17 (NH17) alone and in the presence of *L. reuteri* CRL1324 (CRL1324). (a) CRL1324 inoculation: balanced vaginal ecosystem. (b) NH17 challenge. Day 1 post-challenge: decrease in IL-10 levels and increase in TNF-α secretion; neutrophil activation and recruitment. Day 4 post-challenge: IL-10 increase, inhibition of TNF-α secretion and neutrophils recruitment. (c) NH17 challenge after CRL1324 inoculation: increase in IL-10 to basal levels (inhibition of TNF-α production and neutrophils recruitment); increase in IFN-α secretion (promotion of macrophage proliferation and activation); IL-6 secretion by macrophages; B-cell maturation to IgG- and IgA-secreting plasma cells; and NH17 opsonization by antibodies (contribution to pathogen removal).

participating cells (TNF-α, IFN-γ, IL-10, and IL-6) might be secreted primarily by cells involved in innate immunity. The suggested mechanisms by which *L. reuteri* CRL 1324 could exert its inhibitory effect against GBS NH17 vaginal colonization are summarized in Fig. 5.

Lactobacillus predominance in vaginal microbiota was associated with reduced GBS colonization, in pregnant and non-pregnant women.[60,61] Several probiotic products have been administered during clinical trials to more than 2000 pregnant women, in which side-effects or negative sequelae were not reported.[62] Additionally, the prenatal probiotic supplementation to reduce GBS colonization has been recommended as a safe strategy, but there are not enough clinical trials to support this practice.[63,64] For example, Hanson et al.[63] observed that pregnant women that were administered with oral probiotic (from the 28th week of pregnancy) had lower GBS vaginal load at 36 weeks.

The results of this work suggest that *L. reuteri* CRL1324 could be used as a prenatal probiotic supplement with potential to reduce GBS colonization. This promising strategy could decrease the numbers of women that require intrapartum antibiotic prophylaxis and the numbers of fetuses that are exposed to adult doses of antibiotics before birth. However, the requirement of *L. reuteri* CRL1324 to be administered at least seven times prior to GBS in the murine model could be considered as one of the major obstacles when applying this probiotic as a treatment for GBS-positive pregnant women. Therefore, further studies should be carried out to design novel strategies to include *L. reuteri* CRL1324 as a probiotic alternative to prevent or treat GBS colonization in pregnant women.

Conclusion

The results obtained in this work show that the preventive effect of *L. reuteri* CRL1324 against GBS challenge could be explained by a modulatory effect of this strain on the immune cells and mediators involved in innate immunity, resulting in an increase in the activation of macrophages and B lymphocytes.

Acknowledgments

This work was supported by CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina) (PIP 632 and 744) and ANPCyT (Agencia

Nacional de Promoción Científica y Tecnológica) (PICT 2007-543 and PICT 2012-1187). We wish to thank Elena Bru for her help with the statistical analysis of experimental results and Dr. Virginia Ocaña for the human urogenital pathogens isolated at the Nuevo Hospital 'El Milagro' (Salta, Argentina).

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