Research in Veterinary Science 115 (2017) 211–220

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Intramammary inoculation of *Panax ginseng* plays an immunoprotective role in *Staphylococcus aureus* infection in a murine model

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ARTICLE INFO

Keywords:
Panax ginseng
Murine mastitis model
Staphylococcus aureus
Immunoprotective role

ABSTRACT

The immunoprotective effect of *Panax ginseng* (Pg) extract was investigated in a mouse mastitis model. Lactating female mice were intramammarily inoculated with Pg or placebo, and then were challenged with *S. aureus*, while other group was inoculated with *S. aureus* alone. The number of bacteria recovered from mammary glands was significantly lower in Pg-treated *S. aureus*-infected mice (group I) compared with placebo-treated *S. aureus*-infected mice (group II) and *S. aureus*-infected mice (group III). The mRNA expression of TLR2, TLR4, IL-1α and TNF-α was influenced by treatment; being the transcript levels for all genes higher in group I compared with group II and III. Activation of NF-κB and the number of monocytes-macrophages in mammary gland tissue was significantly increased in group I compared with group II and III. Pg extract was able to trigger an adequate immune response to confront an infection demonstrating its protective effect and potential for preventing bovine intramammary infections.

1. Introduction

Bovine mastitis is the most significant limiting factor for profitable dairying worldwide (Viguier et al., 2009; Hogeveen et al., 2011). Several organisms can infect the bovine mammary gland; among them *Staphylococcus aureus* is one of the most prevalent major pathogens in dairy herds worldwide (Zecconi et al., 2006; Persson et al., 2011). Control of *S. aureus* intramammary infection (IMI) is based on hygiene, antibiotic therapy and culling of chronically infected cows (Zecconi et al., 2006). However, cure rates following antibiotic therapy are variable (Barkema et al., 2006). In addition, extensive antibiotic usage for treatment and prevention of IMI has become a serious concern for dairy industry and public health (De Vliegher et al., 2012; Oliver and Murinda, 2012).

Several factors associated with host, pathogen, and treatment regimen that affect the probability of cure of *S. aureus* IMI have been identified (Barkema et al., 2006; Bradley and Green, 2009). Among them, the ability of *S. aureus* to invade and persist within certain types of host cells, such as mammary epithelial cells, has been associated with recurrence of these IMI (Almeida et al., 1996). The ability of *S. aureus* to escape from the immune response and survive inside different cells is considered a fundamental trait for this bacterium to persist within the mammary gland and establish chronic IMI (Oviedo-Boyso et al., 2007). These limitations led to the search of novel approaches to complement classical control measures. Among them, biological response modifiers have been proposed for enhancement of specific aspects of the host immune response, leading to development and evaluation of new compounds (Takahashi et al., 2004; Dallard et al., 2009).

Ginseng, the root of *Panax ginseng* (Pg) C.A. Meyer, has been utilized in China as a safe traditional medicine for at least 2000 years (Li and Li, 1973). Ginseng contains several pharmacological components including a series of tetracyclic triterpenoid saponins (ginsenosides), polyacetylenes, polyphenolic compounds, and acidic polysaccharides (Kim et al., 2005). Ginsenosides are believed to be the main pharmacologically active constituents (Kiefer and Pantuso, 2003). Several reports support that Pg extract has been widely used as an herbal remedy for various disorders (Akerele, 1992; Kang and Min, 2012). This extract can stimulate the natural resistance against infections in humans and

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http://dx.doi.org/10.1016/j.rvsc.2017.05.010
Received 13 April 2016; Received in revised form 20 April 2017; Accepted 6 May 2017

animals through lymphocyte proliferation enhancement, cytokine production following macrophage stimulation, and improvement phagocytic activity of macrophages and polymorphonuclear leukocytes (Scaglione et al., 1990; Hu et al., 2001; Larsen et al., 2004).

Previous studies have demonstrated that the dry root extract of Pg has immunostimulant properties in noninfected cows at the end of lactation and may enhance early mammary involution (Baravalle et al., 2010, 2011, 2015; Dallard et al., 2013). However, the potential immunoprotective effects of Pg against *S. aureus* IMI and its putative molecular mechanisms are unknown. In an attempt to elucidate the mechanism of action of Pg, we found in a previous study that bovine mammary cells responded to Pg extract components affecting the TLR2 and TLR4 gene and protein expression (Baravalle et al., 2015). Moreover, activation of MyD88 and NF-κB was verified in Pg inoculated bovine mammary glands. Hence, we proposed that activation of MyD88-dependent pathway leads to a release of transcription factors, like NF-κB, that translocates into the nucleus and regulates the expression of cytokines, playing a crucial role in immune response regulation and mammary gland remodeling (Baravalle et al., 2015).

The mouse mastitis model is still considered a very suitable tool to study pathogenesis mechanisms, immune responses and efficacy of antibiotic compounds against pathogens (Chandler, 1970; Malouin et al., 2005; Breyne et al., 2014). In this study, we have used an experimental mastitis model, in which mice were subjected to *S. aureus* infection, in order to evaluate the effects of Pg and examine the protective role of this extract in murine mammary gland.

2. Materials and methods

2.1. Preparation of Pg inoculum

The Pg extract containing saponins equivalent to 27% ginsenoside Rg1, was provided by Indena Company (Indena® SpA, Milan, Italy). The Pg stock solution (50 mg/ml) was prepared and examined for endotoxin contamination as previously described (Baravalle et al., 2015).

2.2. Experimental animals

All the procedures were carried out according to the Guide for the Care and Use of Laboratory Animals (ILAR, 2010) and approved by the Committee of Animal Ethics and Security of the Facultad de Ciencias Veterinarias, UNL. Female BALB/cJ lactating mice were used 10–15 days after birth of the offspring and at the beginning of experiments these mice weighed 30–35 g. The animals were provided by Centro de Medicina Comparada, Instituto de Ciencias Veterinarias del Litoral, (UNL-CONICET). During the experiments, the animals were kept with a controlled cycle of light-darkness (lights on between 8:00 AM and 8:00 PM), and at a temperature of 20–24 °C with free access to water and commercial balanced food.

2.3. Dose-response Pg trials

Prior to experimental challenge with *S. aureus* a dose-response assay was carried out to select a Pg extract dose. Three lactating mice were used for each condition of inoculum and time. Three concentrations of Pg extract were tested: 3 mg/ml, 10 mg/ml and 50 mg/ml. Intramammary inoculation was based on the method described by Brouillette and Malouin (2005). Briefly, the litter was removed 1–2 h before inoculation of the mammary glands and a mixture of 87 mg/kg ketamine (Holliday-Scott S.A., Argentina)/13 mg/kg xylazine (Rompun, Bayer, Argentina), was used for anesthetizing the lactating mice. Teats ducts of both the L4 (on the left) and R4 (on the right) abdominal mammary glands were exposed under a binocular stereoscopic microscope and 100 µl of three different concentrations of Pg extract per gland were intramammarily delivered using a syringe with a 30 G blunt needle. In parallel, other mammary glands were inoculated with 100 µl of placebo (saline solution). The litter was returned to their mothers 3 h later. Mice were euthanized at 6, 24, 48 and 72 h post inoculation (PI) and the mammary glands were aseptically removed. A tissue sample was taken, immediately fixed in 4% neutral buffered formalin for 8 h at 4 °C, washed in phosphate buffered saline (PBS, pH 7.4; 0.01 M) and embedded in paraffin wax. Tissue sections (4 µm thick) were stained with hematoxylin and eosin, and then examined with an optical microscope for histological analysis. Another mammary tissue portion was immediately frozen at − 80 °C in a freezing vial until further use in mRNA genes expression assays. We examined the gene expression of IL-1α and TNF-α (the main acute phase cytokines) up to 72 h based on previous studies (Trigo et al., 2009; Wu et al., 2009; Zhao et al., 2015).

2.4. Bacterial strain and growth conditions

For the intramammary inoculation, *S. aureus* Newbould 305 (ATCC 29740) (Newbould, 1974), a bovine mastitis strain widely used for experimental IMI of cows (Hensen et al., 2000; Atalla et al., 2009; Kim et al., 2011) was used. For inoculation, bacteria were activated from frozen stocks (− 80 °C) by culture in Columbia agar overnight at 37 °C. Then, plated on Columbia agar with 2.5% NaCl, incubated overnight at 37 °C for CP expression induction and finally suspended in saline solution to the appropriate density using a densitometer. The number of bacteria was adjusted to 1×10^8 colony-forming units (CFU)/ml.

2.5. Staphylococcus aureus infection profile

To determine the minimal tissue inflammation dose of *S. aureus* inoculum, three groups of lactating mice were infected through the teat canal of L4 and R4 mammary glands with 100 µl of 1×10^4, 1×10^5 and 1×10^6 CFU/gland. The animals were euthanized at 6, 12, 24, 48 and 72 h post infection (pi) and mammary glands were aseptically removed. A portion of tissue samples was harvested, weighted and homogenized in a final volume of 2 ml of PBS. For each gland homogenate, six serial dilutions were performed in duplicate. Each series of dilutions was plated on Columbia agar plates, incubated overnight at 37 °C and the number of *S. aureus* colonies counted. Raw bacterial CFU counts were transformed to based-10 logarithm values and data were expressed as CFU/g of mammary gland. Another portion of the mammary gland was processed as described previously and tissue sections were subjected to Gram stain and examined by optical light microscopy for presence of Gram-positive cocci.

2.6. Inoculation of Pg and subsequent challenge with S. aureus

Once pilot dose–response trials were carried out to select the Pg and *S. aureus* dose, another cluster of lactating-BALB/cJ mice was divided in three groups. A group of lactating mice was inoculated with 100 µl of Pg extract (50 mg/ml) by intramammary route as described previously (group I) and another group was inoculated with 100 µl of placebo (saline solution) (group II). At 72 h pi with Pg extract and placebo, the L4 and R4 mammary glands of lactating mice from groups I and II were challenged through the teat canal with 100 µl of *S. aureus* suspension (10^5 CFU/gland). Besides, at this time point, another set of animals was infused with only the same dose of bacteria (group III). At 6, 24, 48, 72 and 96 h pi, the inoculated glands from the three groups of mice were harvested, weighted and homogenized as described previously, serial dilutions were performed, plated on Columbia agar, enumerated and expressed as CFU/g of mammary gland. Portions of the mammary gland sections from the three groups of mice were immediately frozen at − 80 °C until further use for gene expression and Western blot assays. Additional sections of the mammary tissue were fixed in formalin and processed as previously described.

Table 1
Antibodies, conditions and commercially purchased reagents used.

	Source	Type of antibody	Dilution IHC	Dilution WB
Anti-NF-κB-p65[a,b]	Santa Cruz Biotechnology	Polyclonal	1:500	1:1000
Anti-CD14 (clone RPA-M1)[a]	Zymed	Monoclonal	1:150	
Biotinylated anti-rabbit IgG[a]	Zymed	Polyclonal	1:100	
Streptavidin-peroxidase solution[a]	BioGenex			
Diaminobenzidine[a]	Dako Corp			
Anti-rabbit IgG peroxidase[b]	Amershan	Polyclonal		1:200

[a] Used for IHC staining.
[b] Used for Western blot study.

2.7. Immunohistochemical analysis

Histological paraffin sections from murine mammary glands were prepared and mounted on slides previously treated with 3-aminopropyltriethoxysilane (Sigma–Aldrich, St. Louis, MO, USA) for use in immunohistochemistry (IHC) procedures. An extravidin biotin immunoperoxidase method, previously described by Baravalle et al. (2015) was used. Briefly, after deparaffinization and hydration, endogen peroxidase activity was quenched with 3% (vol/vol) H_2O_2 in methanol for 20 min, and non-specific binding sites were blocked with 10% (vol/vol) normal goat serum for 15 min. Sections were incubated with primary antibodies (Table 1) for 18 h at 4 °C. Slides were washed with PBS and incubated at room temperature for 30 min with a biotinylated secondary antibody (Table 1). The antigens were visualized by ExtrAvidin-Peroxidase, and 3.3-diaminobenzidine was used as chromogen. Subsequently, samples were rinsed in distilled water, counterstained with hematoxylin, dehydrated, and mounted. Negative control sections in which primary antibody was replaced by non-immune rabbit serum were included. To validate endogenous peroxidase activity blockage, some sections were incubated with DAB alone. CD-14 positive cells were visualized by brown cytoplasmic staining. Active complexes of NF-κB were identified by nuclear staining of NF-κB-p65 subunit in parenchymal and stromal cells of the mammary gland (Notebaert et al., 2008).

2.8. Image analysis and immunohistochemistry quantification

Images from paraffin sections were captured using a colour video camera (Motic 2000, Motic China Group, China) mounted on top of a conventional light microscope (Olympus BH-2, Olympus Co., Japan), using an objective magnification of 40 × as described and validated previously (Baravalle et al., 2011; Dallard et al., 2011).

Positive nuclear cells for NF-κB-p65 were quantified in the epithelium (parenchyma) and stroma of the mammary gland as described previously (Baravalle et al., 2015). For measuring number of monocytes-macrophages stained with anti-CD14 antibody, 30–40 random images from each slide were digitized at 40 × and number of cells per mm^2 was obtained (Baravalle et al., 2011).

2.9. Western blotting

To test the specificity of the NF-κB-p65 primary antibody used in the IHC technique, murine mammary gland tissue sections were homogenized in a radio-immunoprecipitation assay lysis buffer and a protease inhibitor cocktail as previously described (Baravalle et al., 2015). The homogenate was then centrifuged at 12000 × *g* at 4 °C for 30 min and the supernatant was frozen at − 80 °C. Protein concentration in the supernatants was estimated using fluorescence methods (QubitTM, Invitrogen). For the Western blot analysis, 20 µg of protein, along with pre-stained molecular weight markers (Bio-Rad, Hercules, CA, USA), was separated in SDS-PAGE containing 12% (w/v) acrylamide–polyacrylamide and subsequently transferred electrophoretically to a nitrocellulose membrane (Hybond ECL Nitrocellulose Membrane, GE Healthcare, Buckinghamshire, UK). The membranes were then blocked with Tris-buffered saline (TBS) containing 0.05% (v/v) Tween20 (Sigma–Aldrich Corp.) and 5% (w/v) non-fat milk for 5 h at room temperature with gentle agitation, and then incubated overnight at 4 °C with specific primary antibodies (Table 1). Bound antibody was detected using anti-rabbit IgG peroxidase antibody (Santa Cruz Biotechnology, Inc., CA, USA). Immunoblots were developed with an enhanced chemiluminescence detection system (GE Healthcare) and the exposed films were then scanned at 1200 dpi (scanner HP Officejet J5, 780).

2.10. Quantitative reverse transcriptase PCR (qRT-PCR)

Total RNA from the murine mammary gland sections from Pg dose-response trials and final assays were extracted by Trizol LS reagent (Invitrogen, Life technology, CA, USA) according to the manufacturer's instructions. Quantity and quality of RNA of all samples was determined using a fluoroscopic method (Qubit, Invitrogen, CA, USA) and by visualization on a denaturing agarose gel. RNA samples were treated with DNase I (Invitrogen, Life Technology, CA, USA) in accordance with the manufacturer's instructions. RNA was reverse-transcribed into cDNA as described previously (Baravalle et al., 2015). Absence of genomic DNA was validated running for each sample and gene, non-transcriptional controls (without the reverse transcriptase and with RNA as sample).

Subsequently, relative quantification of gene expression (qPCR) was performed using a StepOne Real Time PCR System (Applied Biosystems, Life Technology, CA, USA). Briefly, each PCR was performed in a total volume of 20 µl containing: 4 µl of cDNA (500 ng/ml), 4 µl of 5 × Phire reaction buffer, 0.5 µl of each 10 µM forward/reverse primers, 0.2 mM dNTPs, 1 µl SYBR Green I (Invitrogen), 0.05 µl Phire Taq polymerase (Thermo Fisher Scientific Company, Finland) and 14 µl of sterilized DEPC treated water.

Sequences of specific primer sets used to amplify genes are listed in Table 2. Optimal conditions for PCR amplification of cDNA were established using routine methods. Each qPCR was performed using the comparative Ct method ($2^{-\Delta\Delta Ct}$) and β-actin as an internal control (Livak and Schmittgen, 2001; Baravalle et al., 2015).

Table 2
Quantitative reverse transcriptase PCR (qRT-PCR) primers. Probe sequences (5′ to 3′).

Molecule	Primer sequence (5′to 3′)	Reference sequence
TLR2[c]	For[a] CAAGTACGAACTGGACTTCTCC Rev[b] CAGGTAGGTCTTGGTGTTCATT	NM_011905
TLR4	For ATTCAGAGCCGTTGGTGTATC Rev CCAGGTAGGTGTTTCTGCTAAG	NM_021297
IL-1α	For CTCTGAGAACCTCTGAAACGTC Rev GAAACTCAGCCGTCTCTTCTT	NM_010554
TNF-α	For GTCGTAGCAAACCACCAAGT Rev TTGAAGAGAACCTGGGAGTAGA	NM_013693
INF-γ	For CAAGTGGCATAGATGTGGAAGA Rev GACGCTTATGTTGTTGCTGATG	NM_010548
IL-10	For ATACTGCTAACCGACTCCTTAAT Rev TCAAATGCTCCTTGATTTCTGG	NM_008337
β-actin	For AACTCCATCATGAAGTGTGA Rev ACTCCTGCTTGCTGATCCAC	NM_007393

[a] Forward (For).
[b] Reverse (Rev).
[c] References: TLR2: toll-like receptor 2; TLR4: toll-like receptor 4; IL-1α: interleukin 1 alpha; TNF-α: Tumor necrosis factor alpha; INFγ: Interferon alpha; IL-10: interleukin 10; β-actin: Beta-actin.

Specificity of the PCR products was verified by DNA sequencing using the Macrogen Sequencing Service (Macrogen, Korea). Oligonucleotide primers and amplification products were then tested using BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

2.11. Statistical analysis

All statistical analyses were performed using a software package (SPSS 11.0 for Windows, SPSS Inc., Chicago, Illinois). The general linear model (GLM) procedure was used for data analysis. The model tested for main effects of treatment (Pg, placebo, and *S. aureus* alone), time (6, 24, 48, 72 and 96 h pi) and the treatment x time interaction. Individual means were compared by Duncan test. The level of significance was set at $P < 0.05$. Results were expressed as mean ± SEM (standard error of the mean).

3. Results

3.1. Analysis of endotoxin levels of Pg extract

The endotoxin levels in Pg extract at 3 mg/ml, 10 mg/ml and 50 mg/ml were lower than the detection limit of the test (< 0.05 ng/ml), indicating that the biological effects of Pg were not due to endotoxin contamination.

3.2. Dose-response Pg trials

3.2.1. Histological examination

Murine mammary gland tissues were harvested at 6, 24, 48 and 72 h after treatment with different Pg doses. Macroscopically, no pathological changes were observed in the mammary gland inoculated with different Pg extract doses and controls at any sampling time. Histological examination of the mammary glands of mice inoculated with 3 and 10 mg/ml of Pg extract showed a slight neutrophil and mononuclear cell infiltration in the intra and interlobular stroma and in the lumen of alveoli and ducts at every sampling time. With a 50 mg/ml Pg extract concentration, at 24 h pi, a moderate infiltration of neutrophils, macrophages and lymphocytes was observed. At 48 h pi a pronounced infiltration of inflammatory cells (neutrophils, eosinophils and mast cells) was observed surrounding the alveoli and ducts. At 72 h pi a massive infiltration of neutrophils, macrophages and eosinophils in the intra and interlobular stroma and in the lumen of alveoli and ducts was observed (Fig. 1A). A large number of lymphocytes and plasma cells were present throughout the parenchyma and stroma. The mammary tissue architecture was conserved at this sampling time indicating a mild inflammatory reaction. In control mice, no histopathological changes were observed (Fig. 1B). The dose of 50 mg/ml of Pg extract was considered appropriate to trigger and adequate inflammatory response to confront an experimental infection.

3.2.2. Cytokine mRNA expression

To investigate the magnitude of inflammatory response induced by intramammary inoculation of 50 mg/ml of Pg extract, we examined the gene expression of IL-1α and TNF-α at different time points compared with placebo-inoculated glands.

The IL-1α mRNA expression level was higher in Pg inoculated glands (50 mg/ml dose) than control glands ($P < 0.001$) and was affected by sampling time ($P = 0.003$). There was a significant interaction between treatment and sampling time ($P = 0.022$), being the mRNA expression level higher in Pg-inoculated glands than control glands at 48 and 72 h pi ($P < 0.05$ and $P < 0.001$, respectively). The highest IL-1α mRNA expression in Pg-inoculated group was observed at 48 h pi (Fig. 1C).

The TNF-α mRNA expression level was higher in Pg-inoculated glands than placebo-inoculated glands ($P < 0.001$) and was affected by sampling time ($P = 0.007$). There was no interaction between treatment and sampling time ($P = 0.088$) (Fig. 1D). The relative expression of TNF-α was higher in Pg-inoculated glands compared with placebo-inoculated glands at every evaluated time, reaching the greatest gene expression at 24 h pi ($P < 0.05$).

The identity of the qRT-PCR products for all evaluated genes was confirmed by sequencing (99% homology with murine sequences).

3.3. Bacterial challenge

A preliminary experiment was designed to evaluate three inoculum doses, 1×10^4, 1×10^5 and 1×10^6 CFU/gland of *S. aureus* that induced mastitis in the mice. No animals died during the experiment for all evaluated doses. With an inoculum of 1×10^6 CFU/gland, growth of *S. aureus* in the mammary glands peaked at 24 h pi. At 48 h a sharp decrease in bacterial load occurred, followed by a new peak at 72 h pi. Mice challenged with 1×10^5 CFU/gland showed an increase of CFU recovered from the mammary glands at 12 and 24 h pi, followed by bacterial clearance at 48 h pi. No bacterial colonization was observed with a 1×10^4 CFU/gland dose (data not shown).

Bacteria in the mammary tissue from mice challenged with 1×10^5 CFU/gland were detected by Gram staining. Gram-stained tissues showed large numbers of bacteria in the lumen of alveoli. Also, bacteria were visualized in mammary epithelial cells and the cytoplasm of neutrophils and macrophages (Fig. 2).

3.4. Recovery of bacteria from the Pg-treated S. aureus-infected mice

Fig. 3 shows the *S. aureus* log CFU recovered from the mammary glands of the three study groups at different time points. Mice treated with Pg plus *S. aureus* demonstrated an enhanced bacterial clearance, compared with placebo-infected mice and mice challenged only with the bacteria ($P < 0.001$), which was affected by sampling time ($P < 0.001$). The interaction between treatment and sampling time was significant ($P < 0.001$). The number of bacteria recovered in Pg-treated *S. aureus*-infected mice (group I) was lower than the number found in placebo-treated *S. aureus*-infected mice (group II) and in *S. aureus*-infected mice (group III) at 6, 48 and 96 h pi ($P < 0.001$; $P < 0.01$ and $P < 0.001$ respectively). These data suggested that the pretreatment of mice with 50 mg/ml of Pg extract enhanced *S. aureus* clearance from infected mammary gland.

3.5. Toll-like receptor 2 and 4 mRNA expression in Pg-treated S. aureus-infected mice

To study whether pretreatment with Pg could modulate the expression of TLR2 and TLR4 in *S. aureus* infected mammary glands, we analyzed the genes profiles of these receptors at different pi times.

The mRNA expression of TLR2 in the mammary tissue was influenced by treatment ($P < 0.001$), but not by sampling time ($P = 0.226$). No interaction between treatment and sampling time was observed ($P = 0.751$). At 48 h pi the transcript levels for TLR2 in the mammary glands of group I were higher than the transcript levels observed in groups II and III ($P = 0.007$) (Fig. 4A).

The mRNA expression for TLR4 in mammary tissue was affected by treatment and sampling time ($P < 0.001$ and $P = 0.002$, respectively). There was interaction between treatment and sampling time ($P < 0.001$). At 48 h pi the transcript levels for TLR4 in group I were higher than the transcript levels observed in group II and III ($P < 0.001$) (Fig. 4B). Although TLR4 mRNA peaked at 72 h pi, no differences were observed between groups ($P > 0.05$).

3.6. Cytokine mRNA expression in Pg-treated S. aureus-infected mice

In order to evaluate whether pretreatment with Pg extract could modulate the expression of pro- and anti-inflammatory cytokine production in *S. aureus*-infected mammary gland we analyzed by qRT-

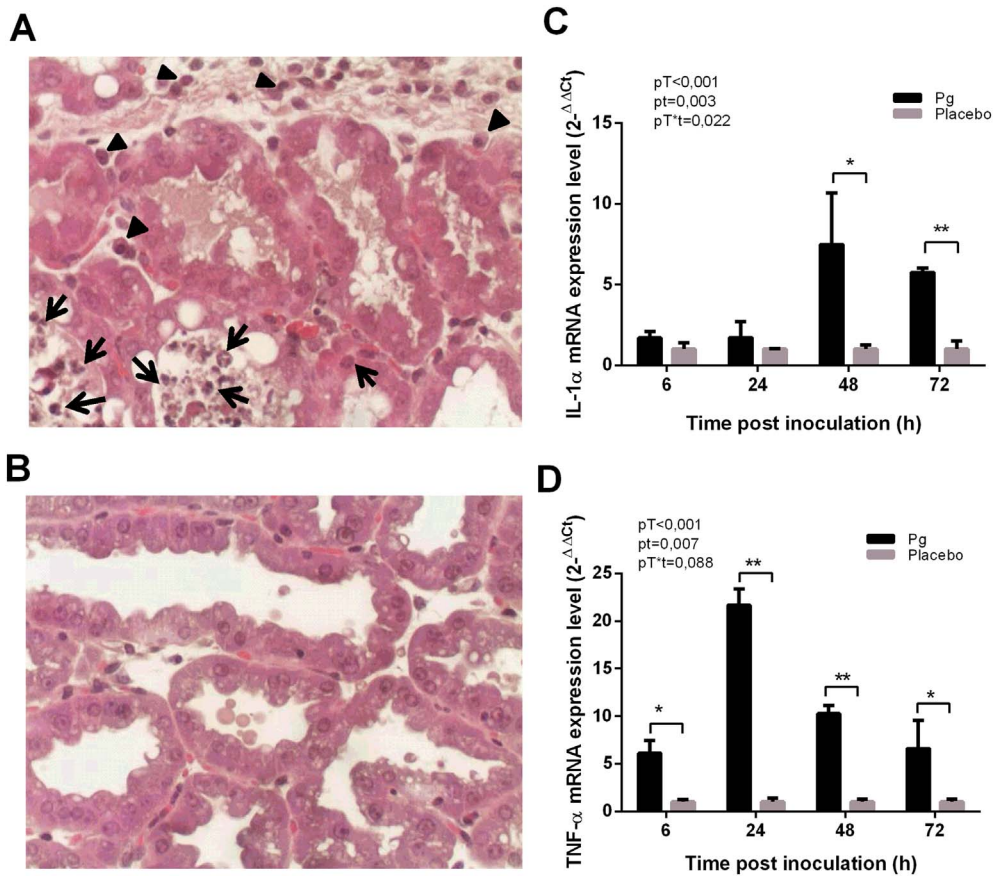

Fig. 1. A) Representative section of mammary tissue exhibiting mild inflammation at 72 h post inoculation with 50 mg/ml of Pg. The black arrows indicate neutrophils and the arrowheads macrophages in the lumen of alveoli and ducts. B) Representative section of mammary tissue at 72 h post inoculation exhibiting no changes after inoculation with placebo. Hematoxylin-eosin stain. Magnification 40 ×. (C) Relative expression of IL-1α mRNA and (D) TNF-α mRNA in mammary gland after inoculation with 50 mg/ml Pg and with placebo at different sampling times. Values are expressed as mean ± standard error of the mean (SEM) (*, $P < 0.05$; **, $P < 0.01$).

PCR the relative expression of IL1-α, TNF-α, INF-γ and IL-10 at different pi times.

We found transcripts for cDNA of IL1-α, TNF-α, INF-γ and IL-10 in mammary tissue from the three groups at every sampling period. However, mRNA relative levels for INF-γ and IL-10 could not be examined in mammary tissue since these samples fall outside of the dynamic linear range.

The IL-1α mRNA expression level in mammary tissue was affected by treatment ($P = 0.002$) but not by sampling time ($P = 0.140$). There was interaction between treatment and sampling time ($P = 0.023$) (Fig. 4C); being the transcripts levels for IL-1α higher in group I than in group II and III at 6 h pi ($P = 0.042$).

The TNF-α mRNA expression level in mammary tissue was affected by treatment ($P < 0.001$) but was not affected by sampling time ($P = 0.240$). There was a significant treatment × sampling time interaction ($P < 0.001$); being the transcript levels for TNF-α higher in group I than in group II and III at 6 and 24 h pi ($P < 0.008$ and $P = 0.002$, respectively). The highest TNF-α mRNA expression in group I was observed at 6 h pi (Fig. 4D).

3.7. NF-κB activity in Pg-treated S. aureus-infected mice

NF-κB-mediated transcription is only possible through translocation of its subunits to the nucleus. In order to evaluate the NF-κB activity in Pg exposed mammary glands following *S. aureus* challenge, mammary tissue was harvested and subjected to IHC technique.

Antibody specificity for IHC was confirmed by Western blot assay by the presence of one unique band for anti-NF-κB-p65 at 65 kDa (Fig. 5D). Activated NF-κB was detected in stroma and parenchyma mammary cells by the nuclear localization of p65 subunit in all evaluated groups (Fig. 5 A, B and C).

The immunoexpression of NF-κB-p65 in parenchyma mammary cells was higher in group I than group II and III ($P < 0.001$) and was affected by sampling time ($P < 0.001$). There was a significant interaction between treatment and sampling time ($P < 0.001$); being the percentages of positive cells for NF-κB-p65 higher in group I than in group II and III at 6, 24, 48 and 72 h ($P < 0.001$) pi (Fig. 5E).

In stroma mammary cells from group I, a significant increase of the immunoexpression of NF-κB-p65 was observed ($P < 0.001$) and was affected by sampling time ($P = 0.002$). There was a significant interaction between treatment and sampling time ($P < 0.001$); being the percentages of positive cells for NF-κB-p65 higher in group I than in group II and III at 6 ($P < 0.001$), 24 ($P < 0.05$) and 48 h ($P < 0.001$) pi (Fig. 5F).

3.8. Monocytes-macrophages recruitment in Pg-treated S. aureus-infected mice

In order to evaluate whether pretreatment with Pg could modulate the monocytes-macrophages recruitment in *S. aureus*-infected mammary glands, we detected and quantified these cells by IHC at different times pi.

Monocytes-macrophages were found within lining epithelial cells and in the lumina of alveoli and ducts. They were large, with pale nuclei and vacuolated cytoplasm and strongly stained at their surfaces. Immunopositive cells were observed infiltrating inter and intra-lobular stromal tissue in the mammary glands from all groups (Fig. 6 A, B and C). Monocytes-macrophages recruitment was affected by treatment

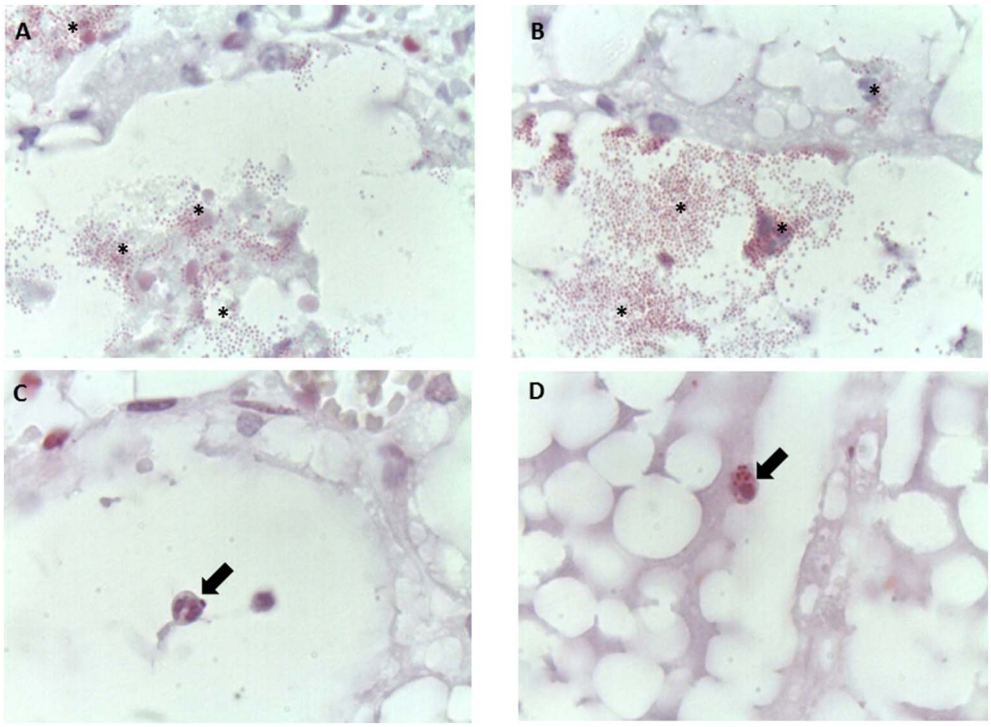

Fig. 2. Gram-stained sections of mammary tissue after challenge with *S. aureus* Newbould 305. (A) and (B) murine mammary gland at 24 h post-infection with 1×10^5 CFU/gland. Bacteria present in the gland are stained in blue. The presence of large numbers of *S. aureus* in the lumen of alveoli are indicated with *. Gram stain: bacteria within neutrophil (C) and macrophage (D) cytoplasm (black arrows). Magnification for all panels is 100 ×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

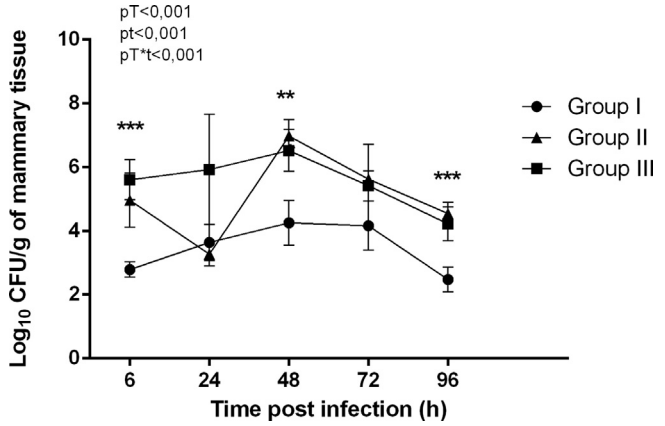

Fig. 3. Infection profiles of *S. aureus* Newbould 305 after intramammary infection in murine mammary gland. The number of recovered bacteria in each sample is presented as the $\log_{10}$ median of CFU/g of mammary tissue^{-1} ± standard error of the mean (SEM) (**, $P < 0.01$; ***, $P < 0.001$). Mice inoculated with Pg prior to *S. aureus* challenge, group I; mice inoculated with placebo prior to *S. aureus* challenge, group II; and mice challenged with *S. aureus*, group III.

($P < 0.001$) and by sampling time ($P < 0.001$). There was a significant treatment x sampling time interaction ($P < 0.001$). The number of monocytes-macrophages was higher in group I compared with group II and III at 6 ($P < 0.001$), 24 ($P < 0.001$) and 48 h ($P < 0.001$) pi (Fig. 6 E).

4. Discussion

Previous studies reported that Pg extract had an immunomodulatory effect on healthy bovine mammary glands (Baravalle et al., 2010, 2011, 2015). In order to further evaluate the potential protective role of this extract, a mouse mastitis model (Brouillette and Malouin, 2005) was used in this study inoculating lactating mice intramammarily with Pg extract or placebo and challenging those glands 72 h later with *S. aureus*. For this study, we chose the mouse mastitis model both to obtain standardized experimental infections post Pg administration and to achieve a large number of repeats per treatment at different time points (6, 24, 48, 72 and 96 h pi), that would have been extremely difficult and expensive conditions to be accomplished in cows.

Based on the histopathological observation, a moderate inflammatory response was induced by Pg extract at 72 h pi in the mammary gland tissue. A massive afflux of inflammatory cells, including neutrophils, monocytes-macrophages, and plasma cells was observed in the lumen of alveoli and ducts and in the stromal tissue previous to *S. aureus* challenge. Besides, a significant difference in the mRNA levels of IL-1α and TNF-α between Pg-treated and control mice were observed previous to infection. These results demonstrate that Pg stimulates innate immunity and could exert an immunomodulatory effect as shown previously in bovine mammary gland at drying off (Baravalle et al., 2010, 2011, 2015).

In the present study, the number of bacteria recovered from the mammary glands was significantly lower in Pg-treated *S. aureus*-infected mice than in placebo-treated *S. aureus*-infected mice and mice treated only with the bacteria, indicating that Pg partially inhibited bacterial multiplication within the mammary gland and can thus play a protective role. These results agree with those reported by Song et al. (1997) where ginseng-treated rats challenged with *Pseudomonas aeruginosa* showed a significantly improved bacterial clearance in lung. In addition, Hu et al. (2001) reported that following subcutaneous injections of ginseng solution a tendency to decrease bacterial growth was observed in bovine mammary quarters naturally infected with *S. aureus*.

Toll-like receptors recognize pathogen molecular patterns and mediate innate antimicrobial immune responses in various systems (Sabroe et al., 2005). These receptors also recognize nonpathogenic molecules like plant derived and stress related proteins such as heat shock proteins and β-defensin (Nakaya et al., 2004). In a previous study carried out in bovine mammary glands, we have demonstrated that

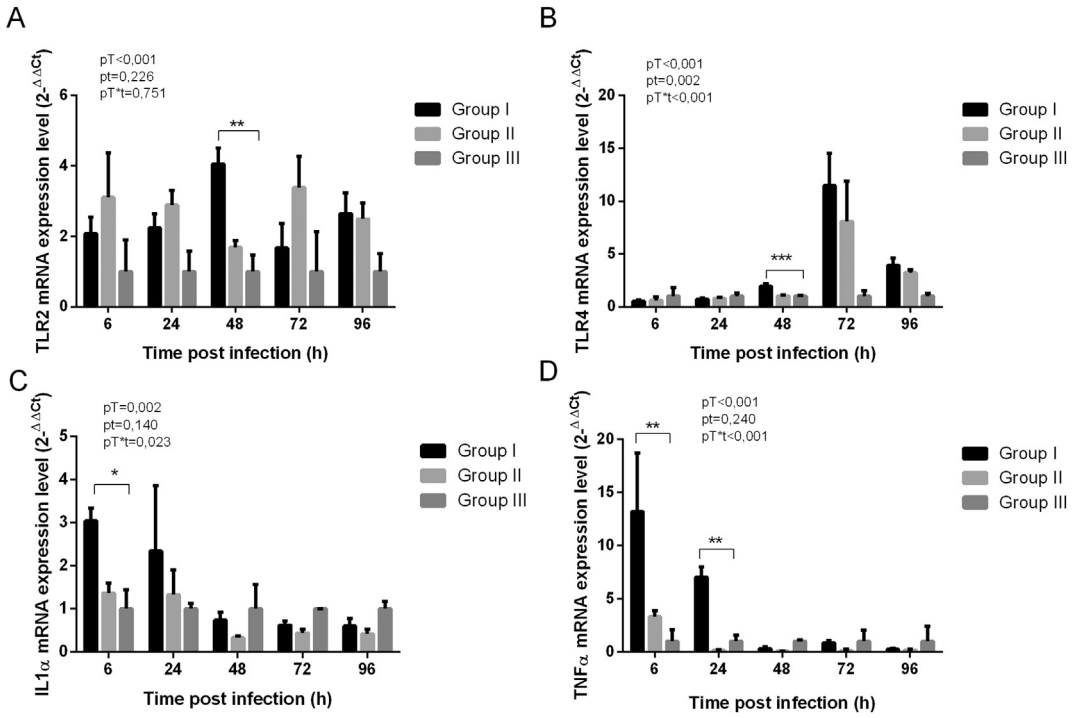

Fig. 4. Relative expression of TLR-2 mRNA (A), TLR-4 mRNA (B), IL-1α mRNA (C) and TNF-α mRNA (D) in mammary glands obtained from mice inoculated with Pg prior to *S. aureus* infection (group I), with placebo prior to *S. aureus* infection (group II) and with *S. aureus* only (group III) at different sampling times. Values are expressed as mean ± standard error of the mean (SEM) (* $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$).

inoculation of Pg extract at drying off significantly increased the expression of TLR2 and TLR4 at mRNA level and protein level in mammary tissue. To further characterize the effect of Pg in infected mice on TLR2 and TLR4 expression, mRNA levels were examined. The results showed that the TLR2 and TLR4 mRNA levels were significantly increased in Pg-treated *S. aureus*-infected mice compared with placebo-treated *S. aureus*-infected mice and mice treated only with the bacteria at 48 h pi. Although discrimination of the effect on TLR2 and TLR4 of previous Pg extract stimulation on group I was beyond the objective of this study, both our previous results in bovine mammary gland as well as the evidence that Pg extract increased the expression of TLR4 in peritoneal macrophages and the production of pro-inflammatory cytokines, like TNF-α and IL-1β, in mice under physical stress (Pannacci et al., 2006), support the role of activation of these receptors by Pg extract treatment on the response modification to *S. aureus* infection.

Upon bacterial recognition, macrophages activate the immune system by release of cytokines and other proinflammatory mediators and facilitate the innate immune response, including neutrophil migration and bactericidal functions (Aitken et al., 2011).The inflammatory responses are regulated by the cytokine network during an infection (Lee et al., 2006), and among them, TNF-α and IL-1β have been reported to play an important role in the inflammatory response during bovine *S. aureus* IMI (Bannerman et al., 2004; Aitken et al., 2011). In the Pg-treated *S. aureus*-infected mice, we found higher mRNA expression levels of IL-1α and TNF-α after the challenge (6–24 h pi) than in placebo-treated *S. aureus*-infected mice and mice treated only with the bacteria. These findings confirm that the inflammatory response was more pronounced in mammary glands treated with Pg and that the increased transcriptional levels of these proinflammatory cytokines was associated with increased *S. aureus* clearance from these glands. Using a rat mastitis model, Wang et al. (2015) found that *S. aureus* inoculation elicited a peak in TNF-α mRNA levels at 48 pi. In contrast, we observed that TNF-α increased sharply after inoculation and peaked at 6 h pi in Pg-treated *S. aureus*-infected mice. Although a different experimental model was used, our results suggest that Pg contributed to an early increase of TNF-α, which has been determined to be delayed in *S. aureus* IMI in bovines and rats (Lee et al., 2006; Wang et al., 2015).

The higher expression of proinflammatory cytokines was associated with a significant increase in the number of monocytes-macrophages infiltrating the mammary tissue in Pg-treated *S. aureus*-infected mice compared with placebo-treated *S. aureus*-infected mice and mice treated only with the bacteria at 6, 24 and 48 h pi. These results agree with those from our previous study showing that the number of monocytes-macrophages was significantly increased in bovine mammary quarters infused with Pg extract compared with placebo and noninoculated control quarters at 7 day of drying off (Baravalle et al., 2011). In the present study, the high number of monocytes-macrophages in mice treated with Pg prior to challenge with *S. aureus* could contribute to enhance innate immune response and accelerate bacterial elimination. In agreement with our findings Shin et al. (2002) found that surface expression of CD14 on murine peritoneal macrophages was increased after treatment with different Pg extract doses.

IL-10 is often associated with suppression or resolution of inflammatory responses (Redpath et al., 2001). Although cDNA transcripts for IL-10 in mammary tissue from the three studied groups was found at all sampling times (up to 96 h pi), levels were too low to be quantified. In contrast, Zhao et al. (2015) found that IL-10 mRNA expression was significantly increased on day 1 and 7 pi in mammary glands from mice challenged with 4×10^6 CFU/gland of *S. aureus*. Discrepancies between studies may rely in the different inoculum concentration used, which was lower in the present study.

IFN-γ is an important mediator for activation of recruited neutrophils and macrophages as well as for enhancement of their phagocytic activity (Riollet et al., 2000; Wedlock et al., 2000). In this study, although the number of monocytes-macrophages was higher in Pg-treated *S. aureus*-infected mice (up to 48 h), the transcript levels for IFN-γ were too low to be quantified in all experimental groups. Alluwaimi et al. (2003) evaluated cytokine transcription in milk cells from bovine mammary glands experimentally infected with *S. aureus* at 7, 24 and 32 h pi. These authors found that IFN-γ transcription continuously declined with time pi. In addition, a report from challenge studies with *Streptococcus agalactiae* in a mouse mastitis model indi-

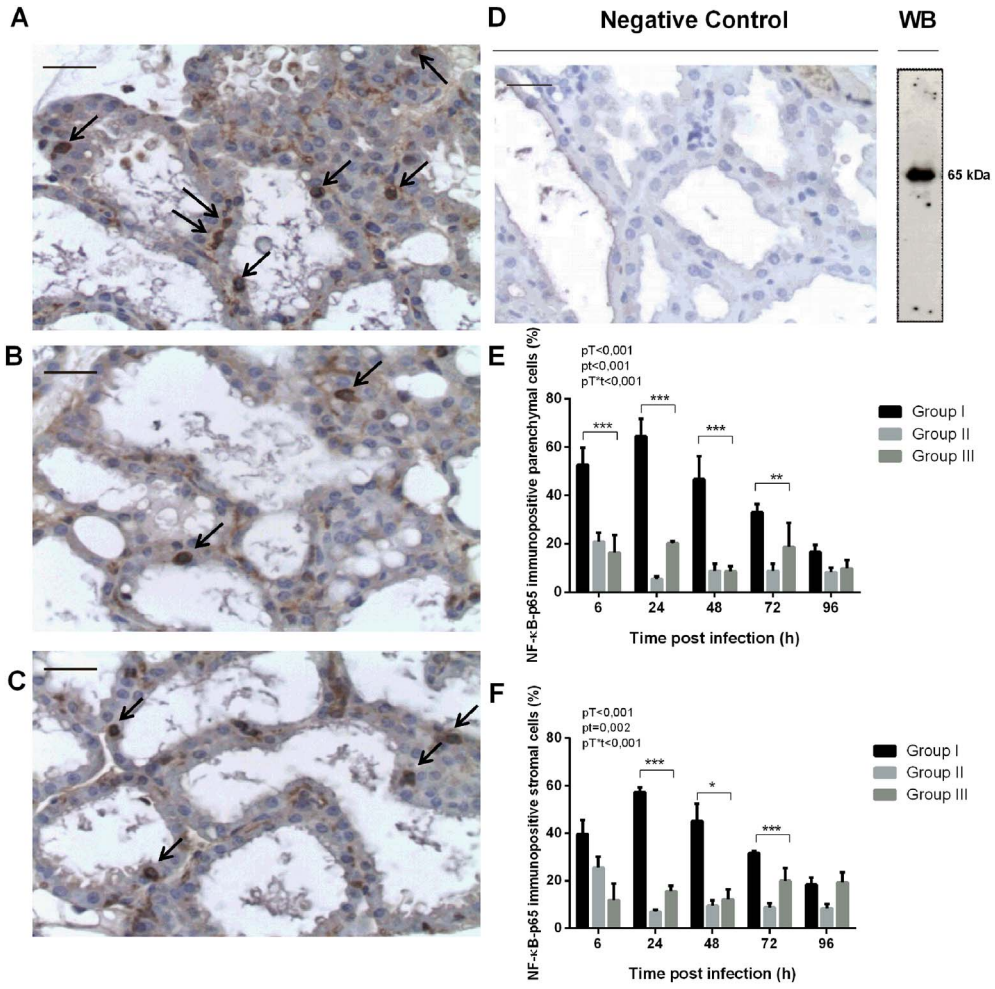

Fig. 5. Representative images of immunohistochemical staining of the NF-κB-p65 subunit following Pg intramammary inoculation prior to *S. aureus* infection (group I) (A), with placebo prior to *S. aureus* infection (group II) (B) and with bacteria only (group III) (C) at 24 h pi. Nuclear immunoreactivity of mammary epithelial and stromal cells to antibody against NF-κB-p65 is indicated by black arrows. Bars, 25 μm. Verification of antibody specificity by Western blot (WB) analysis of mammary gland homogenates and negative controls for immunostaining are shown in (D). Relative expression (measured as % of immunopositive cells) of NF-κB-p65 in parenchyma (E) and stroma (F) of mammary tissue groups I, II and III at different sampling times. Values represent the mean ± standard error of the mean (SEM) (* $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$).

cated that IFN-γ was not detected or was below the level of detection in the infected animals at any point after infection (Trigo et al., 2009). In this study, both Pg extract treatment and *S. aureus* infection were insufficient to stimulate the IFN-γ expression to quantifiable levels.

In a previous study, Yang et al. (2008) demonstrated that both *E. coli* and *S. aureus* bind mammary epithelial TLR2 and TLR4; however, these pathogens modulate NF-κB in a different fashion. Expression of inflammatory mediators is regulated by the NF-κB nuclear translocation (Notebaert et al., 2008). To further assess the mechanism by which Pg promotes cytokine production in *S. aureus*-infected mice, the effects of this extract on the NF-κB activation were examined. Upon phosphorylation, the NF-κB subunit, p65, dissociates from IκB and the inhibitory protein of NF-κB and translocates into the nucleus, where it initiates the transcription of inflammatory cytokines (Li and Verma, 2002; Godowski, 2005). In agreement with a previous report (Breyne et al., 2014) that compared the kinetics of the induced cytokine protein profiles and their underlying pathways following mice mammary gland challenge with *E. coli* and *S. aureus*, in the present study the protein expression of NF-κB-p65 subunit was detected in the nucleus of mammary epithelial and stromal cells and immune cells after *S. aureus* challenge. In this regard, the activation of NF-κB-p65 was significantly increased in the Pg-treated *S. aureus*-infected mammary glands at 6, 24, 48 and 72 h pi for parenchymal tissue and at 24, 48 and 72 h pi for stromal tissue. Simultaneously, an increase of pro-inflammatory cytokines expression was found at 6 and 24 h pi in Pg-treated *S. aureus*-infected. These results demonstrate that Pg extract can increase the dissociation of IκB and NF-κB promoting the subsequent translocation of NF-κB-p65 into the nucleus and the cytokines gene expression.

Taken together, results of this study allow to postulate that Pg extract components, like ginsenosides, may interact with a putative receptor, trigger an intracellular signal transduction cascade resulting in the up-regulation of cytokine genes associated with the mobilization and activation of monocytes-macrophages and polymorphonuclear granulocytes, hence limiting infection. Other studies performed in mice (Nakaya et al., 2004; Pannacci et al., 2006) do indicate the triggering of intracellular signal transduction cascades. Although, the exact receptor that Pg utilizes is currently under investigation; our results support the involvement of TLR2 and TLR4. In this regard, we showed that the protective effect of Pg in this mouse mastitis model was associated with activation of the mRNA expression of TLR2 and TLR4, presence of NF-κB-p65 in mammary cell nuclei and proinflammatory cytokine expression in murine mammary tissue.

In conclusion, our findings using a murine mastitis model, demonstrated that intramammary inoculation of Pg extract prior to *S. aureus* challenge triggered an adequate immune response to confront an infection demonstrating its protective effect. In addition, a putative mechanism by which Pg extract stimulates innate immune response was proposed.

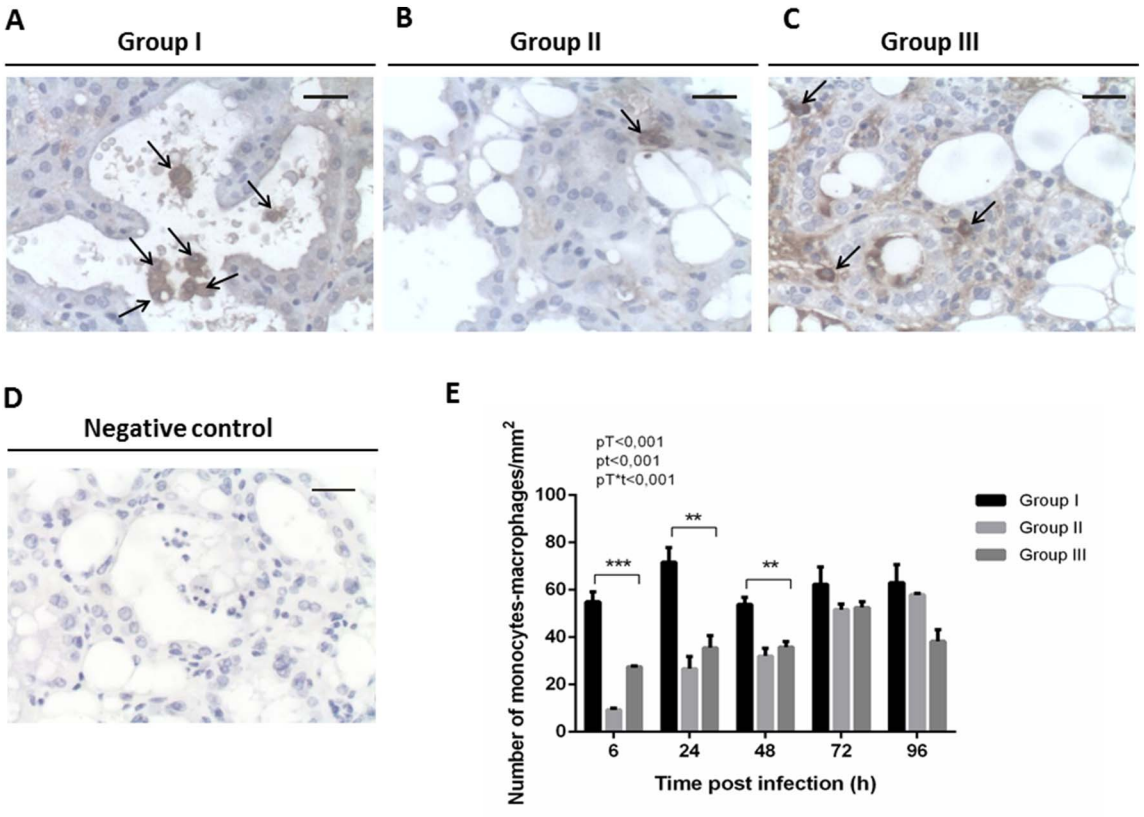

Fig. 6. Representative images of CD14 + cells immunostaining in murine mammary gland following Pg intramammary inoculation prior to *S. aureus* infection (group I) (A), with placebo prior to *S. aureus* infection (group II) (B) and with bacteria only (group III) (C) at 24 h pi. Positive staining is shown as a brown staining of cytoplasm of cells (black arrows). Negative controls for immunostaining are shown in (D). Bars, 25 μm. Number of monocytes/macrophages detected with CD14 antibody in mammary gland from groups I, II and III (E). Values represent mean numbers of monocytes-macrophages/mm^2 across zones within the gland ± standard error of the mean (SEM) (**, P < 0.01; *** P < 0.001). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Acknowledgments

This work was supported by CAI + D (Universidad Nacional del Litoral) (50120110100448LI). We thank Dr. M. Zayas for technical assistance and express our appreciation to Leandro Neme for his assistance in the Center Of Comparative Medicine.

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