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ORIGINAL RESEARCH PAPER

Nasal priming with immunobiotic *Lactobacillus rhamnosus* modulates inflammation–coagulation interactions and reduces influenza virus-associated pulmonary damage

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Abstract

Objective To evaluate the effect of the nasal administration of live and heat-killed *Lactobacillus rhamnosus* CRL1505 (Lr1505) on immune-coagulative response during influenza virus (IFV) infection to improve survival and reduce lung injury.

Methods Six-week-old BALB/c mice were treated with live or heat-killed Lr1505 by the nasal route during two consecutive days. Treated and untreated control mice were then nasally challenged with IFV.

Results Both viable and non-viable Lr1505 protected infected mice by reducing pulmonary injury and lung viral loads trough several mechanisms: (a) Inflammatory cytokines were efficiently regulated allowing higher clearance of virus and reduction of inflammatory lung tissue damage, associated to higher levels of the regulatory cytokine IL-10. (b) The antiviral immune response was enhanced with improved levels of type I interferons, CD4$^+$IFN-γ^+ lymphocytes, and lung CD11c$^+$CD11b^{low}CD103$^+$ and CD11c$^+$CD11b^{high}CD103$^-$ dendritic cells. (c) The procoagulant state was reversed mainly by downregulating tissue factor expression and restoring thrombomodulin levels in lung. The capacity of Lr1505 to improve the outcome of IFV infection would be related to its ability to beneficially modulate lung TLR3-triggered immune response.

Conclusions Our work is the first to demonstrate the ability of an immunobiotic strain to beneficially modulate inflammation–coagulation interactions during IFV infection. Interestingly, non-viable *L. rhamnosus* CRL1505 was as effective as the viable strain to beneficially modulate respiratory antiviral immune response.

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Abbreviations

APTT	Activated partial thromboplastin time
BAL	Bronchoalveolar lavage
DCs	Dendritic cells
ELISA	Enzyme-linked immunosorbent assay
IFV	Influenza virus
IL	Interleukin

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IFN	Interferon
LAB	Lactic acid bacteria
LDH	Lactate dehydrogenase
Lr1505	*Lactobacillus rhamnosus* CRL1505
HK-Lr1505	Heat-killed Lr1505
MDCK	Madin–Darby canine kidney cells
MCP	Monocyte chemotactic protein
MRS	Man–Rogosa–Sharpe
PAI	Plasminogen activator inhibitor
PT	Prothrombin time
RSV	Respiratory syncytial virus
TATc	Thrombin–antithrombin complexes
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor (),
TM	Thrombomodulin
TNF	Tumor necrosis factor
vWF	von Willebrand factor

Introduction

Influenza virus (IFV) is a highly infectious (−)ssRNA virus of the *Orthomyxoviridae* family, which causes mild to severe respiratory illness. For most individuals, the flu is self-limiting, but in susceptible populations, including individuals with pre-existing pulmonary or cardiac conditions as wells as infants and the elderly, fatal complications such as severe pneumonia may arise. Viral induced pneumonia results from a combination of factors derived from both the extensive viral replication in tissues of the respiratory tract and the antiviral host immune response [1]. The general progression to viral pneumonia starts with viral replication in lung epithelial cells and the alveolar epithelium. When epithelial cells recognize a viral particle, pro-inflammatory cytokines and chemokines are induced and released to the extracellular milleu [2]. Circulating leukocytes are recruited to the lungs and activated in response to chemokines and cytokines. As a result, there is an infiltration of mononuclear cells, which together with the resident alveolar macrophages lead to the overproduction of pro-inflammatory cytokines that contributes to lung tissue injury and alterations of the function of the lungs [3]. Thus, balancing the generation of immune responses capable of controlling virus replication with those causing immunopathology is critical for the survival of the host and resolution of influenza-induced inflammation.

Recent evidence also suggests that endothelial cell activation, loss of barrier function, and consequent microvascular leak may also serve important mechanistic roles in the pathogenesis of severe influenza pneumonia [4]. IFV may exert both direct and indirect effects on lung endothelium by activating it and inducing microvascular leak. In this regard, acute respiratory tract infections have been associated with an increased risk of acute ischemic heart disease, stroke and venous thromboembolism [5, 6]. A transient change in local hemodynamic factors, coagulation activation, reduced generation of anticoagulant activated protein C, inhibition of fibrinolysis and endothelial cell perturbation as a result of systemic inflammation might be some of the underlying mechanisms [7]. Indeed, it has been shown that IFV is able to activate coagulation, causing a reduction in clotting time, and increasing the expression of tissue factor (TF) and thrombin generation, the latter by reduced levels of protein C. Moreover, it was showed that these hemostatic changes are risk factors for thrombotic diseases during respiratory infections [8, 9].

Overall, these data suggest that the exacerbated disease due to immune- and coagulation-mediated pulmonary injury during acute respiratory IFV infections results in severe morbidity and mortality. Then, identifying novel approaches to modulate IFV-induced inflammation–coagulation interactions could be important alternatives for preventing or treating acute influenza infections.

Certain probiotic lactic acid bacteria (LAB) strains, termed immunobiotics, can exert their beneficial effect on the host through their immunomodulatory activity [10]. We recently reported that viable *Lactobacillus rhamnosus* CRL1505 (Lr1505) in contrast to *L. rhamnosus* CRL1506 administered orally, was able to protect mice from respiratory syncytial virus (RSV) and IFV challenges [11]. We showed that the early induction of pro-inflammatory cytokines accompanied by an increase in antiviral interferon (IFN)-γ, were responsible for the increased survival to both respiratory viruses tested. In addition, we unraveled the key role of enhanced interleukin (IL)-10 production in resolving inflammation and limiting lung injury, which was strain-specific. This was the first report showing that an orally administered LAB strain modulated the coagulation state triggered by viral infections [11].

There is also evidence that some nasally administered LAB do have the ability to stimulate respiratory immunity and increase resistance to viral infections, including IFV, RSV and murine pneumonia virus [10]. Moreover, during the last decade, there has been a significant progress in the knowledge of the cellular and molecular mechanisms involved in the protective effect induced by immunobiotics in the respiratory tract. It has been demonstrated that nasally administered probiotics are able to increase NK cells and macrophages activities, modulate type I IFN and IFN-γ production and, antigen presenting cells functions in lungs infected with viruses, allowing an improved immune response and a higher resistance to infection [10]. In addition, other studies showed that it is possible to achieve these immunoregulatory effects with non-viable immunobiotics

[12–14]. Therefore, the aim of the present work was to evaluate whether the nasal administration of live and heat-killed immunobiotic strain *L. rhamnosus* CRL1505 were able to beneficially modulate the immune-coagulative response during IFV infection to improve survival and reduce lung injury.

Materials and methods

Microorganisms

Lr1505 was obtained from the CERELA culture collection. It was kept freeze-dried and then rehydrated using a standard medium [15]. For experiments, it was cultured for 12 h at 37 °C (final log phase) in Man–Rogosa–Sharpe broth (MRS, Oxoid). Bacteria suspensions were prepared by harvesting them by centrifugation at $3000 \times g$ for 10 min, washing three times with sterile 0.01 mol/l phosphate buffer saline (PBS), pH 7.2.

Heat-killed Lr1505 (HK-Lr1505) was prepared by tyndallization in a water bath at 80 °C for 30 min and the lack of bacterial growth was confirmed using MRS agar plates.

Animals and treatments with lactobacilli

All experiments were approved by the Ethical Committee for Animal Care of Reference Centre for Lactobacilli (CERELA-CONICET, Tucuman, Argentina). Endpoints were used in survival experiments to euthanize animals. IFV infection in this animal model induces mortality between days 4 and 9. In that period of time, animals were checked for sings of suffering and euthanized if considered appropriate, before the end of experiments on day 14. Signs of pain, suffering, and especially moribund conditions were used for the decision to euthanize mice.

Six-week-old BALB/c male mice were obtained from a closed colony kept at CERELA. They were housed in plastic cages at room temperature. Parameters were studied in 5–6 mice per group for every time point. Groups were housed separately according the received treatment. Lr1505 or HKLr1505 was administered for two consecutive days at a final dose of 10^8/mouse/day inoculated via nostrils. All groups were fed a conventional balanced diet ad libitum.

Intranasal administration of poly(I:C)

Administration of the viral pathogen molecular pattern poly(I:C) was performed on day 3, after the 2 days treatments with lactobacilli. Mice were lightly anesthetized and 100 µl of PBS, containing 250 µg poly(I:C) (equivalent to 10 mg/kg body weight), was administered dropwise, via the nares. Control animals received 100 µl of PBS. Mice received three doses of poly(I:C) or PBS with 24 h rest period between each administration.

Virus and infection

IFV A/PR/8/34 (H1N1) infection was performed as described previously (10). Briefly, IFV was propagated in Madin–Darby canine kidney (MDCK) cells, and virus titers in the stock solution were determined by a plaque assay [16]. MDCK cells were grown and maintained in Eagle's minimum essential medium supplemented with 2 and 5 % heat-inactivated fetal bovine serum, respectively. Infection was performed on day 3, after the 2 days treatments with lactobacilli. Mice were intranasally infected or mock-infected with 500 PFU of the A/PR/8/34 strain in 25 µl of PBS.

Tissue and fluids sampling

Blood samples were obtained through cardiac puncture. Bronchoalveolar lavage (BAL) samples were obtained as described previously [17]. Briefly, the trachea was exposed and intubated with a catheter, and two sequential BAL were performed in each mouse by injecting sterile PBS; the recovered fluid was centrifuged for 10 min at $900 \times g$; smears were done with the cellular pellet and stained for determining cell counts. The supernatant fluids were frozen at -70 °C for subsequent enzymatic, cytokines and chemokines analyses.

Cytokines and chemokines analysis

IFN-γ, IFN-β, TNF-α, IL-8, IL-6, MCP-1, and IL-10 in serum and BAL were determined with commercially available enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's instructions (R&D Systems, MN, USA).

Determination of cell populations

Total number of leukocytes in blood and BAL were determined using a hemocytometer. Differential cell counts in blood and BAL were obtained by microscopically counting cells in smears stained with May Grünwald-Giemsa as described before [17].

Lung injury parameters

Protein content was measured by the bicinchoninic protein assay (Pierce Biotechnology Inc., Rockford, IL, USA). Albumin content was determined colorimetrically based on albumin binding to bromocresol green using an albumin diagnostic kit (Wiener Lab, Buenos Aires, Argentina).

LDH activity, expressed as units per liter of BAL fluid, was determined by measuring the formation of the reduced form of nicotinamide adenine dinucleotide using the Wiener reagents and procedures (Wiener Lab). The lung wet:dry weight ratio was measured as previously obtained and described by Aeffner et al. [18]. Briefly, mice were euthanized and exsanguinated, and their lungs removed, weighed, and dried in an oven at 55 °C for 7 days. After drying, the lungs were weighed again. Wet:dry weight ratio was then calculated as an index of intrapulmonary fluid accumulation, without correction for blood content.

Whole-lung samples from all experimental groups were excised and washed out with PBS. Then, tissues were immersed in 4 % (v/v) formalin saline solution. Once fixed, samples were dehydrated and embedded in Histowax (Leica Microsystems Nussloch GmbH, Nussloch, Germany) at 56 °C. Finally, lungs were cut into 4 μm serial sections and stained with hematoxylin–eosin for light microscopy examination. All slides were coded and evaluated blindly.

Flow cytometry

Cell suspensions obtained from lung were incubated with anti-mouse CD32/CD16 monoclonal antibody (Fc block) for 15 min at 4 °C. Then, cells were incubated with the respective antibody mixes (anti-mouse CD3-FITC, anti-mouse CD4-PE, anti-mouse CD8-PE, anti-mouse IFN-γ-APC, anti-mouse CD11b-FITC, anti-mouse CD11c-PE, anti-mouse IFN-γ-PE, anti-mouse MHC-II-PE, anti-mouse IL-10-PE and anti-mouse CD103-biotin, BD PharMingen) for further 30 min at 4 °C and washed with FACS buffer. After staining, cells were acquired on a BD FACSCalibur™ flow cytometer (BD Biosciences) and the data were analyzed with FlowJo software (TreeStar). The total number of cells in each population was determined by multiplying the percentages of subsets within a series of marker negative or positive gates by the total cell number determined for each tissue.

Haemostatic tests

Coagulation tests

Blood samples were collected by cardiac puncture in a 3.2 % sodium citrate (ratio 9:1). Plasma was obtained according to Agüero et al. [19]. Prothrombin time (PT) and APTT were performed manually on fresh plasma samples. PT was determined to evaluate the extrinsic coagulation pathway; it was determined by a one-step method (Thromborel S, Behningwerke AG, Marburg, Germany). Results are expressed as percentage of prothrombin activity (%) from a calibration curve made from a pool of fresh

plasma from normal mice [20]. APTT was determined to evaluate the intrinsic pathway of coagulation. APTT was determined by mixing plasma with calcium chloride and a partial thromboplastin reagent (STA APTT Reagent, Stago, Asnières, France), and timing initial clot formation. Results are expressed in seconds [20]. TATc (markers of coagulation system activation) were measured in BAL and plasma samples by the ELISA technique, according to the manufacturer's instructions (TAT Complexes Mouse ELISA Kit, Abcam Inc., UK).

Platelet counts

Blood samples were obtained as described before and collected in tubes containing EDTA as an anticoagulant. Manual platelet counting was performed by visual examination of diluted whole blood with 1 % (w/v) aqueous ammonium oxalate [20].

Determination of vWF in plasma and BAL

vWF was measured in plasma and BAL samples by ELISA as described before [11].

Quantitative expression analysis by real-time PCR

Two-step real-time quantitative PCR was performed to characterize the expression of TF, TFPI, PAI-1, and TM mRNAs in lung. Total RNA was isolated from each sample using TRIzol reagent (Invitrogen). All cDNAs were synthesized using a Quantitect reverse transcription (RT) kit (Qiagen, Tokyo, Japan) according to the manufacturer's recommendations. Real-time quantitative PCR was carried out using a 7300 real-time PCR system (Applied Biosystems, Warrington, United Kingdom) and the Platinum SYBR green qPCR SuperMix uracil-DNA glycosylase (UDG) with 6-carboxyl-X-rhodamine (ROX) (Invitrogen). The following primers were used: TF (sense: 5′-CAA TGA ATT CTC GAT TGA TGT GG-3′; antisense: 5′-GGA GGA TGA TAA AGA TGG TGG C-3′); TFPI (sense: 5′-ACT GTG TGT CTG TTG CTT AGC C-3′; antisense: 5′-GTT CTC GTT CCC TTC ACA TCC C-3′); PAI-1 (sense: 5′-AGG TCA GGA TCG AGG TAA ACG AG-3′; antisense: 5′-GGA TCG GTC TAT AAC CAT CTC CGT-3′); TM (sense: 5′-AGT GTG CCA GTT CAT AAG AAT C-3′; antisense: 5′-AGT GTG CCA GTT CAT AAG AAT C-3′). The PCR cycling conditions were 2 min at 50 °C, followed by 2 min at 95 °C, and then 40 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. The reaction mixtures contained 5 μl of sample cDNA and 15 μl of master mix, which included the sense and antisense primers. Expression of β-actin was used to normalize cDNA levels for differences in total cDNA levels in the samples.

Statistical analysis

Experiments were done in triplicate and results were expressed as mean ± standard deviation (SD). Data were normally distributed, and therefore, a 2-way ANOVA could be used. Tukey's test (for pairwise comparisons of the means) was used to test for differences among groups. Differences were considered significant at $P < 0.05$.

Results

Live and heat-killed *L. rhamnosus* CRL1505 increase survival of mice infected with H1N1 IFV

Fifty percent of the control mice infected with 500 PFU of H1N1 IFV strain A/PR/8/34 died already at day 4 post-infection (Fig. 1). Only 30 % of the control mice survived up to day 11 and this percentage remained unchanged until day 14 post-infection (Fig. 1). The nasal administration of Lr1505 and HK-Lr1505 *L. rhamnosus* CRL1505 significantly increased survival to 70 and 60 %, respectively, by the end of the second week. The protective effect of the immunobiotic strain can already be seen on day 4 post-infection with survival rates increased up to 80 and 70 % for infected mice previously treated with live Lr1505 or HK-Lr1505, respectively (Fig. 1). To investigate whether the increased survival related to a better control of virus replication, lungs from control and lactobacilli-treated mice were recovered and virus titers at five time-points post-infection was assessed. The highest titers were observed in control mice between days 3 and 4. Furthermore, this increase in survival rates inversely correlated with a reduction in the IFV virus titers during the first

Fig. 1 Effect of lactobacilli on the survival and the lung damage induced by influenza virus infection. Effect of live *Lactobacillus rhamnosus* CRL1505 (Lr1505) and heat-killed Lr1505 (HK-Lr1505) nasal treatments on survival, virus titers in lungs, albumin, lactate dehydrogenase (LDH), and protein concentration in bronchoalveolar lavages (BAL), and lung wet:dry weight ratio. The results represent data from three independent experiments. Results are expressed as mean ± SD. *Different letters* indicate significant differences ($P < 0.05$)
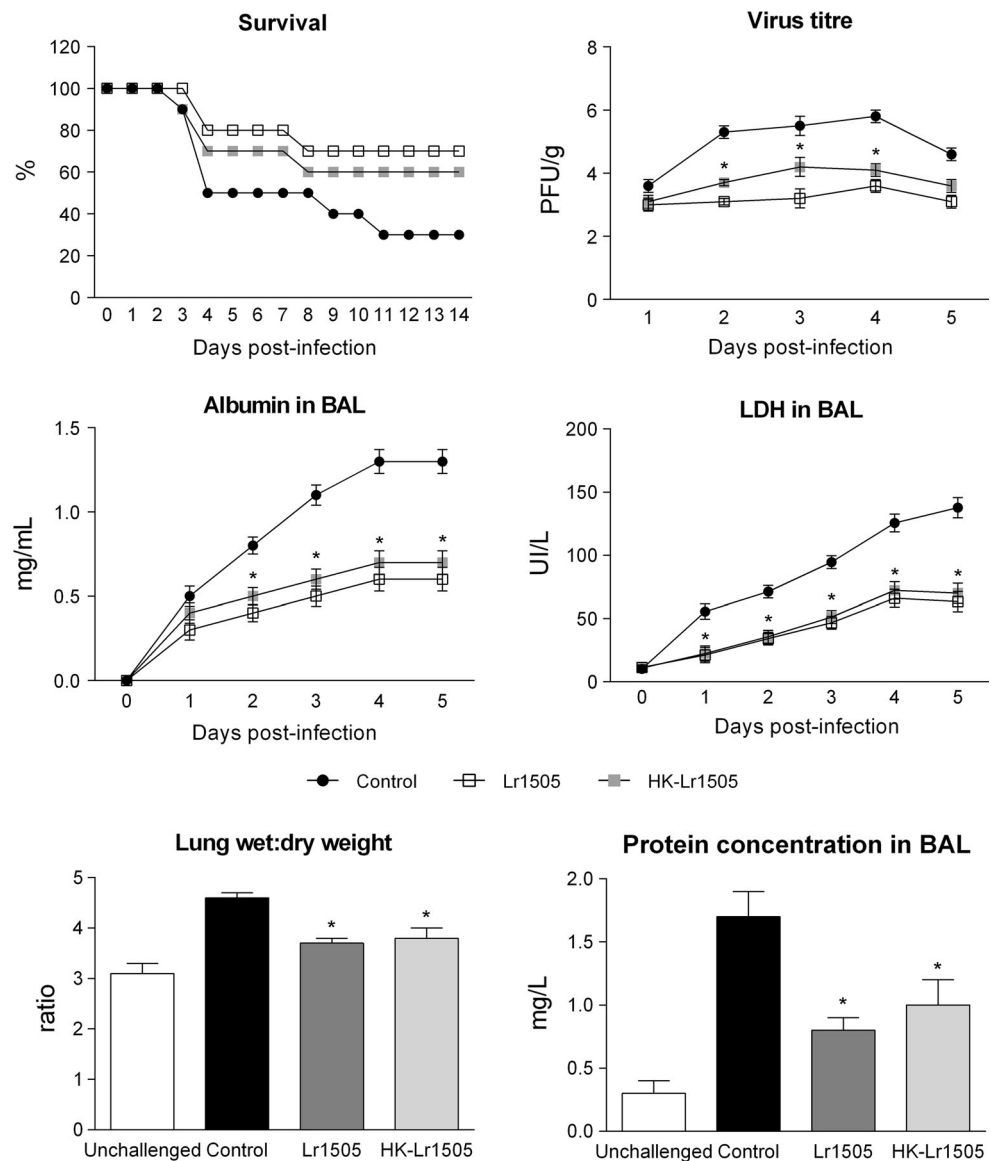
5 days of infection (Fig. 1). Thus, a significant 2-log virus titer reduction was observed already on day 2 and continued on time until day 4 in mice treated with live Lr1505 (Fig. 1). A similar but smoother effect was also induced by HK-Lr1505 (Fig. 1).
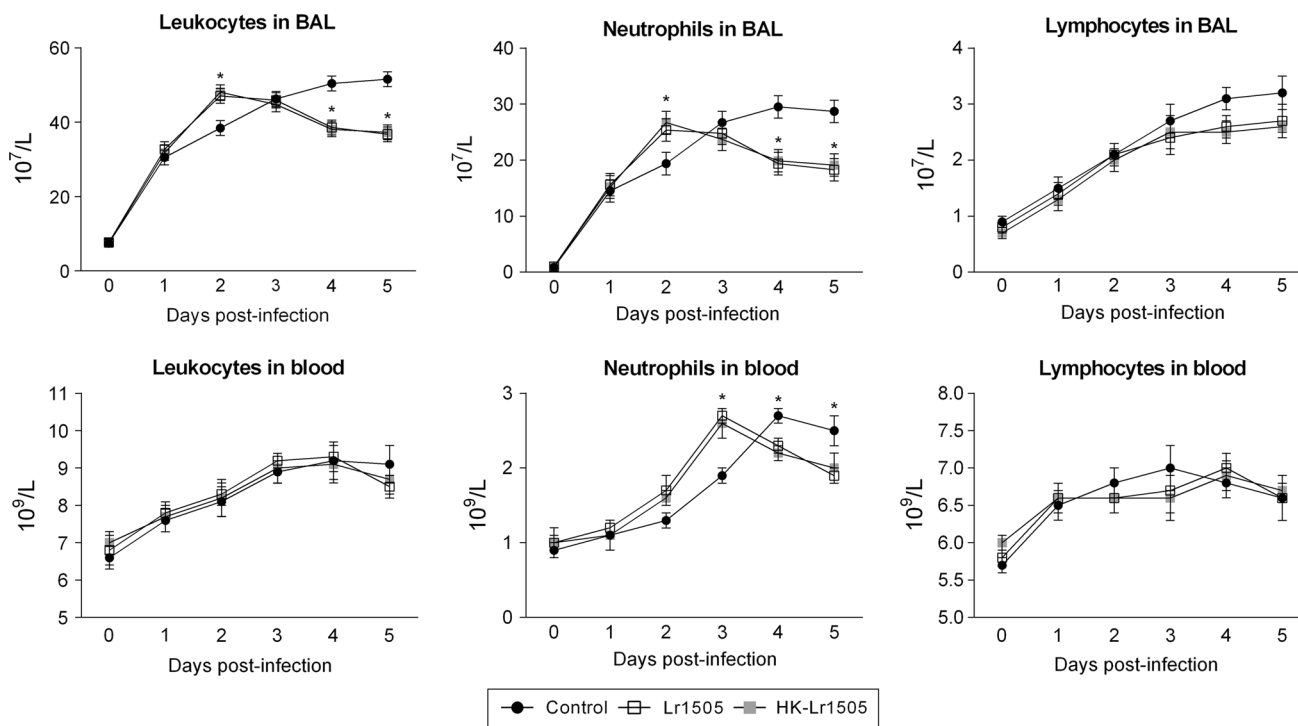
Live and heat-killed *L. rhamnosus* CRL1505 diminish acute lung injury

Four approaches were chosen to evaluate pulmonary damage, i.e.: albumin, lactate dehydrogenase (LDH) and total protein concentrations in BAL as well as the lung wet to dry weight ratio (Fig. 1). When cell lysis or membrane disruptions occur, cytoplasmatic enzymes such as LDH are released to the extracellular alveolar space and can be measured to determine the degree of cytotoxicity. On the other hand, albumin and total protein reflect an increased alteration of the alveolar capillary barrier. Last, the lung wet:dry weight ratio represents a more general parameter of augmented permeability and pulmonary edema as it is an estimate of the total water content of the lung. In general, IFV infection resulted in cellular damage and increased permeability of lung and vascular epithelia (Fig. 1). Strikingly, both Lr1505 and HK-Lr1505 significantly reduced pulmonary cell damage and edema as assessed by

all four parameters studied (Fig. 1). Both treatments resulted in similar effects, with a slightly stronger protective effect for live Lr1505.

Live and heat-killed *L. rhamnosus* CRL1505 reduce pulmonary inflammation during IFV infection

IFV causes an acute inflammatory response in the first 5 days post-infection in mice, which is characterized by an increased influx of leukocytes into the lung. The quantification of leukocytes in BAL clearly shows that there is a migration from blood to lung airspaces, represented by a ~fourfold increase in total leukocyte counts in BAL (Fig. 2) as observed before in IFV mouse infection models. Within this study, we report for the first time, a modulation of the leukocyte efflux by nasal priming with Lr1505 and HK-Lr1505 before IFV infection. It has been reported that the main population responsible for the efflux to the airspaces of the lung in IFV infections are neutrophils. The behaviors of the curves depicted in Fig. 2 indicate, that in our hands, the main component of the efflux are also neutrophils, and that the main effect of Lr1505 is on the kinetics and total counts of this cell population. Thus, in Lr1505- and HK-Lr1505-treated mice, neutrophils highest counts were

Fig. 2 Effect of lactobacilli on leukocytes in broncho-alveolar lavages and blood during influenza virus infection. Effect of live *Lactobacillus rhamnosus* CRL1505 (Lr1505) and heat-killed Lr1505 (HK-Lr1505) nasal treatments on the number of leukocytes, neutrophils, and lymphocytes in broncho-alveolar lavages (BAL) and blood. The results represent data from three independent experiments. Results are expressed as mean ± SD. *Different letters* indicate significant differences ($P < 0.05$)

reached on day 3 post-infection, 24 h earlier than in control mice. In addition, treatments reduced $\sim$twofold the leukocyte counts in BAL (Fig. 2), $\sim$1.5-fold the neutrophils counts (Fig. 2) and $\sim$0.5-fold the lymphocytes counts (Fig. 2) by day 4 post-infection. A similar effect was observed when analyzing blood neutrophils, while Lr1505 treatments did not result in significant differences regarding blood lymphocytes and total number of leukocytes in blood (Fig. 2).

Systemic and pulmonary chemokines and cytokines of mice infected with H1N1 IFV are modulated by *L. rhamnosus* CRL 1505

In previous studies using poly(I:C) (a dsRNA analog) as model for RNA virus infections, we reported the modulation of pro-inflammatory and regulatory cytokines, as well as certain IFN molecules by administration of Lr1505 [21]. In this study, we use a similar approach to evaluate the actual effect of Lr1505 in the context of an IFV infection. As expected, there is a correlation between the levels of pro-inflammatory cells, lung injury, and chemokines and cytokines levels. Thus, key pro-inflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α were induced by IFV infection as described before [22], and their levels in blood and BAL were higher during the first 48 h post-infection in mice nasally primed with Lr1505 or HK-Lr1505 than in control mice (Fig. 3a, b). In all groups, there was a rise in IL-8, IL-6, and monocyte chemotactic protein (MCP)-1 levels in BAL after infection, which reached a maximum on day 3. The kinetics of TNF-α, IL-8 and MCP-1 followed a similar pattern in BAL for both live and killed Lr1505 treated mice; after day 3, the cytokines levels were gradually down-regulated. In contrast, these cytokines remained high in the control group. In the case of IL-6, there was a state increment in BAL levels which continued upon day 3 in control mice, whereas in HK-Lr1505, it was less pronounced and in live Lr1505 treated mice, IL-6 reached a plateau at day 3 (Fig. 3). In serum, there were no significant differences in the level of these molecules between treated mice and the control group.

IL-10 is an important regulatory cytokines, whose levels augment gradually upon IFV infection, however, treatment with HK-Lr1505 and Lr1505 resulted in higher IL-10 levels in BAL and serum (Fig. 3a, b).

In addition, IFN-β and IFN-γ are crucial for antiviral response and may be modulated by certain lactobacilli strains such as Lr1505. Already at 24 h post-infection, both IFN-β and IFN-γ were significantly higher in BAL and serum of mice nasally primed with Lr1505 and HK-1505, being the effect of live bacteria significantly stronger than that of non-viable bacteria (Fig. 3a, b).

L. rhamnosus CRL1505 modulate lung immune cells in H1N1 IFV-infected mice

In lungs, two populations of dendritic cells (DCs) can be defined using CD11c, CD11b, CD103 and MHC-II antibodies: $CD11c^+CD11b^{low}CD103^+$ and $CD11c^+CD11b^{high}CD103^-$ cells. Twelve hours post-infection, these populations of antigen presenting cells were analyzed. Both $CD11c^+CD103^+CD11b^{low}$ and $CD11c^+CD103^+CD11b^{high}$ were significantly increased in Lr1505 and HK-Lr1505 groups when compared to controls (Fig. 4). Furthermore, the expression of MHC-II in both antigen presenting cells populations was also up-regulated in *L. rhamnosus* CRL1505 treated mice.

When analyzing further, the cellular response of mice infected with H1N1 IFV, we observed that administration of lactobacilli, either live or heat-killed, significantly increased the number of effector $CD3^+CD4^+IFN\gamma^+$ and regulatory $CD3^+CD4^+IL-10^+$ lymphocytes, whereas $CD3^+CD8^+IFN\gamma^+$ cells in infected mice pre-treated with lactobacilli did not suffer modifications when compared to the control group (Fig. 5).

Pro-coagulant state in the context of H1N1 IFV infection is modulated by *L. rhamnosus* CRL1505

To evaluate the local activation and regulation of coagulation cascades, we measured several parameters such as thrombin–antithrombin complexes (TATc) in BAL and TF, tissue factor pathway inhibitor (TFPI), plasminogen activator inhibitor (PAI)-1, and thrombomodulin (TM) expressions in lung tissue. In infected mice, coagulation was notably activated as reflected by the high values of TATc, which is a marker of the activation of coagulation. In addition, a higher ratio of TF and lower ratio of TM are detected in lungs (Fig. 6). In contrast, these values were significantly reduced and increased, respectively, when mice were previously treated with lactobacilli. A slightly more pronounced difference was seen when live Lr1505 was administered. The coagulation regulatory molecule TFPI and the fibrinolytic inhibitor PAI-1 reached similar values in lactobacilli treated and untreated mice infected with IFV (Fig. 6).

Lung inflammation and injury induced by poly(I:C) resembles IFV infection and is modulated by *L. rhamnosus* CRL1505

During replication, IFV generates dsRNA intermediates, which activate the innate immune system via TLR3. Therefore, we next aimed to test whether *L. rhamnosus* CRL1505 modulated lung inflammation triggered by poly(I:C) administration. We and others proved that the
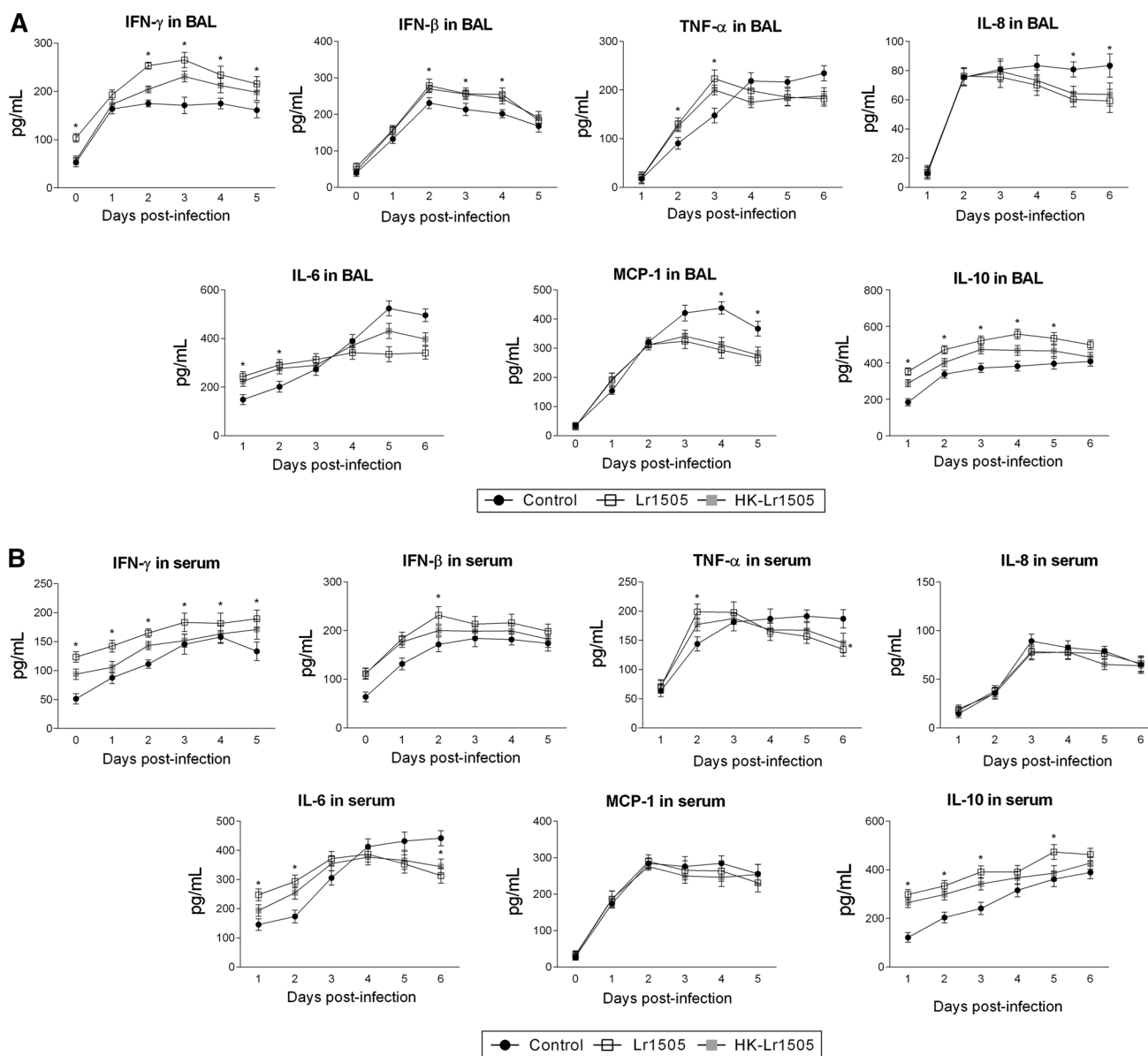
Fig. 3 Effect of lactobacilli on cytokines and chemokines in broncho-alveolar lavages and serum during influenza virus infection. Effect of live *Lactobacillus rhamnosus* CRL1505 (Lr1505) and heat-killed Lr1505 (HK-Lr1505) nasal treatments on interferon (IFN)-γ, IFN-β, tumor necrosis factor (TNF)-α, interleukin (IL)-8, IL-6, monocyte chemoattractant protein (MCP)-1, and IL-10 concentrations in broncho-alveolar lavages (BAL) and serum. The results represent data from three independent experiments. Results are expressed as mean ± SD. *Different letters* indicate significant differences ($P < 0.05$)

pathogenic mechanisms triggered by poly(I:C) resemble in many aspects the immune pathogenic components observed in dsRNA virus infections [18, 23] as it was the case of the IFV mouse infection model of this study. Indeed, Lr1505 and HK-Lr1505 were able to reduce both lung inflammation and injury to a similar extent as reflected by albumin, LDH, and protein contents in BAL, and lung wet/dry weight ratio (Fig. 7). Even though Lr1505 and HK-Lr1505 increased the levels of TNF-α at 12 h after

challenge, they also up-modulated the anti-inflammatory cytokine IL-10, as observed in day 3 of IFV-infected mice (Fig. 7).

As we demonstrated previously [11], poly(I:C) induced a clear tissue inflammation around alveoli and blood vessels in lung, with a significant reduction of gas exchange space in some regions of lungs (Fig. 7). Lr1505 and HK-Lr1505 treatments significantly reduced inflammation and lung tissue alterations (Fig. 7).

Fig. 4 Effect of lactobacilli on lung dendritic cells during influenza virus infection. Effect of live *Lactobacillus rhamnosus* CRL1505 (Lr1505) and heat-killed Lr1505 (HK-Lr1505) nasal treatments on CD11c$^+$CD103$^+$CD11b^{low}, and CD11c$^+$CD103$^-$CD11b^{high} dendritic cells from lung, as well as their expression of MHC-II. The results represent data from three independent experiments. Results are expressed as mean ± SD. *Different letters* indicate significant differences ($P < 0.05$)
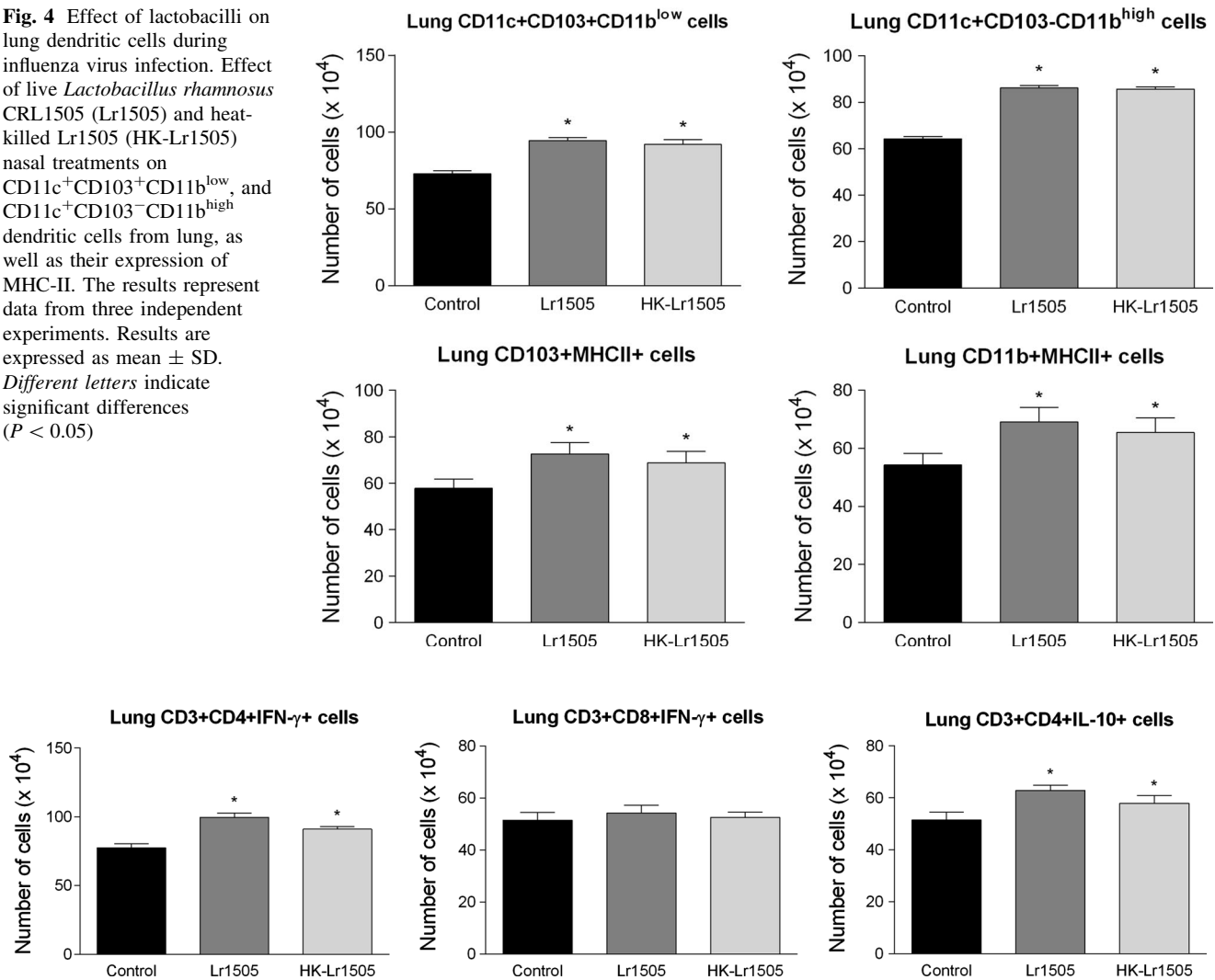
Fig. 5 Effect of lactobacilli on effector and regulatory lymphocytes during influenza virus infection. Effect of live *Lactobacillus rhamnosus* CRL1505 (Lr1505) and heat-killed Lr1505 (HK-Lr1505) nasal treatments on CD3$^+$CD4$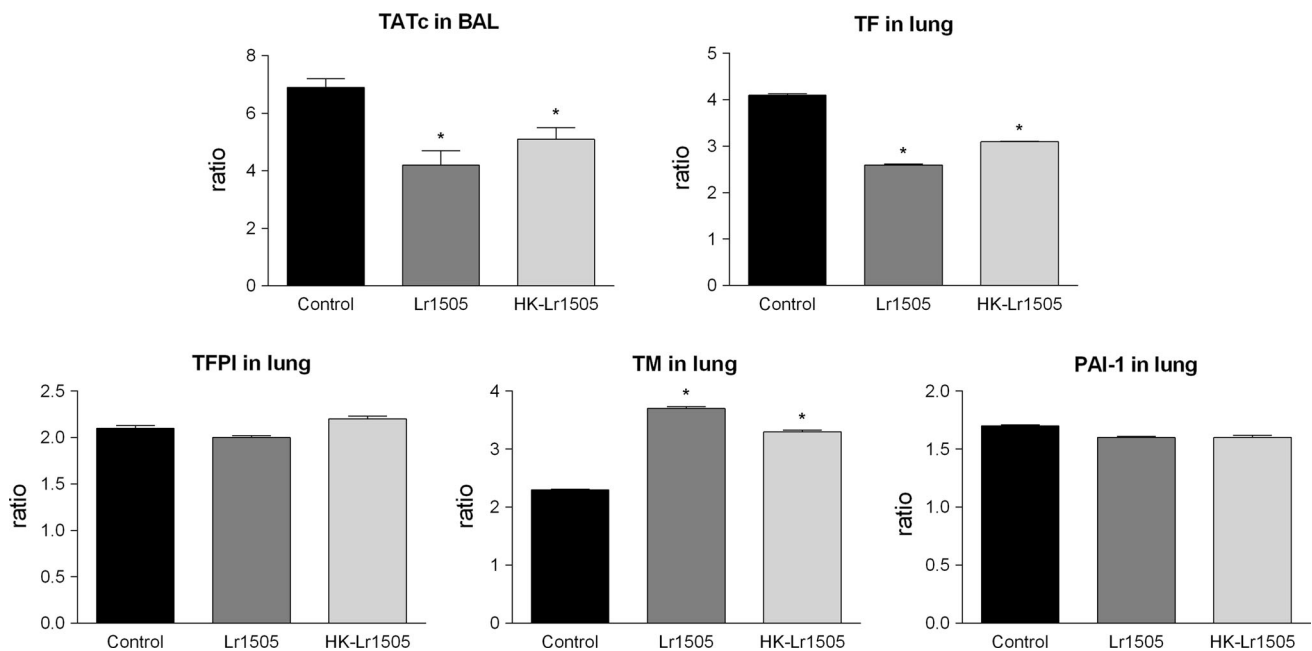^+$IFN-γ$^+$, CD3$^+$CD8$^+$IFN-γ$^+$, and CD3$^+$CD4$^+$IL-10$^+$ T cells from lung. The results represent data from three independent experiments. Results are expressed as mean ± SD. *Different letters* indicate significant differences ($P < 0.05$)

Pro-coagulant state induced by poly(I:C) mimics IFV infection and is modulated by *L. rhamnosus* CRL1505

Certain respiratory viruses such as RSV and IFV produce a shift to a hypercoagulation state possibly due to a procoagulant cytokines status and the direct stimulation of TF and repression of TM via TLR3 [24]. To study the systemic hemostasis, we evaluated some parameters such as global coagulation tests, von Willebrand factor (vWF) in plasma, and platelet counts in blood. Challenge with poly(I:C) significantly decreased prothrombin activity in the control group, as we demonstrated previously [11], however, Lr1505 and HK-Lr1505 treatments induced an improvement of this parameter (Fig. 8). On the contrary, no

modifications in the activated partial thromboplastin time (APTT) test and platelet counts were observed at hour 12 post-challenge with poly(I:C) in all the experimental groups (Fig. 8). On the other hand, the nasal challenge with poly(I:C) increased vWF in plasma, indicating systemic endothelial activation (Fig. 8). Lr1505 and HK-Lr1505 treated mice showed significant lower vWF levels, being live Lr1505 more effective than HK-Lr1505 to achieve this effect (Fig. 8).

Finally, we determined TF, TFPI, PAI-1 and TM lung expressions to determine the pulmonary hemostatic alterations induced by the nasal challenge with the TLR3 agonist. Poly(I:C) induced increased expression in lungs of these factors in all the experimental groups (Fig. 8). However, Lr1505 and HK-Lr1505 treated mice showed

Fig. 6 Effect of lactobacilli on the hemostatic alterations in lung during influenza virus infection. Effect of live *Lactobacillus rhamnosus* CRL1505 (Lr1505) and heat-killed Lr1505 (HK-Lr1505) nasal treatments on thrombin–antithrombin complexes (TATc) in broncho-alveolar lavages (BAL). Tissue factor (TF), Tissue factor pathway inhibitor (TFPI), Plasminogen activator inhibitor (PAI)-1, and Thrombomodulin (TM) mRNA expressions in lung was examined using RT-qPCR. The results represent data from three independent experiments. Results are expressed as mean ± SD. *Different letters* indicate significant differences ($P < 0.05$)

significant lower TF and higher TM expressions than control (Fig. 8). In addition, no significant differences in TFPI and PAI-1 expressions were observed in all the experimental groups (Fig. 8).

Discussion

Five to fifteen percent of global population is affected by seasonal influenza yearly. Thus, influenza represents a major public health problem. Symptoms are variable ranging from mild respiratory distress to massive organ failure resulting in death [25]. The severity of the infection mostly depends on the general health and immune state of the patient, and on the virulence of the specific influenza strain. Seasonal influenza is usually self-limiting but in susceptible patients, it may progress to acute lung injury, which is characterized by augmented pulmonary microvascular permeability leading to pulmonary edema, hypoxemia and respiratory failure [4, 26, 27]. Available drugs are of limited efficacy in this setting, therefore, the development of novel therapeutic or preventive alternatives are a milestone in influenza research.

Based on previous results using the immunobiotic strain *L. rhamnosus* CRL1505 for enhancing antiviral immunity against RSV and modulating inflammation [21, 28], we explored the effectiveness of this strain (alive or heat-killed) to reduce the influenza burden of disease. IFV-infected mice receiving Lr1505 or HK-Lr1505 showed a milder disease course than non-treated mice, and presented a significantly higher survival rate. In line with the increased survival, virus titers in lungs of treated mice were significantly declined in comparison to viral titers in control animals. Furthermore, when IFV-infected mice were nasally administered with Lr1505, pulmonary damage was significantly lessened as reflected by the improvement of all pulmonary parameters analyzed. Thus, the reduced levels of albumin and proteins in BAL reflect a reduction of microvascular leakage, which is a hint towards improving acute lung injury at the first steps of infection.

The process of microvascular leakage is postulated to result from both the direct effect of virus replication and virus induced pro-inflammatory cytokines, especially IFN-α and TNF-α [29]. More recently, it has been reported that IL-6 is necessary not only for the development of a virus-specific T cell response but also for orchestrating inflammatory resolution. In fact, Lauder et al. [22] reported striking differences in virus clearance, lung immunopathology and generation of heterosubtypic immunity between IL-6 deficient and wild type mice. Thus, IL-6$^{-/-}$ knock-out mice were not capable to resolve sublethal dosis of IFV and died because of their inability to control inflammation. These mice showed an exacerbated influx of leucocytes into pulmonary tissues and vascular
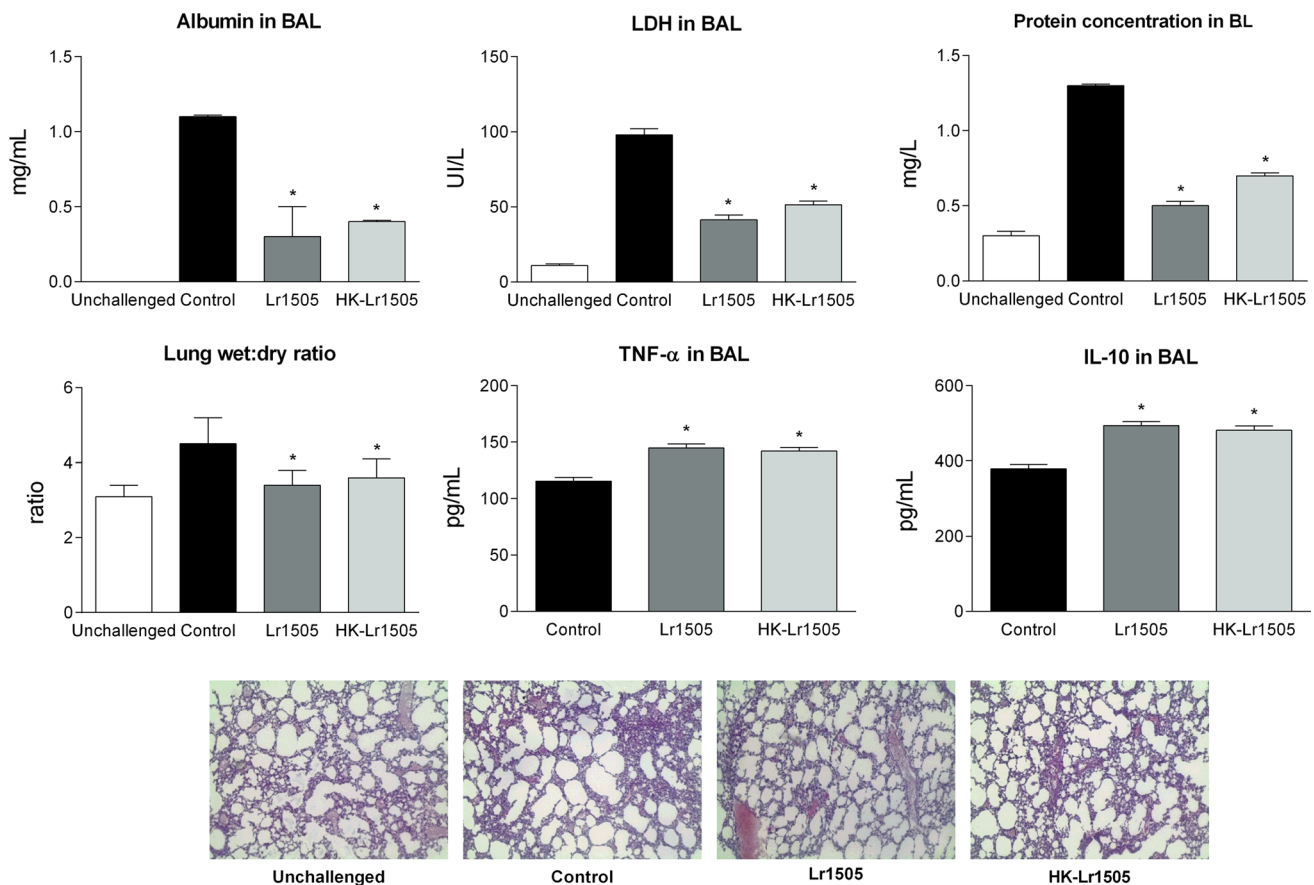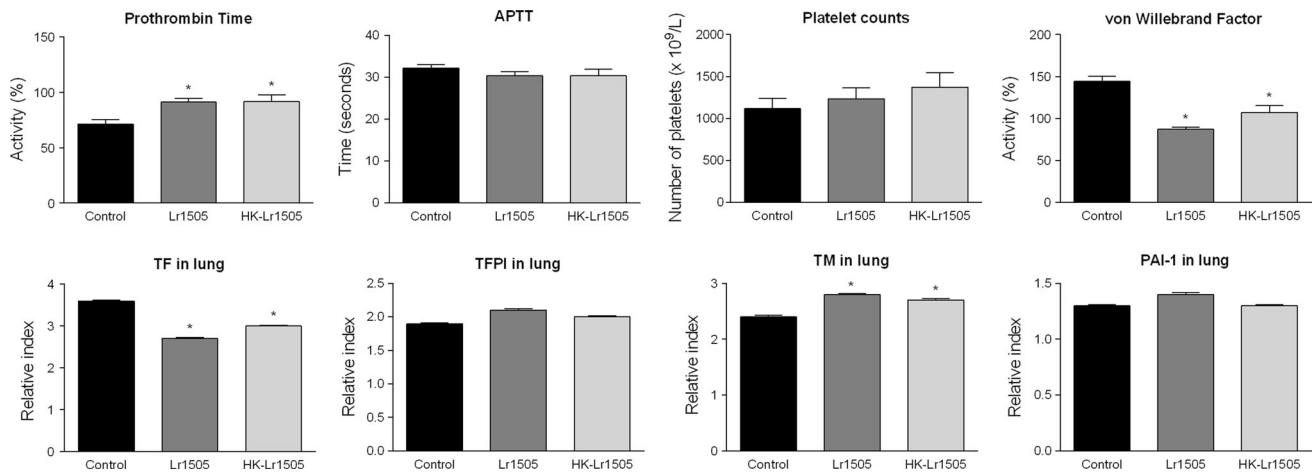
Fig. 7 Effect of lactobacilli on the lung damage induced by the nasal administration of the viral pathogen-associated molecular pattern poly(I:C). Effect of live *Lactobacillus rhamnosus* CRL1505 (Lr1505) and heat-killed Lr1505 (HK-Lr1505) nasal treatments on the lung damage. Albumin, lactate dehydrogenase (LDH), protein concentration in bronchoalveolar lavages (BAL), lung wet:dry weight ratio, and tumor necrosis factor (TNF)-α, and IL-10 in bronchoalveolar lavages. The lungs were removed fixed, and stained with hematoxylin and eosin for histological analysis. Light micrographs, original magnification ×100. The results represent data from three independent experiments. Results are expressed as mean ± SD. *Different letters* indicate significant differences ($P < 0.05$)

leakage, which finally resulted in pulmonary damage [22]. In our experimental models, there were increased levels of IL-6 in serum and BAL in Lr1505 treated animals until day 3 post-infection, after which IL-6 started to decrease in contrast to IL-6 levels in control mice, which continued increasing. A similar kinetics was also observed for pro-inflammatory TNF-α. Hussel et al. [30] treated IFV-infected mice with TNF-α antagonists and reported significant reduction of virus-specific lung histopathology without directly affecting viral replication. Therefore, the trend toward lower TNF-α levels registered from day 4 post-infection in mice treated with Lr1505 and HK-Lr1505, may be in part responsible for the reduced severity of pulmonary damaged observed in these animals with respect to control mice. In parallel, the recruitment of neutrophils to the lungs also showed a similar behavior as IL-6 and TNF-α. Thus, we hypothesize that the earlier increase in neutrophil counts in BAL and serum of treated mice may contribute to limit virus replication during acute infection

as suggested by Dienz et al. [31]. On the other hand, the influx of neutrophils into the lung is controlled by day 3, as evidenced by the reduction of neutrophil numbers counted in BAL of treated mice. In contrast, neutrophil migration continued upon day 3 in control mice. Treatment with lactobacilli also seems to have controlled the influx of monocytic cells into the lungs upon day 3 in contrast to control mice, which showed a continuous increment in monocytic cell numbers in BAL. According to Lauder et al. [22], an enhanced monocytic cell infiltration in pulmonary tissues is deleterious.

Type I IFNs upon binding to their cognate receptors, trigger the expression of antiviral genes via activation of the JAK-STAT pathway. Apart from their antiviral functions, type I IFNs modulates both innate and adaptive immune cells by inducing IL-10 [32]. Therefore, these cytokines are important in protection against acute IFV infection because of both their direct antiviral and anti-inflammatory activities [33]. In addition, IFN-γ is produced

Fig. 8 Effect of lactobacilli on the hemostatic parameters after nasal administration of the viral pathogen-associated molecular pattern poly(I:C). Effect of live *Lactobacillus rhamnosus* CRL1505 (Lr1505) and heat-killed Lr1505 (HK-Lr1505) nasal treatments on the hemostatic alterations at systemic level. Prothrombin time, Activated partial thromboplastin time (APTT), and von Willebrand factor (vWF) concentration were evaluated in plasma. In addition, platelet counts were performed in blood. Tissue factor (TF), Tissue factor pathway inhibitor (TFPI), Plasminogen activator inhibitor (PAI)-1, and Thrombomodulin (TM) mRNA expressions in lung was examined using RT-qPCR. The results represent data from three independent experiments. Results are expressed as mean ± SD. *Different letters* indicate significant differences ($P < 0.05$)

by immune cells, especially Th1 lymphocytes, and it further enhances antiviral immune response. IFN-γ induces macrophage activation, activation of NK cell effector functions, and enhancement of specific cytotoxic immunity. Our studies support the possibility to modulate these cytokines during an IFV infection using the immunobiotic strain *L. rhamnosus* CRL1505 live or heat-killed. Moreover, the modulation of type I IFNs and IFN-γ would be responsible for the reduction of viral loads in IFV-infected mice previously treated with the CRL1505 strain through the activation of adaptive immunity. In this regard, it was demonstrated that DCs are key players in clearance of influenza virions and also participate in maintaining the immune reactivity of bronchial-associated lymphoid tissue (BALT) [34–36]. Therefore, the accelerated airway (CD103^{+}) and parenchymal (CD11b^{+}) DCs migration to the lungs, and the up-modulation of maturation markers such as MHC-II may actively contribute to the enhanced clearance of IFV observed in mice receiving Lr1505 or HK-Lr1505 nasally. These results are consistent with previous data obtained by our group in experiments using immunobiotics in the context of other viral and non-viral respiratory infections. Moreover, Richert et al. [37] described the phenomenon of innate imprinting, which is closely related to the process of immune modulation with immunobiotics. Thus, administration of certain antigens (VLPs and *Pneumocystis murina*) veering no antigenic similarity to influenza, were protective upon IFV challenge.

Both Lr1505 and HK-Lr1505 significantly increased the levels of the regulatory cytokine IL-10 in infected mice when compared to infected mice without treatment. These results are in line with previous reports of our group using this strain in the context of pulmonary infections with other pathogens [11, 21, 38, 39]. The delicate balance between pro-inflammatory cytokines and anti-inflammatory cytokines is pivotal for a proper and regulated immune response. Thus, insufficient inflammation may result in virus escape, whereas exacerbated inflammation may conduce to bystander pulmonary damage, leakage, loss of functionality and the concomitant severe prognose. *L. rhamnosus* CRL505 alive or heat-killed has the ability to modulate this process activating innate immunity pathways while modulating inflammation.

There is epidemiological evidence that influenza may trigger vascular diseases including myocardial stroke in susceptible individuals [40–42]. Goeijenbier et al. [43] associated the pro-coagulant state observed in ferrets infected with influenza with the development of infarction. They also observed hemostatic alterations at both vascular and tissue levels during IFV infections. The magnitude of these alterations correlated with the severity of infection. Hence, although activation of the coagulation cascade, contributes on the one hand, to controlling infection by trapping virus particles in clots, on the other hand, it also participates in acute lung injury, both as an initiating factor and as a consequence. For instance, TF plays not only a role in coagulation but it also has a strong pro-inflammatory activity. When IFV infects endothelial cells, dsRNA replication intermediates are recognized by TLR3, and a cascade of events occur, including the initiation of a pro-coagulant state [24]. In vivo studies showed that poly(I:C) could up-regulate the expression of pro-inflammatory and antiviral cytokines [44], influence vascular permeability

[45], and increase circulating D-dimer levels indicating that both coagulation and fibrinolysis are stimulated. Shibamiya et al. [24] reported that two of the mechanisms underlying the pro-coagulant activity are TF up-regulation and TM repression at mRNA, and protein levels in endothelial cells triggered by TLR3 ligands. Besides, in a pro-coagulant sate, TATc values are elevated and platelets are reduced in numbers as a consequence of increased clot formation. Therefore, we evaluated in this study, if any of these pro-coagulant mechanisms were modulated by lactobacilli both, in the context of IFV infection and using poly(I:C) for mimicking viral nucleic acid recognition via TLR3. As a result, we show that nasally administered Lr1505 or HK-Lr1505 significantly down-regulated TF and restored the levels of TM. Moreover, TATc levels and platelet counts were reversed to near normal values. Armstrong et al. [4] suggested that a novel target to improve the outcome of influenza is restraining or reversing increased vascular leak. In this regard, our results provide evidence that *L. rhamnosus* CRL1505 is effective in regulating the pro-coagulant state in mice infected with IFV or challenged with poly(I:C).

Further, we show that live and heat-killed *L. rhamnosus* CRL1505 display very similar modulatory activities at distinct humoral and cellular levels of the immune response, as well as in the pro-coagulation state. Therefore, we hypothesize that components of the cell-wall such as peptidoglycan, which is not denaturalized by heat, are mainly responsible for reducing inflammation and reverting the pro-coagulant state, which resulted in better survival and reduced lung injury of infected mice. This offers advantages in safety concerns, because of the reduced risk of live bacteria trespassing the brain-blood barrier, and in biotechnology, because there is no need to maintain a bacterial culture in an exponential phase and so conservation is easier.

Summarizing, we examined the effect of nasal administration of live and heat-killed *L. rhamnosus* CRL1505 on influenza infection and found that both treatments were able to protect mice challenged with IFV. We unraveled three mechanisms by which this immunobiotic strain protected infected mice by reducing pulmonary injury and lung viral loads: (a) Inflammatory response was improved early after IFV infection and efficiently regulated later, allowing higher clearance of virus together with a reduction of inflammatory lung tissue damage. This latter effect was associated to the higher levels of the regulatory cytokine IL-10. (b) Innate and adaptive antiviral immune responses were enhanced as shown by the improved levels of type I IFNs, $CD4^+IFN\text{-}\gamma^+$ lymphocytes, and lung $CD11c^+CD11b^{low}CD103^+$ and $CD11c^+CD11b^{high\text{-}}CD103^-$ DCs. (c) The pro-coagulation state induced by IFV infection was reversed mainly by down-regulating lung TF and restoring TM levels. In addition, our results

demonstrated that the capacity of *L. rhamnosus* CRL1505 to improve the outcome of IFV infection would be related to its ability to beneficially modulate lung TLR3-triggered immune response. Although several other works have demonstrated the capacity of immunobiotic lactobacilli to improve defenses against influenza, our work is the first in demonstrating the ability of an immunobiotic strain to beneficially modulate inflammation–coagulation interactions during IFV infection. Moreover, we demonstrated here that non-viable *L. rhamnosus* CRL1505 was as effective as the viable strain to beneficially modulate respiratory antiviral immune-coagulative response.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Ruuskanen O, Lahti E, Jennings LC, Murdoch DR. Viral pneumonia. Lancet. 2011;377:1264–75.
2. Herold S, von Wulffen W, Steinmueller M, Pleschka S, Kuziel WA, Mack M, et al. Alveolar epithelial cells direct monocyte transepithelial migration upon influenza virus infection: impact of chemokines and adhesion molecules. J Immunol. 2006;177:1817–24.
3. Geiler J, Michaelis M, Sithisarn P, Cinatl J Jr. Comparison of pro-inflammatory cytokine expression and cellular signal transduction in human macrophages infected with different influenza A viruses. Med Microbiol Immunol. 2011;200:53–60.
4. Armstrong SM, Mubareka S, Lee WL. The lung microvascular endothelium as a therapeutic target in severe influenza. Antiviral Res. 2013;99:113–8.
5. Smeeth L, Thomas SL, Hall AJ, Hubbard R, Farrington P, Vallance P. Risk of myocardial infarction and stroke after acute infection or vaccination. N Engl J Med. 2004;351:2611–8.
6. Smeeth L, Cook C, Thomas S, Hall AJ, Hubbard R, Vallance P. Risk of deep vein thrombosis and pulmonary embolism after acute infection in a community setting. Lancet. 2006;367:1075–9.
7. Esmon CT. Crosstalk between inflammation and thrombosis. Maturitas. 2004;47:305–14.
8. Keller TT, van der Sluijs KF, de Kruif MD, Gerdes VE, Meijers JC, Florquin S, et al. Effects on coagulation and fibrinolysis induced by influenza in mice with a reduced capacity to generate activated protein C and a deficiency in plasminogen activator inhibitor type 1. Circ Res. 2006;99:1261–9.
9. Visseren FL, Bouwman JJ, Bouter KP, Diepersloot RJ, de Groot PH, Erkelens DW. Procoagulant activity of endothelial cells after infection with respiratory viruses. Thromb Haemost. 2000;84:319–24.
10. Villena J, Salva S, Barbieri N, Alvarez S. Immunobiotics for the prevention of bacterial and viral respiratory infections. In: Kitazawa H, Villena J, Alvarez S, editors. Probiotics: Immunobiotics and Immunogenics: Science Publishers, CRC Press, Taylor & Francis Group company; 2013. p. 128–68.
11. Zelaya H, Tsukida K, Chiba E, Marranzino G, Alvarez S, Kitazawa H, et al. Immunobiotic lactobacilli reduce viral-associated

pulmonary damage through the modulation of inflammation–coagulation interactions. Int Immunopharmacol. 2014;19:161–73.

12. Izumo T, Maekawa T, Ida M, Noguchi A, Kitagawa Y, Shibata H, et al. Effect of intranasal administration of Lactobacillus pentosus S-PT84 on influenza virus infection in mice. Int Immunopharmacol. 2010;10:1101–6.

13. Takeda S, Takeshita M, Kikuchi Y, Dashnyam B, Kawahara S, Yoshida H, et al. Efficacy of oral administration of heat-killed probiotics from Mongolian dairy products against influenza infection in mice: alleviation of influenza infection by its immunomodulatory activity through intestinal immunity. Int Immunopharmacol. 2011;11:1976–83.

14. Kobayashi N, Saito T, Uematsu T, Kishi K, Toba M, Kohda N, et al. Oral administration of heat-killed Lactobacillus pentosus strain b240 augments protection against influenza virus infection in mice. Int Immunopharmacol. 2011;11:199–203.

15. Salva S, Villena J, Alvarez S. Immunomodulatory activity of Lactobacillus rhamnosus strains isolated from goat milk: impact on intestinal and respiratory infections. Int J Food Microbiol. 2010;141:82–9.

16. Sawai R, Kuroda K, Shibata T, Gomyou R, Osawa K, Shimizu K. Anti-influenza virus activity of Chaenomeles sinensis. J Ethnopharmacol. 2008;118:108–12.

17. Villena J, Racedo S, Agüero G, Bru E, Medina M, Alvarez S. Lactobacillus casei improves resistance to pneumococcal respiratory infection in malnourished mice. J Nutr. 2005;135:1462–9.

18. Aeffner F, Traylor ZP, Yu ENZ, Davis IC. Double-stranded RNA induces similar pulmonary dysfunction to respiratory syncytial virus in BALB/c mice. Am J Physiol Lung Cell Mol Physiol. 2011;301:L99.

19. Agüero G, Villena J, Racedo S, Haro C, Alvarez S. Beneficial immunomodulatory activity of Lactobacillus casei in malnourished mice pneumonia: effect on inflammation and coagulation. Nutrition. 2006;22:810–9.

20. Zelaya H, Haro C, Laiño J, Alvarez S, Agüero G. Coagulation activation in an experimental pneumonia model in malnourished mice. Can J Physiol Pharmacol. 2011;89:41–9.

21. Tomosada Y, Chiba E, Zelaya H, Takahashi T, Tsukida K, Kitazawa H, et al. Nasally administered Lactobacillus rhamnosus strains differentially modulate respiratory antiviral immune responses and induce protection against respiratory syncytial virus infection. BMC Immunol. 2013;14:40.

22. Lauder SN, Jones E, Smart K, Bloom A, Williams AS, Hindley JP, et al. Interleukin-6 limits influenza-induced inflammation and protects against fatal lung pathology. Eur J Immunol. 2013;43:2613–25.

23. Kawai T, Akira S. Toll-like receptor and RIG-I-like receptor signaling. Ann N Y Acad Sci. 2008;1143:1–20.

24. Shibamiya A, Hersemeyer K, Schmidt Woll T, Sedding D, Daniel JM, Bauer S, et al. A key role for Toll-like receptor-3 in disrupting the hemostasis balance on endothelial cells. Blood. 2009;113:714–22.

25. Schutten M, van Baalen C, Zoeteweij P, Fraaij P. The influenza virus: disease, diagnostics, and treatment. MLO Med Lab Obs. 2013;45:38–40.

26. Gurfinkel EP, de la Fuente RL. Two-year follow-up of the FLU Vaccination Acute Coronary Syndromes (FLUVACS) registry. Tex Heart Inst J. 2004;31:28–32.

27. Gurfinkel EP, Leon de la Fuente R, Mendiz O, Mautner B. Flu vaccination in acute coronary syndromes and planned percutaneous coronary interventions (FLUVACS) Study. Eur Heart J. 2004;25:25–31.

28. Chiba E, Tomosada Y, Vizoso-Pinto MG, Salva S, Takahashi T, Tsukida K, et al. Immunobiotic Lactobacillus rhamnosus improves resistance of infant mice against respiratory syncytial virus infection. Int Immunopharmacol. 2013;17:373–82.

29. Armstrong SM, Wang C, Tigdi J, Si X, Dumpit C, Charles S, et al. Influenza infects lung microvascular endothelium leading to microvascular leak: role of apoptosis and claudin-5. PLoS One. 2012;7:e47323.

30. Hussell T, Pennycook A, Openshaw PJ. Inhibition of tumor necrosis factor reduces the severity of virus-specific lung immunopathology. Eur J Immunol. 2001;31:2566–73.

31. Dienz O, Rud JG, Eaton SM, Lanthier PA, Burg E, Drew A, et al. Essential role of IL-6 in protection against H1N1 influenza virus by promoting neutrophil survival in the lung. Mucosal Immunol. 2012;5:258–66.

32. Corre B, Perrier J, El Khouri M, Cerboni S, Pellegrini S, Michel F. Type I interferon potentiates T-cell receptor mediated induction of IL-10-producing CD4(+) T cells. Eur J Immunol. 2013;43:2730–40.

33. Arimori Y, Nakamura R, Yamada H, Shibata K, Maeda N, Kase T, et al. Type I interferon limits influenza virus-induced acute lung injury by regulation of excessive inflammation in mice. Antiviral Res. 2013;99:230–7.

34. McGill J, Van Rooijen N, Legge KL. Protective influenza-specific CD8 T cell responses require interactions with dendritic cells in the lungs. J Exp Med. 2008;205:1635–46.

35. Halle S, Dujardin HC, Bakocevic N, Fleige H, Danzer H, Willenzon S, et al. Induced bronchus-associated lymphoid tissue serves as a general priming site for T cells and is maintained by dendritic cells. J Exp Med. 2009;206:2593–601.

36. GeurtsvanKessel CH, Willart MA, Bergen IM, van Rijt LS, Muskens F, Elewaut D, et al. Dendritic cells are crucial for maintenance of tertiary lymphoid structures in the lung of influenza virus-infected mice. J Exp Med. 2009;206:2339–49.

37. Richert LE, Rynda-Apple A, Harmsen AL, Han S, Wiley JA, Douglas T, et al. CD11c(+) cells primed with unrelated antigens facilitate an accelerated immune response to influenza virus in mice. Eur J Immunol. 2014;44:397–408.

38. Haro C, Villena J, Zelaya H, Alvarez S, Agüero G. Lactobacillus casei modulates the inflammation–coagulation interaction in a pneumococcal pneumonia experimental model. J Inflamm (Lond). 2009;6:28.

39. Villena J, Barbieri N, Salva S, Herrera M, Alvarez S. Enhanced immune response to pneumococcal infection in malnourished mice nasally treated with heat-killed Lactobacillus casei. Microbiol Immunol. 2009;53:636–46.

40. Loomba RS, Aggarwal S, Shah PH, Arora RR. Influenza vaccination and cardiovascular morbidity and mortality: analysis of 292,383 patients. J Cardiovasc Pharmacol Ther. 2012;17:277–83.

41. Ciszewski A. Why young healthy adults should become a target group for the influenza vaccination: a cardiologist's point of view. Vaccine. 2008;26:4411–2.

42. Ciszewski A, Bilinska ZT, Brydak LB, Kepka C, Kruk M, Romanowska M, et al. Influenza vaccination in secondary prevention from coronary ischaemic events in coronary artery disease: FLUCAD study. Eur Heart J. 2008;29:1350–8.

43. Goeijenbier M, van Gorp EC, Van den Brand JM, Stittelaar K, Bakhtiari K, Roelofs JJ, et al. Activation of coagulation and tissue fibrin deposition in experimental influenza in ferrets. BMC Microbiol. 2014;14:134.

44. Gitlin L, Barchet W, Gilfillan S, Cella M, Beutler B, Flavell RA, et al. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. Proc Natl Acad Sci USA. 2006;103:8459–64.

45. Wang T, Town T, Alexopoulou L, Anderson JF, Fikrig E, Flavell RA. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. Nat Med. 2004;10:1366–73.