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Lactobacillus rhamnosus (LR) ameliorates pulmonary and extrapulmonary acute respiratory distress syndrome (ARDS) via targeting neutrophils

Leena Sapra [a], Chaman Saini [a], Sneha Das [a], Pradyumna K. Mishra [b], Anurag Singh [a], Asit R. Mridha [c], Pardeep K. Yadav [d], Rupesh K. Srivastava [a,*]

[a] Translational Immunology, Osteoimmunology & Immunoporosis Lab (TIOIL), Department of Biotechnology, All India Institute of Medical Sciences (AIIMS), New Delhi 110029, India
[b] Department of Molecular Biology, ICMR-National Institute for Research in Environmental Health, Bhopal, MP 462001, India
[c] Department of Pathology, All India Institute of Medical Sciences (AIIMS), New Delhi 110029, India
[d] Central Animal Facility, All India Institute of Medical Sciences (AIIMS), New Delhi 110029, India

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ABSTRACT

Pulmonary and extrapulmonary acute respiratory distress syndrome (ARDS) is a life-threatening respiratory failure associated with high mortality. Despite progress in our understanding of the pathological mechanism causing the crippling illness, there are currently no targeted pharmaceutical treatments available for it. Recent discoveries have emphasized the existence of a potential nexus between gut and lung health fueling novel approaches including probiotics for the treatment of ARDS. We thus investigated the prophylactic-potential of *Lactobacillus rhamnosus*-(LR) in lipopolysaccharide (LPS)-induced pulmonary and cecal ligation puncture (CLP) induced extrapulmonary ARDS mice. Our in-vivo findings revealed that pretreatment with LR significantly ameliorated vascular-permeability (edema) of the lungs via modulating the neutrophils along with significantly reducing the expression of inflammatory-cytokines in the BALF, lungs and serum in both pulmonary and extrapulmonary mice-models. Interestingly, our ex-vivo immunofluorescence and flow cytometric data suggested that mechanistically LR via short chain fatty acids (butyrate being the most potent and efficient in ameliorating the pathophysiology of both pulmonary and extra-pulmonary ARDS) targets the phagocytic and neutrophils extracellular traps (NETs) releasing potential of neutrophils. Moreover, our in-vivo data further corroborated our ex-vivo findings and suggested that butyrate exhibits enhanced potential in ameliorating the pathophysiology of ARDS via reducing the infiltration of neutrophils into the lungs. Altogether, our study establishes the prophylactic role of LR and its associated metabolites in the prevention and management of both pulmonary and extrapulmonary ARDS via targeting neutrophils.

1. Introduction

Since it was initially identified in humans over 50 years ago, acute respiratory distress syndrome (ARDS) has become one of the most common chronic illnesses with rising rates of disability and death globally [1]. The hallmarks in the pathophysiology of ARDS are acute and widespread inflammatory damage to the alveolar-capillary barrier, increased vascular permeability, and accumulation of protein-rich edema fluid in the lungs which compromise gaseous exchange resulting in hypoxemia [2]. The major causes of ARDS can be grouped into following two categories: (1) direct pulmonary pathogenesis which includes bacterial and viral pneumonia, inhalation injury, aspiration pneumonitis, or trauma to the lung parenchyma, and (2) indirect extrapulmonary pathogenesis, such as extra-thoracic sepsis, trauma, shock, burn injury, transfusion, and other factors [3]. Depending on the level of oxygenation, ARDS can be categorized into: 'mild', 'moderate' and 'severe'. Lack of a definite diagnostic marker along with the disability to determine direct measurements of lung injury via pathological lung tissue samples in most patients make the diagnosis of ARDS solely based on clinical criteria. Furthermore, neither distal airspace nor blood samples can be used to diagnose ARDS. An important factor involved in the pathogenesis of ARDS is the innate immune system among which neutrophils are the key effectors involved in both pulmonary and extrapulmonary ARDS. These cells induce pathogen

* Corresponding author at: Department of Biotechnology, All India Institute of Medical Sciences (AIIMS), New-Delhi 110029, India.
E-mail address: rupeshk@aiims.edu (R.K. Srivastava).

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clearance by engulfing and killing the bacteria via releasing NETs (network of extracellular DNA with associated proteins). Unfortunately, delayed neutrophils apoptosis and prolonged neutrophilic inflammation mediate further alveolar capillary barrier disruption [4,5]. Treatment for ARDS majorly focuses on lung-protective ventilation since no specific pharmacotherapies have been identified till date. The most widely used adjuvant therapy in normal ARDS includes recruitment techniques, high-dose corticosteroids, and continuous neuromuscular blocking medications. Corticosteroids have anti-inflammatory properties and are observed to be a potential treatment modality for ARDS. Additionally, randomized trials to improve fluid treatment and mechanical ventilation for ARDS have shown promising clinical outcomes. Several immunosuppressive drugs have been tested in clinical trials of patients with ARDS; however, almost none of them has proven beneficial in managing the disease in larger trials. Moreover, handful of these which showed promising outcomes are further associated with serious side effects. Thus, there is an exigent need for a potent immunomodulatory agent which can dampen the excessive inflammatory response in ARDS. As a result, the search for safer and more affordable treatments having the capacity for both prevention and treatment of ARDS is still warranted. Experimental and clinical evidence demonstrates that gut microbiota possesses a crucial role in maintaining lung health. Recent research studies have suggested the existence of crosstalk between the gut and lung and this interplay has a major role in the control of infections [6,7]. Many factors can alter the diversity and composition of the gut microbiota, leading to dysbiosis. Dysbiosis can also occur as a result of infections and chronic inflammatory or metabolic disorders. Changes in intestinal bacterial communities can influence disease outcomes even in distant organs (including the lungs), as demonstrated by transfer experiments with dysbiotic microbiota. Microbiota analyses in various respiratory infections have found a reduced abundance of the Lactobacillus genus (the Bacilli class) in the gut [8,9]. Moreover, analysis of gut microbiome in studies involving COVID-19 patients has recently found that COVID-19 infection appears to blunt the growth of health-promoting gut bacteria such as Lactobacilli [10,11]. Various studies have reported the protective role of probiotics including *Lactobacillus rhamnosus* (LR) in multiple respiratory and inflammatory diseases [12].

According to the Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO), probiotics are "living microbes that impart a health gain on a host when delivered in adequate amounts" [13]. A study reported by Robert et al. demonstrated that LR exhibits antioxidative potential that dampens the ROS production by neutrophils and confer desensitization toward luminal antigens [14]. These findings suggest that probiotics have potential efficacy in dampening the severe inflammatory milieu in ARDS by modulating the immune system and thus would ameliorate respiratory immunity via modulating lung pathophysiology. Recently, a study reported that pretreatment with *Lacticaseibacillus rhamnosus* significantly reduced the pathophysiology of ALI induced ARDS [15]. However, the potential of LR and its associated metabolites such as short chain fatty acids (SCFAs) in dampening inflammatory milieu in both pulmonary and extrapulmonary ARDS via modulating the neutrophils has not been deciphered yet. To our knowledge, this study for the first time reveals the immunomodulatory potential of LR and butyrate in significantly dampening the acute exudative phase (pulmonary edema and vascular permeability) of ARDS. The study thus proposes LR and butyrate administration as a plausible "prophylactic" therapy for managing both pulmonary and extrapulmonary ARDS in various lung pathologies including COVID-19, infectious disease, sepsis etc.

2. Materials and methods

2.1. Reagents and antibodies

The following antibodies/kits were procured from eBiosciences (USA) and Biolegend (USA): BV605 Anti-Mouse-CD45-(103139), PerCp-Cy5.5 Anti-Mouse-CD11b-(45–0112-82), APC Anti-Mouse-Ly6G-(17–9668-80 and RBC lysis buffer (00–4300-54). Lipopolysaccharides (LPS) *Escherichia coli* O111:B4 origin (L2630) and Evans blue (E2129) procured from Sigma, USA. *Lactobacillus rhamnosus* UBLR-58 was procured from Unique Biotech Ltd., Hyderabad, India.

2.2. Animals

All in vivo experiments were carried out in 8–10 wks old male BALB/c and C57BL/6 J mice. Mice were housed under specific pathogen-free (SPF) conditions at the animal facility of All India Institute of Medical Sciences (AIIMS), New Delhi, India and fed with sterilized food and autoclaved drinking water ad-libitum. Mice were randomly allocated into three groups with 6 mice in each group i.e., Control (mice received PBS); LPS (mice instillated with LPS); and LPS + *Lactobacillus rhamnosus* (LR) (mice received LR for 14 days before LPS instillation). All the mice were kept in separate cages with light and dark periods of 12 h each. All the procedures were performed as per the established animal ethical procedures of Institutional Animal Ethics Committee of AIIMS, New Delhi, India (382/IAEC-1/2022; 428/IAEC-1/2023).

2.3. Administration of Lactobacillus rhamnosus (LR)

Probiotic *Lactobacillus rhamnosus* (LR), UBLR-58 was purchased from Unique Biotech. Mice were orally gavaged with LR (10^9 cfu) daily in 1× phosphate buffer saline for 2 weeks.

2.4. Induction of ALI/ARDS with LPS

After two weeks of treatment, ARDS, and ARDS + LR group mice were anesthetized with ketamine (100 mg/kg) and xylazine (10–15 mg/kg) intraperitoneally and received an intranasal instillation of LPS (3 mg/kg). After 24 h of LPS instillation mice were sacrificed and various organs harvested for further analysis.

2.5. Collection of bronchoalveolar lavage fluid (BALF)

For the bronchoalveolar lavage fluid (BALF) collection, tracheotomy was carried out and a cannula was inserted into the trachea after anesthetizing the mice with ketamine (100 mg/kg) and xylazine (10–15 mg/kg). Further lungs were lavaged three times with 1XPBS in a total volume of 3 ml (1 ml × 3). BALF was centrifuged at 1500 rpm for 10 min at 4 °C. The supernatant was aliquoted and stored in a freezer at −80 °C for cytokine analysis by ELISA and the cell pellet was suspended and fixed with 1× fixative solution. The cell suspension was stored in dark at 4 °C for further analysis by flow cytometry.

2.6. Vascular permeability assay

Pulmonary vascular permeability was assessed by Evans blue dye extravasation method. In brief, Evans blue dye (30 mg/kg) was given intravenously (i.v) to mice 60 min before the animals were euthanized. Mice were sacrificed, and both lungs were harvested. Left lung was cut into two halves and each half was weighed (wet weights). One half was dried in a drying oven at 150 °C for 48 h and the other tissue half was placed in 200 μL formamide at 70 °C for 48–72 h. The concentration of Evans blue dye extracted in formamide was determined by spectrophotometry at a wavelength of 620 nm (BioTek Synergy H1). The dry tissue half which has been in the oven was weighed (dry weight). The dry/wet ratio of each lung sample was determined (index of edema) and used in the final calculation of Evans blue extravasation which was expressed as OD_{620}/g of dry weight.

2.7. Histological evaluation

For histological analysis, right lung was harvested, and a portion was

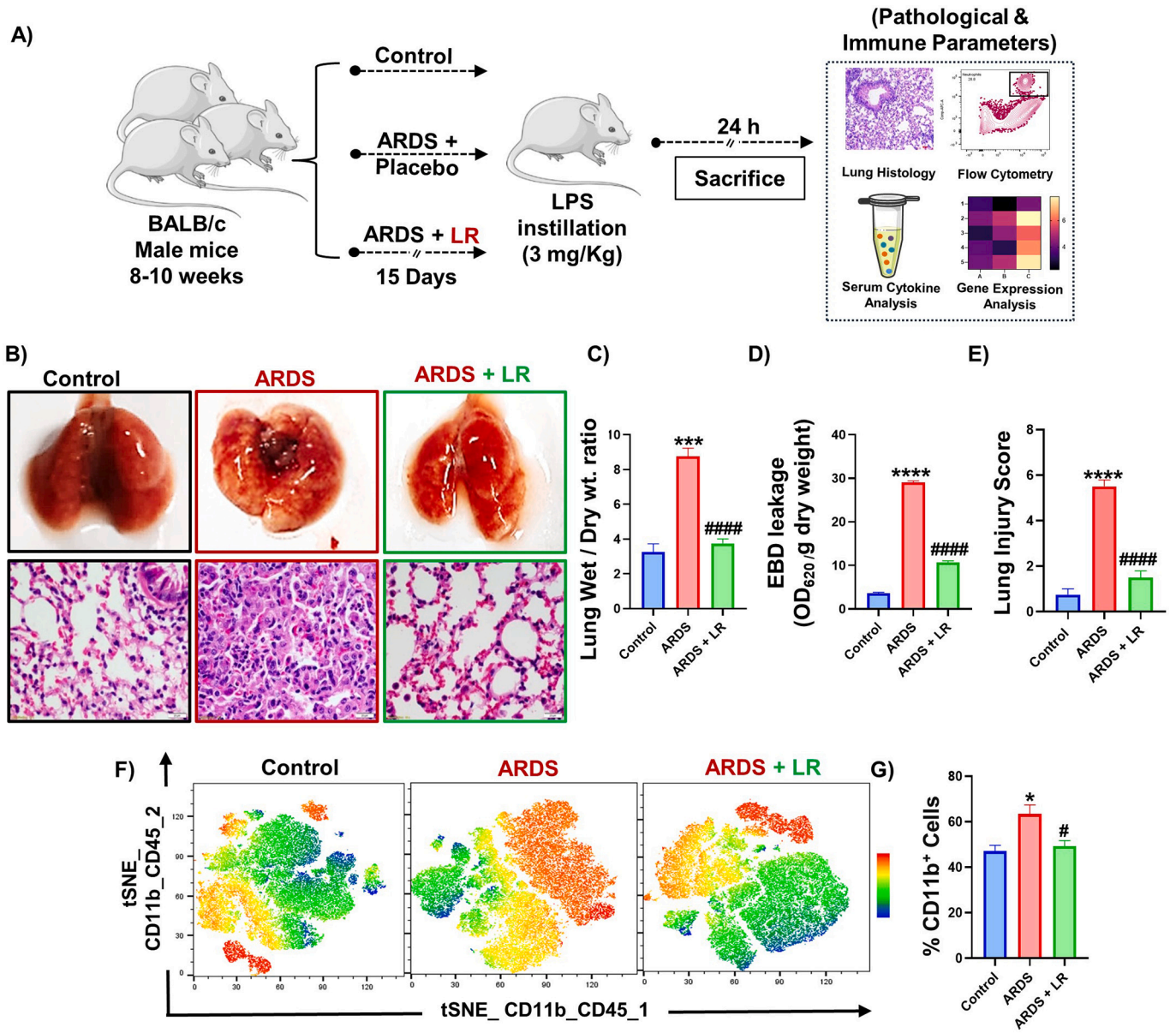

Fig. 1. LR ameliorates pathophysiology of ALI/ARDS: Mice were divided into 3 groups, viz., control, ARDS, and ARDS+LR groups. ARDS + LR group received LR at 10^9 CFU/day (100 μl) orally for 15 days. A) At the end of 15 days, LPS was given via intranasal route (3 mg/kg) and after 24 h mice were sacrificed, and various organs were harvested and analyzed for several immunological and histological parameters. B) Photomicrographs representing morphology of lungs. C) Bar graphs representing lung dry/wet weight ratio. D) Evans blue dye (EBD) leakage/dry weight. E) Lung injury score. F) tSNE representing the cluster of $CD11b^+$ cells in the $CD45^+$ cell population. G) Bar graphs represents the percentage of $CD11b^+$ cells in the lungs. Statistical significance was considered as ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$) with respect to indicated groups (* indicated comparison between control and ARDS; # indicate comparison between ARDS and ARDS+LR). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cut and stored in 10% neutral formaldehyde for 24-48 h. During the process, after following routine procedures for inclusion in paraffin, the lobes were next stained with haematoxylin-eosin (H&E). Analyses of histological sections were performed to determine the pulmonary changes induced by ARDS in the lung parenchyma. Lung injury was scored by a histopathologist as per the following criteria [16]: pulmonary edema, vascular and alveolar features, and bronchiole pathology was graded: 0 (normal), 1 (mild), 2 (moderate), 3+ (severe). Points were added up and are expressed as median ± range of injury score.

2.8. Flow cytometry

Cells were harvested from various tissues (BALF, lung and blood) and stained with antibodies specific for neutrophils and eosinophils. For the innate immune cells (Neutrophils and Eosinophils), cells were stained with anti-CD45-BV-605, anti-Ly6G-APC, anti-Ly6C-BV421, anti-SiglecF-BV786, and anti-CD11b-PerCP-Cy5.5 antibodies for 45 min on ice in dark. Lastly, samples were acquired in the flow cytometry (FACSymphony™, BD, U.S.A).

2.9. q-PCR

Gene expression was measured using quantitative real-time (Applied Biosystems, Quantstudio™-5, USA). Triplicate samples of cDNA from each group was amplified with customized primers viz. TNF-α (NM_013693.3), MCP-1 (NM_011333.3), IL-1β (NM_008361.4), IL-6 (NM_001314054.1), IP-10 (NM_021274.2), ROR-γt (AJ132394.1), IL-17 A (NM_010552.3), IL-17F (NM_145856.2) and normalized with

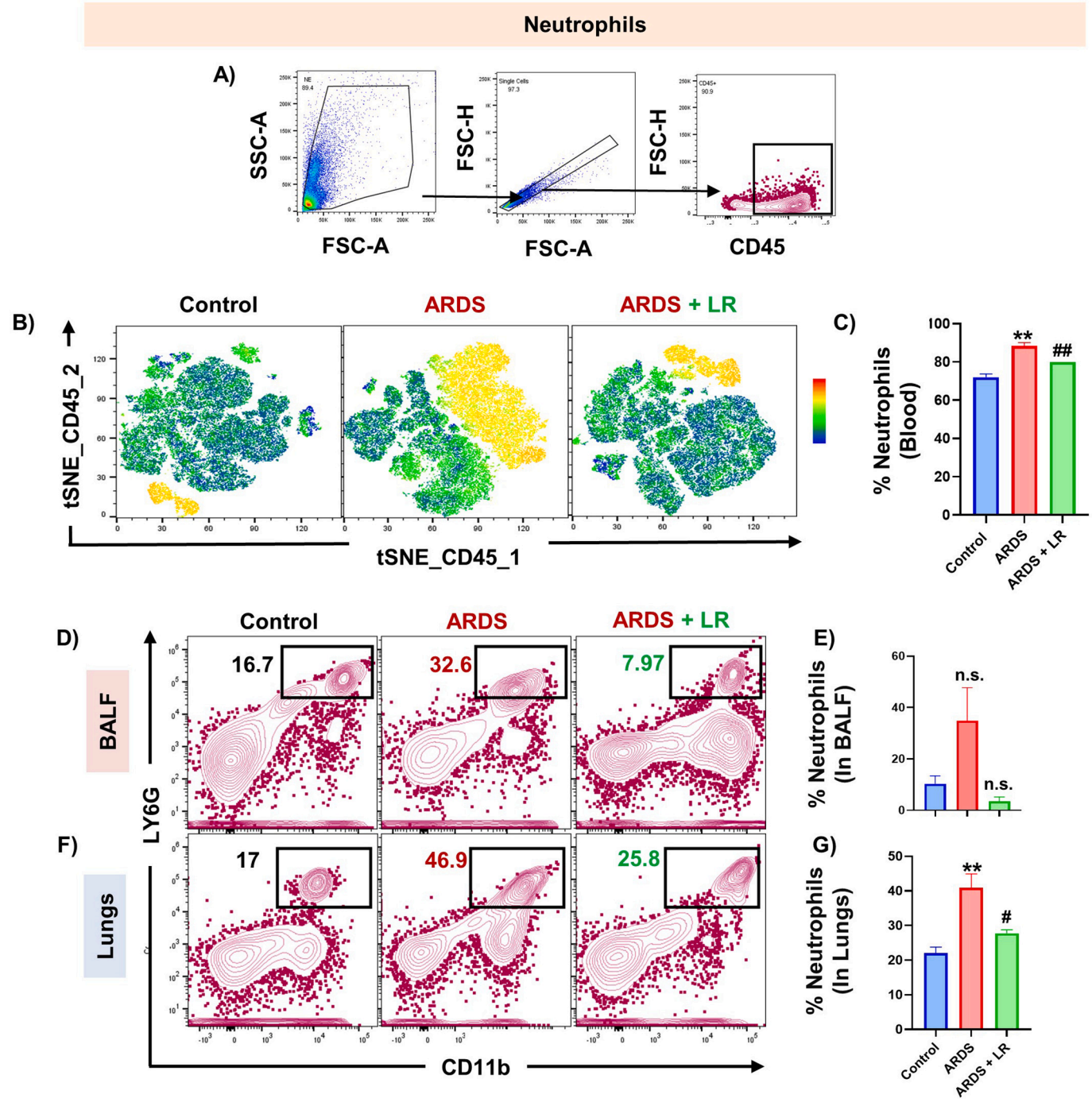

Fig. 2. LR reduces Neutrophils ($CD45^+LY6G^+CD11b^+$) population in BALF and lungs: A) Representative image of gating strategy followed for neutrophils ($CD45^+LY6G^+CD11b^+$) in BALF and lungs. B) tSNE plot representing neutrophils population. C) Bar graphs representing percentages of neutrophils in blood. D) Contour plots representing percentages of $LY6G^+CD11b^+$ neutrophils gated on $CD45^+$ cells in BALF. E) Bar graphs representing percentages of neutrophils in BALF. F) Contour plots representing percentages of $LY6G^+CD11b^+$ neutrophils gated on $CD45^+$ cells in lungs. G) Bar graphs representing percentages of neutrophils in lungs. Statistical significance was considered as (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$) with respect to indicated groups (*indicated comparison between control and ARDS; # indicate comparison between ARDS and ARDS+LR).

arithmetic mean of GAPDH (NM_001289726.2) housekeeping gene. 25 ng of c-DNA was used per reaction in each well containing the 2× SYBR green PCR master mix (Promega, USA) along with appropriate primers. Threshold cycles values were normalized and expressed as relative gene expression.

2.10. Cecal ligation and puncture (CLP) mice model

Briefly, eight weeks old C57BL/6 male mice were orally gavaged with LR (1×10^9 CFU/200 ul/day) or butyrate (200 mM/mice/day/intraperitonially-i.p.) or normal saline (control) for 7 and 14 days prior to the CLP. To develop the septic peritonitis model, mice were anesthetized with ketamine (80–100 mg/Kg body weight) and xylazine (5–15 mg/kg body weight). A small incision (1 cm) was made in the

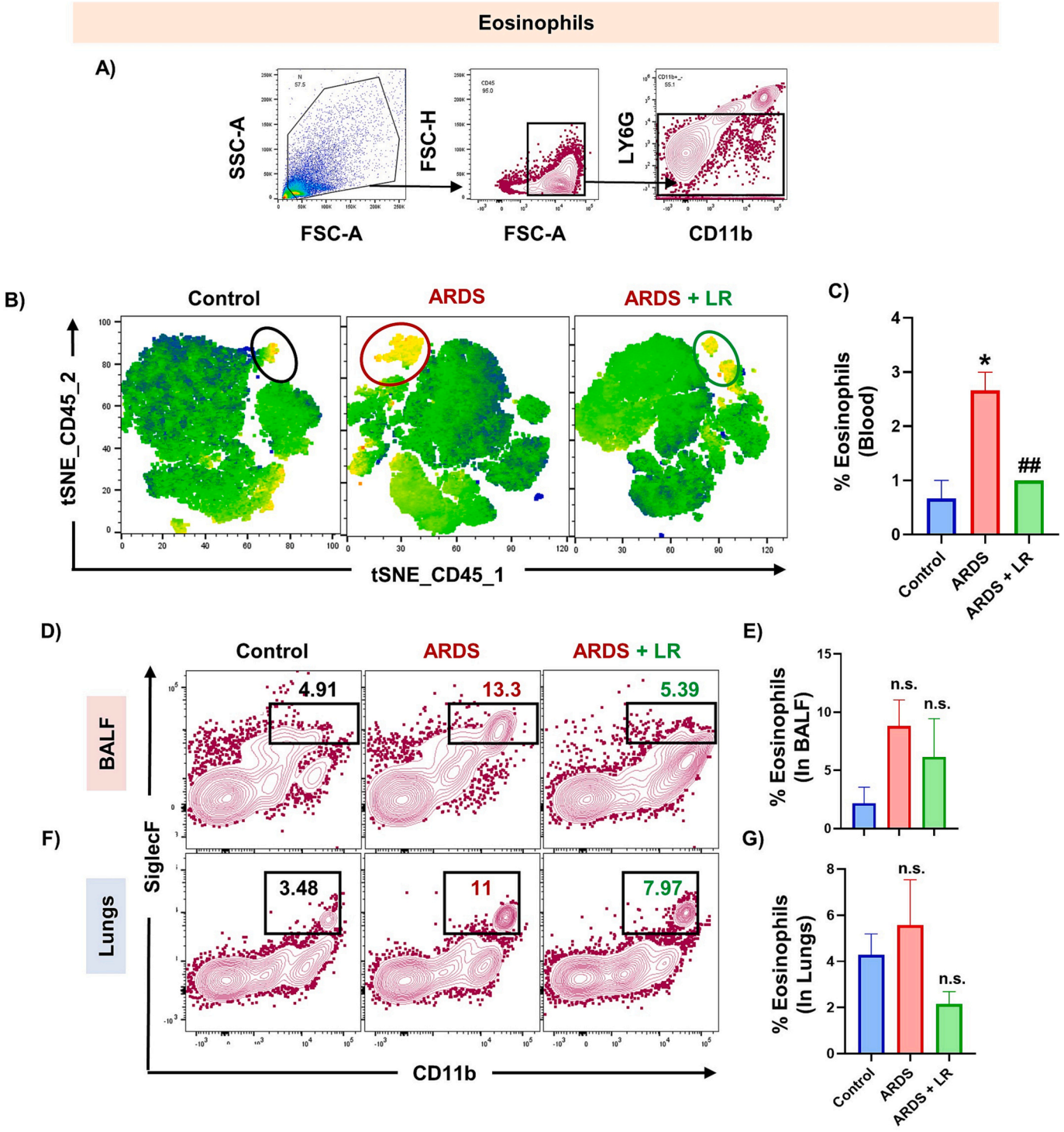

Fig. 3. LR reduces Eosinophils ($CD45^+LY6G^-CD11b^+SiglecF^+$) population in BALF and lungs: A) Representative image of gating strategy followed for eosinophils ($CD45^+LY6G^-CD11b^+SiglecF^+$) in BALF and lungs. B) tSNE plot depicting the cluster of $CD45^+$ population expressing SiglecF. C) Bar graphs representing percentages of eosinophils in blood. D) Contour plots representing percentage of $LY6G^-CD11b^+SiglecF^+$ eosinophils gated on $CD45^+LY6G^-$ cells in BALF. E) Bar graphs representing percentages of eosinophils in BALF. F) Contour plots representing percentage of $LY6G^-CD11b^+SiglecF^+$ eosinophils gated on $CD45^+LY6G^-$ cells in lungs. G) Bar graphs representing percentages of eosinophils in lungs. Statistical significance was considered as (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$) with respect to indicated groups (*indicated comparison between control and ARDS; # indicate comparison between ARDS and ARDS+LR).

midline of abdomen and cecum was exposed carefully via incision. In order to induce mid-grade sepsis, cecum was ligated at the middle of the bottom and distal pole and was punctured with 23-gauge needle from the mesenteric to the antimesenteric direction. Further, a droplet of feces was squeezed out through the holes and then cecum was repositioned into the abdominal cavity. Finally, the peritoneal membrane and the skin were sutured separately in distinct layers. In control group, mice underwent the same surgical procedure with an only difference that cecum in this group remained unmanipulated [17].

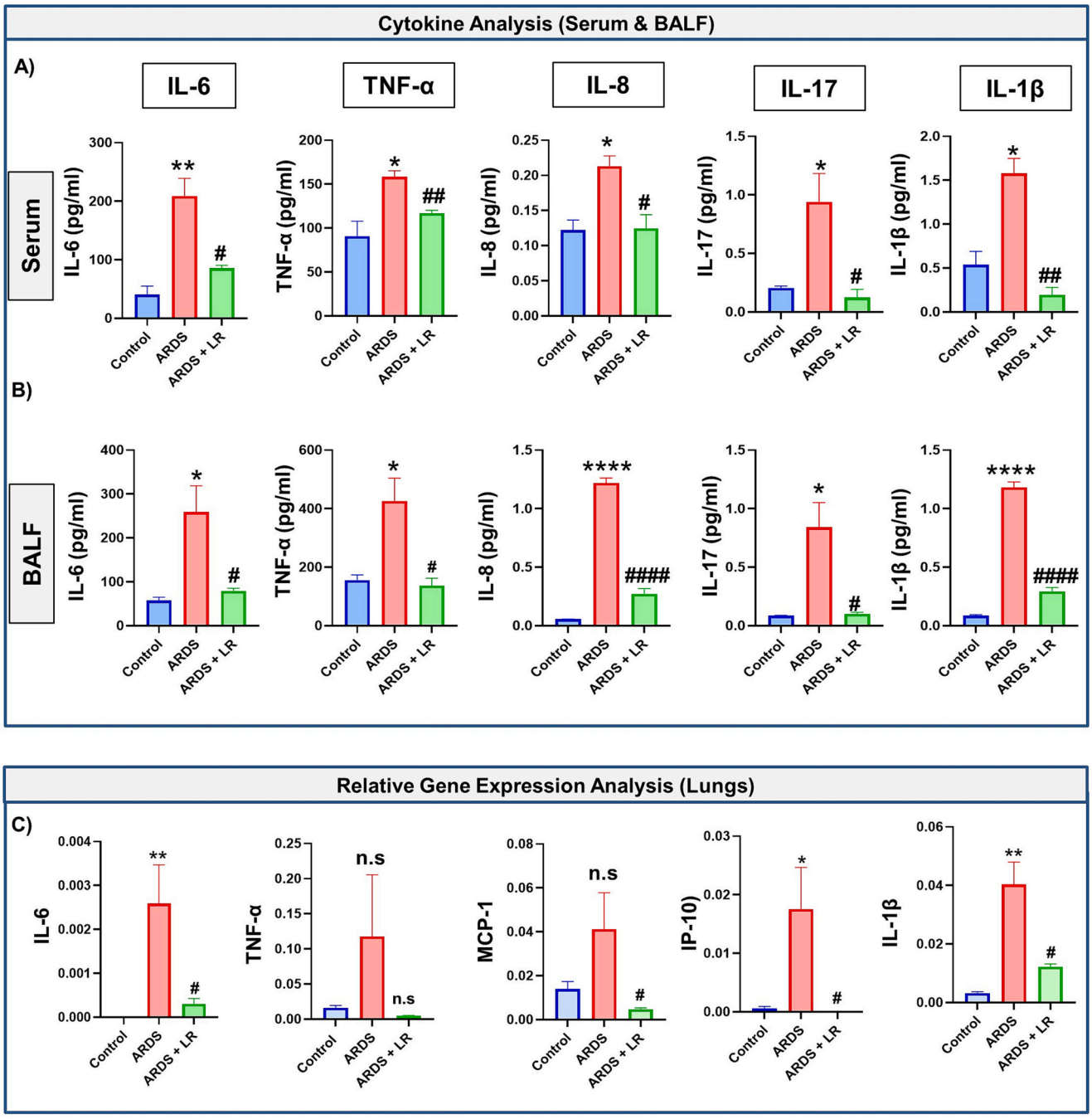

Fig. 4. LR modulates the expression of cytokines in serum and BALF: A) Levels of inflammatory cytokines in sera. B) Levels of anti-inflammatory cytokines in BALF. C) Relative expression of *IL-6, TNF-α, IL-1β, MCP-1 and IP-10* genes. Statistical significance was considered as (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001) with respect to indicated groups (*indicated comparison between control and ARDS; # indicate comparison between ARDS and ARDS+LR).

2.11. Neutrophil isolation from BM

For neutrophil isolation, mouse bone marrow cells (BMCs) were isolated from 8 to 12 wks old C57BL/6 J mice and by flushing the femoral and tibial bones with 1 X HBSS in 0.38% sodium citrate buffer. Further single cell suspension was prepared by gently disaggregating the bone marrow by repeated aspiration with syringe. After centrifugation, cells were layered on percoll gradient (72%, 64% and 52%) and centrifuged at 1545 xg for 30 min at RT (without breaks). Post centrifugation, gently aspirate the neutrophils (at the interface of 64%-72% percoll layer) and transfer the cells into separate tube and after washing cells were assessed for viability (100%) and purity (> 99.8%) and processed for the functional phagocytic assay.

2.12. L. rhamnosus bacterial culture

L. rhamnosus UBLR-58 (MTCC 5402) was cultured overnight at 37 °C in DeMan, Rogosa, Sharpe media (MRS, HiMedia) (Shaking off). After being subcultured onto new MRS media, the culture continued to grow until log phase (OD600nm = 0.4) (shaking off). After harvesting the cells, they were centrifuged for 10 min at 4000 rpm and washed with 1× PBS to eliminate any residual MRS broth. Furthermore, *L. rhamnosus* conditioned media (CM) was produced by resuspending the cells in RPMI-1640 medium and incubating them for 3 h at 37 °C with 60 rpm

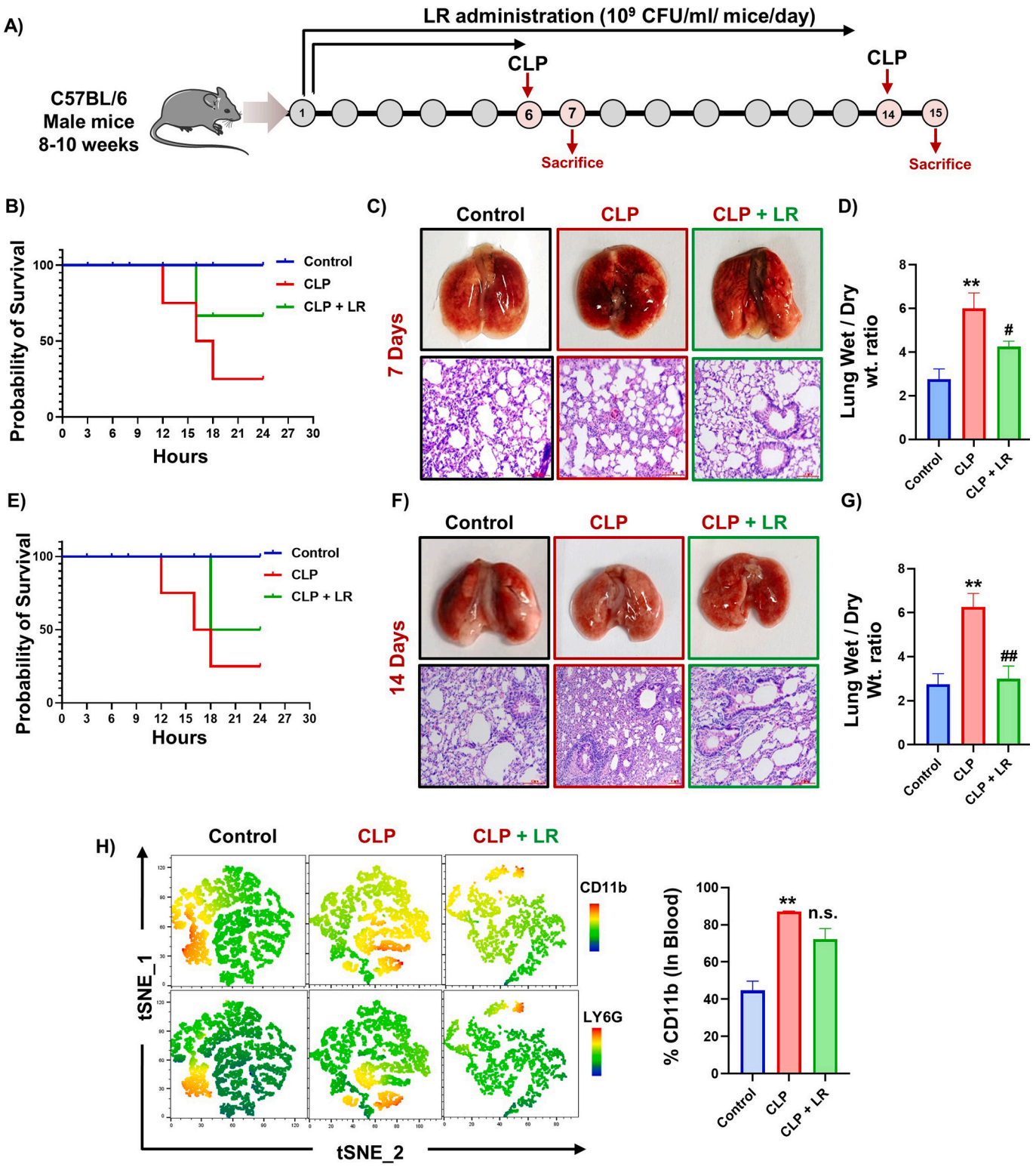

Fig. 5. LR ameliorates pathophysiology of extrapulmonary ARDS in CLP mice model: Mice were divided into 3 groups, viz., control, CLP, and CLP + LR groups. CLP + LR group received LR at 10^9 CFU/day (100 μl) orally for 7 and 15 days. A) At the end of 7 and 15 days, CLP mice model was developed and after 24 h mice were sacrificed, and various organs were harvested and analyzed for several immunological and histological parameters. B) Kaplan-Meier survival curve for 7 days. C) Photomicrographs representing morphology and histopathology of lungs. D) Bar graphs representing lung dry/wet weight ratio at 7 days. E) Kaplan-Meier survival curve for 15 days. F) Photomicrographs representing morphology and histopathology of lungs. G) Bar graphs representing lung dry/wet weight ratio at 15 days. H) tSNE representing the cluster of $CD11b^+$ and $LY6G^+$ Neutrophils cells in the $CD45^+$ cell population. Statistical significance was considered as (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$) with respect to indicated groups (* indicated comparison between control and ARDS; # indicate comparison between ARDS and ARDS+LR).

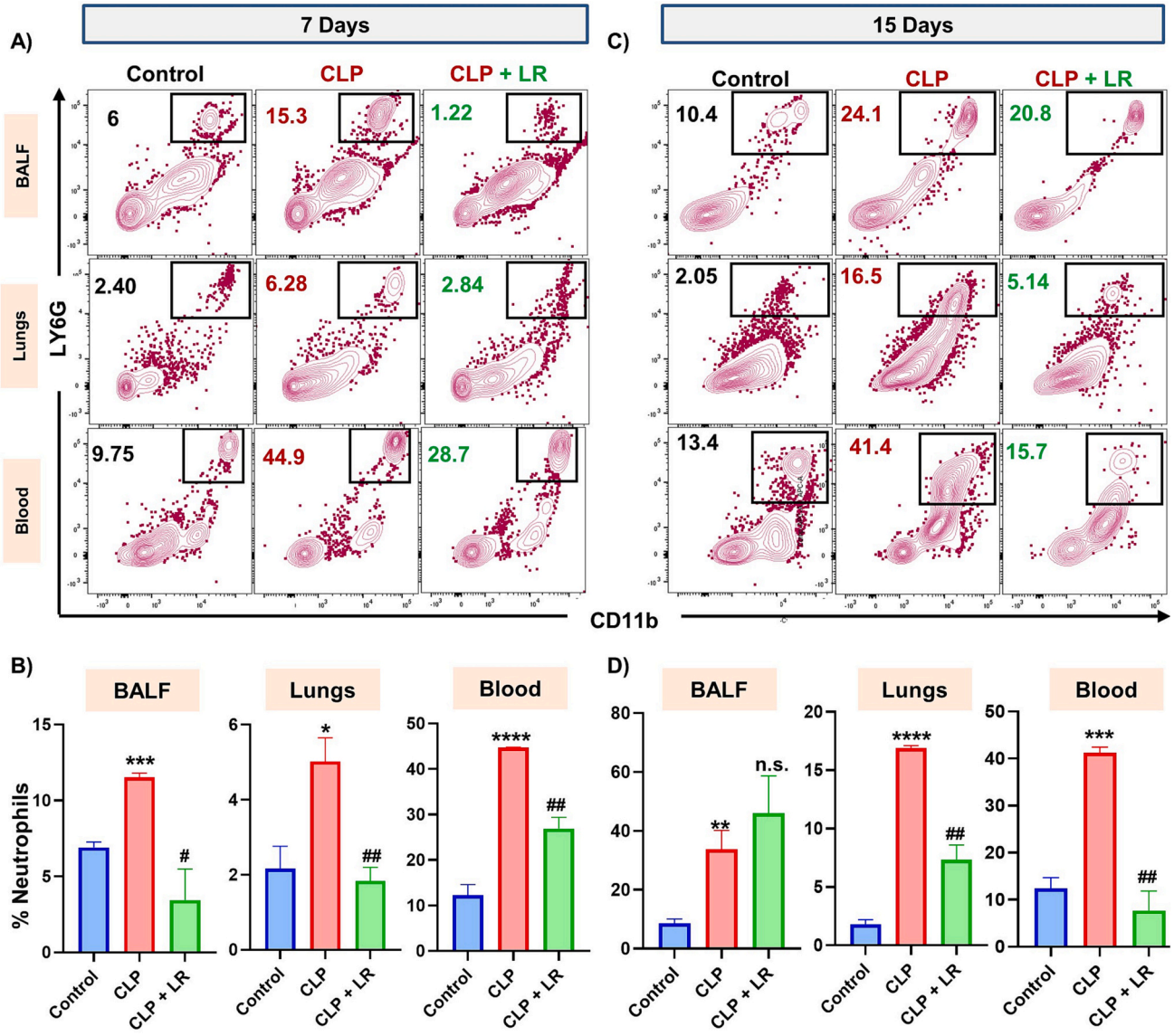

Fig. 6. LR ameliorates extrapulmonary ARDS in CLP mice model via modulating neutrophils: Mice were divided into 3 groups, viz., control, CLP, and CLP + LR groups. CLP + LR group received LR at 10^9 CFU/day (100 μl) orally for 7 and 15 days. A-B) Contour plot and Bar graphs representing percentages of $CD11b^+LY6G^+$ Neutrophils in BALF, lungs and peripheral circulation at 7 days. C—D) Contour plot and Bar graphs representing percentages of $CD11b^+LY6G^+$ Neutrophils in BALF, lungs and peripheral circulation at 15 days. Statistical significance was considered as (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001) with respect to indicated groups (* indicated comparison between control and CLP; # indicate comparison between CLP and CLP + LR).

orbital shaking. After pelleting the cells and neutralizing the pH, the supernatant was collected and filtered through a 0.22 μm filter. CM was used right away or kept in a freezer at −80 °C for further assays.

2.13. Phagocytosis assay

For phagocytosis assay neutrophils (> 99% purity) 2.5×10^5 were seeded on the 96 well plate precoated with the 1× HBSS (without calcium, magnesium, or phenol red) supplemented with the 10% autologous serum and incubated for 60′ at RT to facilitate the adherence of neutrophils. Post incubation, cells were treated with the LR-CM (1:1) and different SCFAs such as acetate, propionate, butyrate, and valerate at 0.5 mM concentration along with an opsonized Alexa Fluor tagged zymosan bioparticles (5 particles per viable neutrophils) and incubated at 37 °C for 60 min. After incubation cells were analyzed for phagocytosis of zymosan beads via fluorescence microscopy (Excitation 495 nm and Emission 519 nm) and flow cytometry.

2.14. NETs quantification

For the quantification of extracellular DNA concentrations in the cell-free supernatants we employed a NanoDrop spectrophotometer (BioTek Synergy H1) for assessing the DNA content in the culture supernatant of neutrophils post activation as per previously established protocols [18].

2.15. Statistical analysis

Statistical differences between the distinct groups were evaluated via student *t*-test paired or unpaired as required. All the values in the data are expressed as Mean ± SEM ($n = 6$). Statistical significance was determined as $p \leq 0.05$ (*p ≤ 0.05, ** $p \leq 0.01$, *** $p \leq 0.001$, *** $p \leq 0.0001$) with respect to the indicated groups.

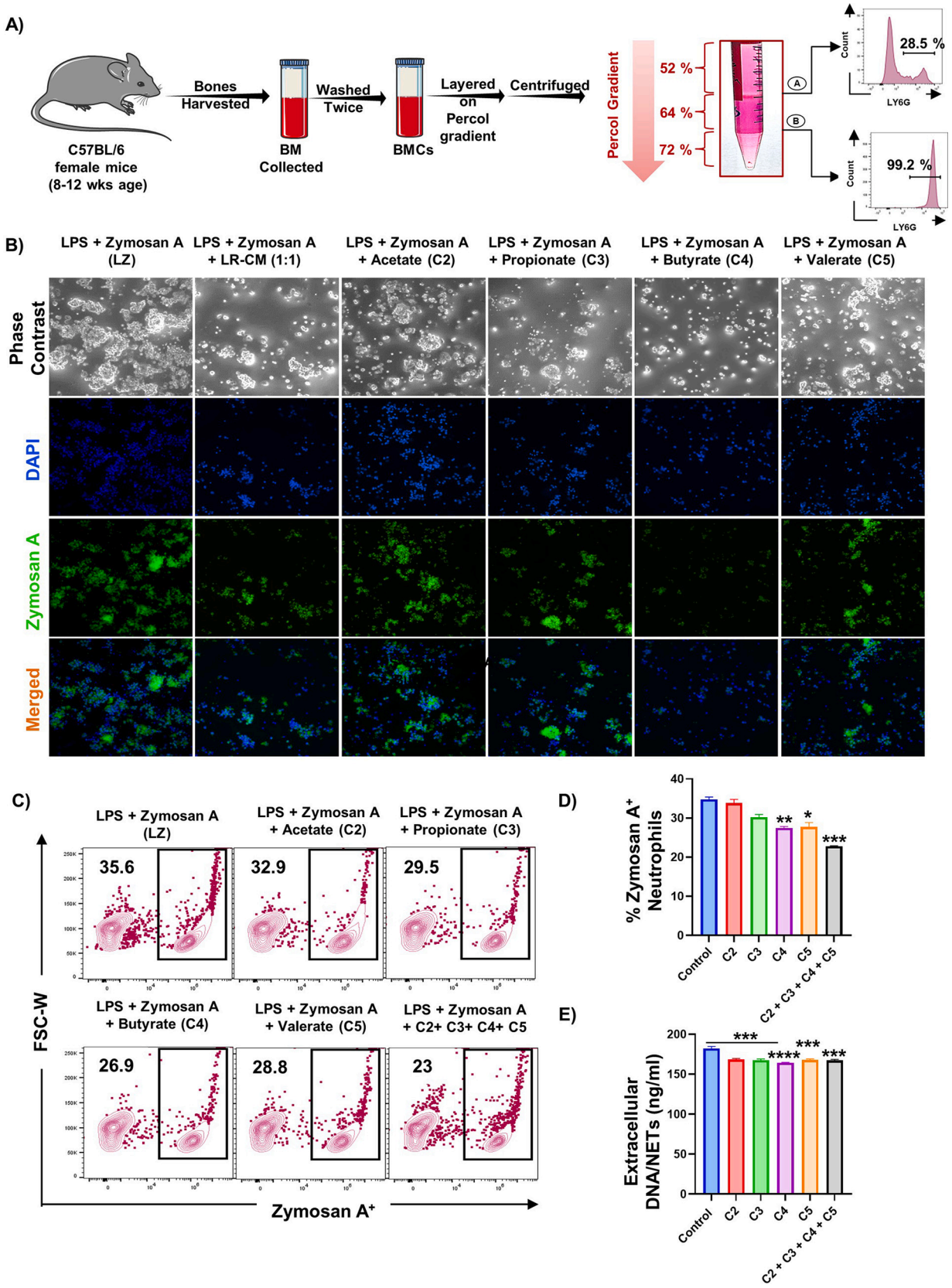

Fig. 7. LR and its active metabolite butyrate reduces the phagocytic potential of neutrophils: A) Experimental layout followed for the isolation of neutrophils. B) Neutrophils with >99% purity was cultured with the zymosan A beads in the absence and presence of LPS, *Lactobacillus rhamnosus*-conditioned media (LR-CM) (1:1), acetate (0.5 mM), propionate (0.5 mM), butyrate (0.5 mM) and valerate/pentanoate (0.5 mM) for phagocytosis assay C—D). Flow cytometry data representing percentage of Zymosan A^{+} Neutrophils. E) Extracellular DNA content in the medium. Statistical significance was considered as (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$) with respect to indicated groups.

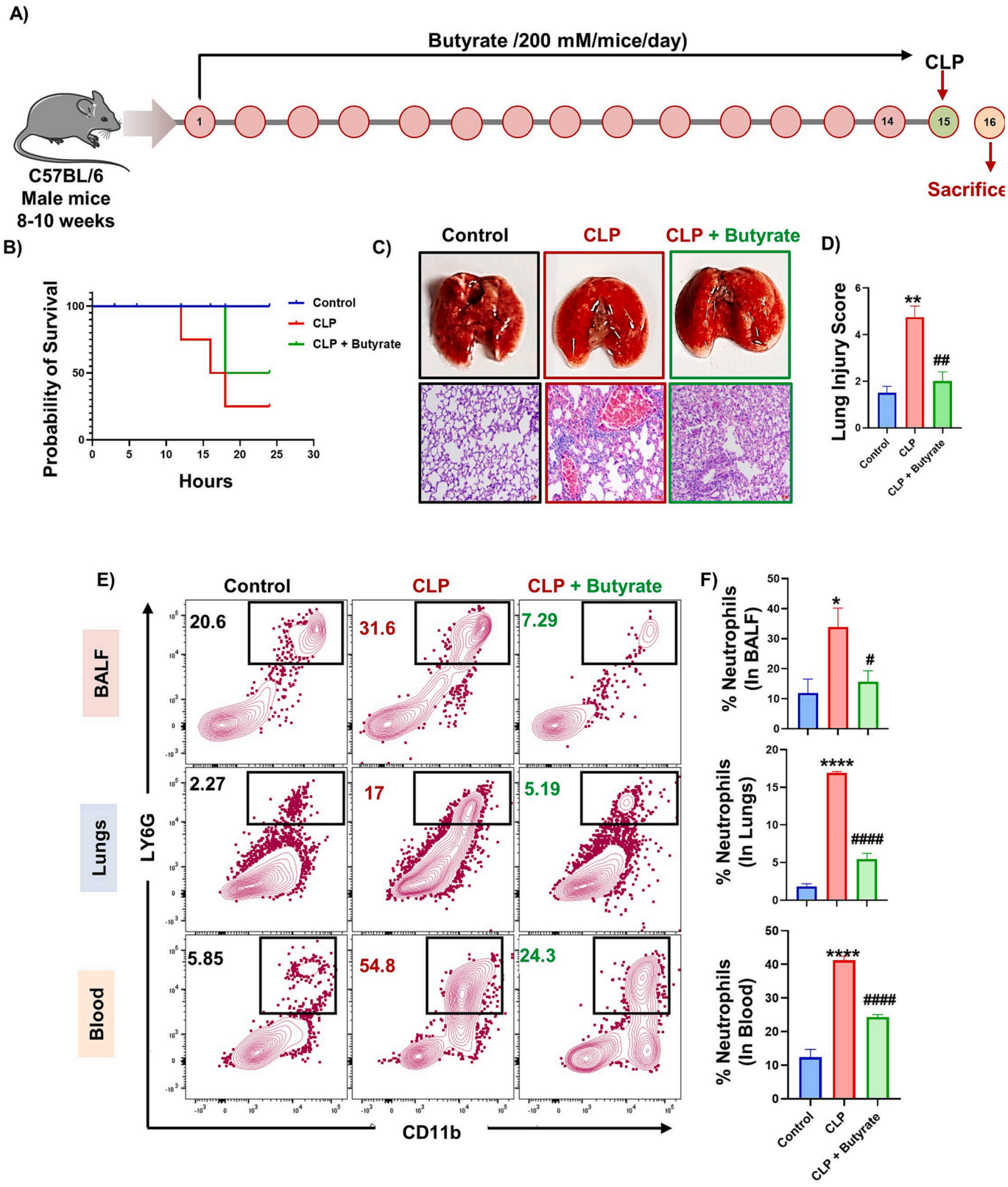

Fig. 8. Butyrate ameliorates extrapulmonary ARDS in CLP mice model: A) Mice were divided into 3 groups, viz., control, CLP, and CLP+ butyrate groups. CLP + LR group received butyrate i.p for 15 days. B) Kaplan-Meier survival curve for 15 days. C) Photomicrographs representing morphology and histopathology of lungs. D) Lung injury score. *E*-F) Contour plot and bar graphs representing the frequencies of neutrophils in BALF, lungs and BALF. Statistical significance was considered as (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001) with respect to indicated groups (* indicated comparison between control and CLP; # indicate comparison between CLP and CLP+ Butyrate).

3. Results

3.1. LR lowers pulmonary edema and maintains vascular permeability in pulmonary ARDS

Firstly, we assessed the prophylactic potential of LR in mitigating ARDS in LPS induced ALI mice model. For accomplishing the same, male BALB/c mice were randomly allocated into three groups viz. control/healthy, ALI/ARDS and ALI/ARDS + LR. In ARDS + LR group, LR (10^9 cfu) was administered orally for 14 days prior to LPS (3 mg/kg) instillation. After 24 h post-LPS instillation, mice were sacrificed and various tissues like blood, BALF and lungs were analyzed for different histological, vascular, and immunological parameters (Fig. 1A). LPS induced ALI/ARDS resulted in the enhanced pulmonary edema (Lung wet/dry weight) in comparison to the control group ($p < 0.05$), and LR administration significantly abrogated this as evidenced by the reduced wet/dry weight ratios in lungs ($p < 0.05$) (Fig. 1B-C). To further examine the status of lungs vascular permeability in response to LR administration we performed Evans-Blue extravasation assay. Interestingly, we observed that LR was able to significantly reduce the

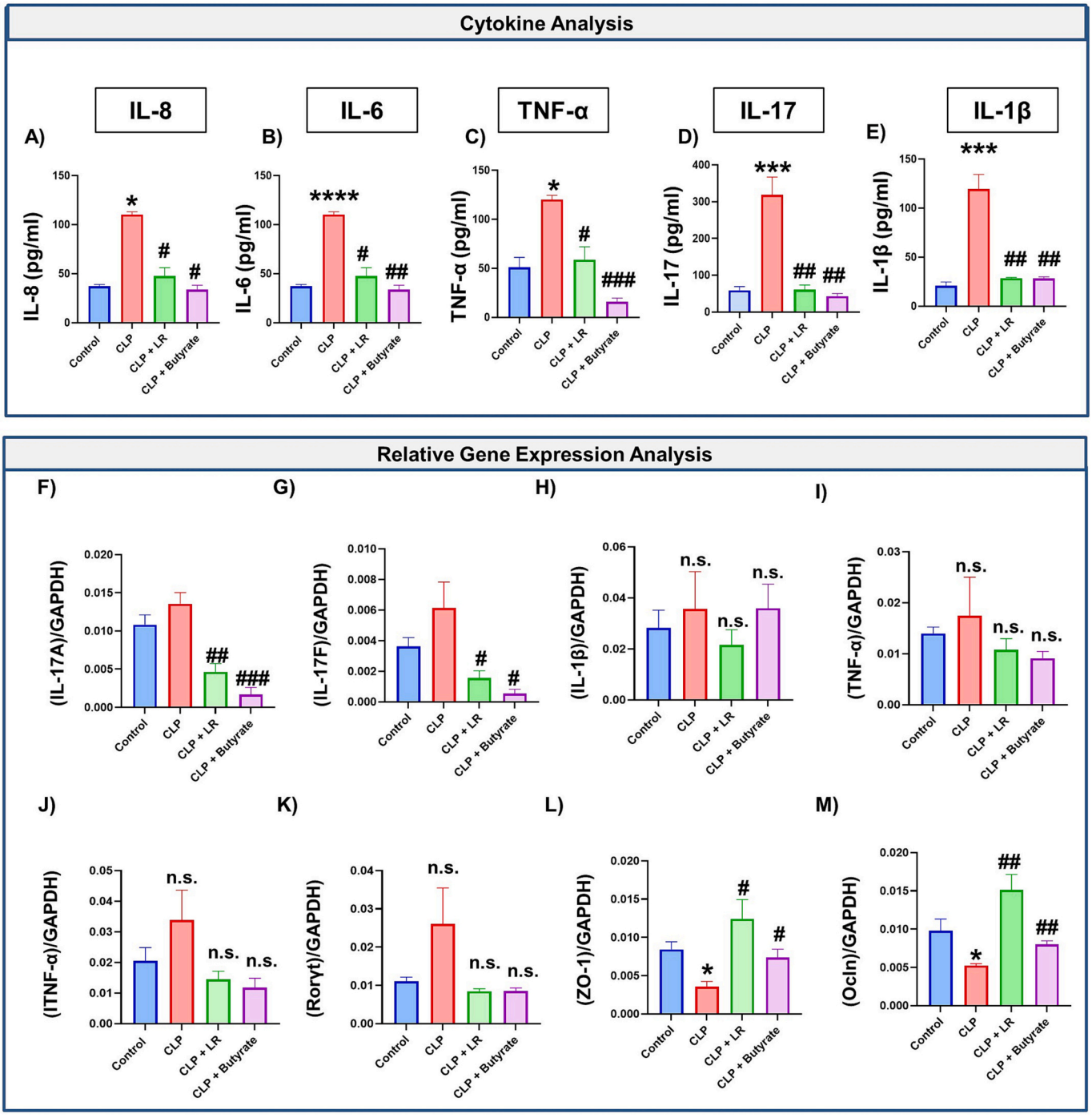

Fig. 9. LR and its active metabolite butyrate improves the gut integrity via modulating tight junction proteins in CLP mice model: A-E) Levels of inflammatory cytokines (IL-6, TNF-α, IL-8, IL-17 and IL-1β) in sera of all the mice of all the groups. F-M) Relative gene expression of inflammatory and tight junctional proteins in the lung tissue and large intestine. Statistical significance was considered as (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001) with respect to indicated groups (* indicated comparison between control and CLP; # indicate comparison between CLP and CLP+ Butyrate).

pulmonary vascular leakage ($p \leq 0.01$) in ARDS group (Fig. 1D). Furthermore, histological (H & E) staining and flow cytometry analysis revealed significant increase in the immune cell's infiltration, accumulation of fluid and alveolar hemorrhage in lungs parenchyma in ALI/ARDS group in comparison to the control. Lung injury score was observed to be significantly enhanced (5-fold; $p < 0.001$) in ALI/ARDS group with respect to the control group (Fig. 1E), and LR administration significantly reduced the lung injury in ARDS group (Fig. 1E). Moreover, tSNE plot flow analysis revealed significant reduction in the $CD11b^+$ cells (inflammatory marker) in LR group with respect to the ARDS group (Fig. 1F-G). These findings undoubtedly demonstrate that LR markedly improved the pulmonary pathophysiology of ALI/ARDS by reducing oedema and alveolar hemorrhage in the lung parenchyma by preventing the infiltration of inflammatory immune cells.

3.2. LR modulates neutrophils in ALI/ARDS

Evidences suggest that acute lung damage is largely caused by activated neutrophils recruited to the lung tissues from the peripheral circulation [19,20]. We thus next assessed the status of neutrophils in the various tissues. Embedded tSNE plot immunoprofiling further led to the identification of a unique cluster of LY6G expressing $CD45^+$ cells in ARDS group and LR administration was able to reduce the cluster of LY6G expressing neutrophils in ARDS group (Fig. 2A-B). Accordingly, it was further observed that the LR-administered ARDS group's differential neutrophil count in the blood was significantly lower than that of the ARDS group (Fig. 2C). Additionally, the percentage of neutrophils ($CD45^+LY6G^+CD11b^+$) in the lungs and BALF were significantly reduced in the ARDS + LR group (Fig. 2D-G). However, no alterations were observed in frequencies of eosinophils in BALF and lung (Fig. 3A-G). These results unequivocally point to LR's preventive potential in preventing ALI-ARDS via influencing neutrophils in the lungs, bronchial alveolar lavage fluid, and the periphery.

3.3. LR administration skews the expression of inflammatory cytokines and chemokines in ARDS

Trafficking of neutrophils and their inflammatory activity is regulated by the various inflammatory mediators such as cytokines and chemokines viz. IL-6, IL-1β, IL-8, IL-17, TNF-α and C-X-C motif chemokine ligand 10-CXCL10 [21–24]. Markedly, we observed that the levels of inflammatory cytokines such as IL-1β ($p < 0.01$), IL-6 ($p < 0.001$), IL-17 ($p < 0.01$) IL-8 ($p < 0.05$) and TNF-α ($p < 0.05$) were significantly reduced in both the serum and BALF of ALI/ARDS + LR administered group (Fig. 4A-B). Of note, the expression of several inflammatory cytokine's genes in lung tissue cells such as *Il-6, TNF-α, Il-1β* and *interferon gamma-induced protein-10 (IP-10)* also known as CXCL10 were observed to be significantly downregulated in ARDS + LR group (Fig. 4C). Moreover, the expression of monocyte chemoattractant protein-1 (MCP-1/CCL2) involved in the recruitment of neutrophils was observed to be upregulated in the ARDS group, and LR treatment significantly downregulated the same (Fig. 4C). These findings thus point to the potent role of LR in diminishing the expression of inflammatory cytokines implicated in neutrophil chemotaxis and activation in the pathophysiology of ALI/ARDS, thereby attenuating neutrophil-induced lung injury.

3.4. LR reduce the mortality and histopathological damage in septic peritonitis mice model

In parallel to pulmonary ARDS, extrapulmonary ARDS is one of the most dangerous and catastrophic consequences of sepsis and has a very high mortality rate. Following encouraging results in pulmonary ARDS, we next asked the question of whether LR could also be employed for the effective prophylactic treatment of extrapulmonary ARDS, observed in septic conditions. In order to determine the same, we next investigated LR's prophylactic potential in CLP mice model (mimics human septic condition). Male C57BL/6 mice were randomly allocated into three groups viz. control/healthy, CLP and CLP + LR. In CLP + LR group, LR (10^9 cfu) was administered orally for 7 or 14 days prior to CLP model development (Fig. 5A). On the following day of CLP, mice were sacrificed and BALF, lungs and blood were analyzed for different histological, vascular, and immunological parameters. Remarkably, we found that LR pre-treatment (7 or 14 days) increased the likelihood of survival following CLP (Fig. 5B & 5E). After CLP surgery, sepsis in the peritonitis mice model led to the development of prominent histological lesions in the lungs (Fig. 5C). Remarkably, we observed that LR administration significantly decreased both the lung wet/dry ratio and lung injury in CLP mice (Fig. 5D, F and G). Embedded tSNE plot further confirmed the significant neutrophilic infiltration into the blood, lungs, and BALF of CLP mice group (Fig. 5H). We observed that pre-treatment of LR significantly normalized the frequencies of $CD11b^+LY6G^+$ neutrophils in the pulmonary system (BALF + Lungs) and peripheral circulation in comparison to the CLP mice model (Fig. 6A-D). These results suggest that LR treatment attenuates the aetiology of extrapulmonary and pulmonary ARDS by altering neutrophils.

3.5. LR suppresses neutrophil phagocytic potential via SCFAs

SCFAs are the crucial immunoregulatory molecules that have potential to dampen exaggerated inflammation [25,26]. Recent advancements demonstrated that probiotics including LR mechanistically mediate its effector functions via inducing the synthesis of SCFAs (mainly butyrate). Thus, we next proceeded to examine whether SCFAs may reduce the inflammatory environment, which would limit neutrophil infiltration and phagocytic capacity in pulmonary and extrapulmonary ARDS. When compared to the other SCFAs (acetate, propionate, and valerate), butyrate was the most effective at significantly lowering neutrophil phagocytic potential, as illustrated by substantially lower zymosan-A $beads^+$ neutrophils in ex vivo settings via both flow cytometry and immunofluorescence microscopy (Fig. 7A-D). Furthermore, we observed a substantial decrease in the release of extracellular DNA/NETs from LPS activated neutrophils in the cell free culture supernatant post-SCFAs treatment (with butyrate being the most effective) (Fig. 7E). Conclusively our data clearly suggest that SCFAs-butyrate being the most potent-are efficient in ameliorating the pathophysiology of both pulmonary and extra-pulmonary ARDS via targeting the phagocytic and NETs releasing potential of neutrophils.

3.6. Butyrate alleviates histopathological damage via restricting infiltration of neutrophils

To determine whether butyrate has the potential to solely mimic the beneficial effects of LR in preventing extra-pulmonary ARDS development, mice were pre-treated with butyrate for 14 days prior to CLP development. It was observed that butyrate treatment significantly enhanced the survival probability of CLP mice (Fig. 8A-B). Lung tissues were collected for further analysis and mice treated with butyrate displayed less severe disease, exemplified by reduced infiltration of inflammatory cells, minimal blood leakage into the interstitium, mild or least RBC obstruction and lower levels of histological lung injury score (Fig. 8C, D). Likewise, flow cytometric analysis further revealed that the frequencies of neutrophils ($CD11b^+$ $Ly6G^+$) were significantly reduced in CLP + butyrate treated mice group (Fig. 8E, F). Moreover, the expression levels of inflammatory cytokines involved in the chemotaxis and activation of neutrophils such as IL-8, IL-6, TNF-α were significantly reduced at both transcriptomic and protein level in the lungs and sera of CLP + LR and CLP + butyrate (Fig. 9A-K). Numerous studies indicate that the emergence of septic diseases may be facilitated by disturbance of the intestinal barrier. Since one of the main causes of extrapulmonary ARDS is sepsis, we next investigated whether LR/butyrate has the potential to restore alterations in the tight junction proteins in a septic environment. Strikingly, we observed that the expression of occludin

(Ocn) and ZO-1 tight junction proteins was significantly enhanced in CLP + LR/ butyrate group in comparison to CLP group (Fig. 9 **L-M).** Altogether, our results for the first time demonstrate the prophylactic and anti-inflammatory potential of LR/butyrate in ameliorating the pathophysiology of both pulmonary and extra pulmonary ARDS.

4. Discussion

The pathophysiology of ALI induced ARDS is initiated by various environmental challenges to the upper respiratory tract including viral (COVID-19, influenza, adenovirus, herpes simplex virus-HSV, cytomegalovirus-CMV), bacterial (*Mycobacterium tuberculosis*-M. tb), pollution (Allergens, Particulate Matter-PMs) etc. [1,27]. ARDS is a major cause of lung trauma, leading to severe morbidity and mortality [1]. Diffuse interstitial and alveolar edema, inflammatory cell infiltration, and the production of proinflammatory factors are characteristic features of ARDS, a severe lung inflammatory illness [1]. An improved prognosis results from reduced alveolar inflammation and restored barrier function. The exudative phase of ARDS is characterized by recruitment of inflammatory cells, especially neutrophils to the site of injury, which play a vital role in host defense [28]. ARDS usually develops in patients with predisposing conditions that induce systemic inflammatory response among which sepsis is the major cause (Matthay 2019). Sepsis occurs primarily due to bacterial (Gram-) infections in which LPS is the major component of their outer membranes [29], that promotes the development of ALI/ARDS [30]. LPS-induced animal models provide novel ways in elucidating mechanisms for multiple diseases underlying ARDS. Chen et al. reported that intranasal administration of LPS mimics the pathophysiology of human ALI/ARDS [31]. Thus, in the present study, we employed both pulmonary (direct) and extra-pulmonary (indirect) models of ARDS to study the immunopathology of ARDS.

Probiotic strains including *Lactobacillus* sp., *Bifidobacterium* sp., and their metabolites have been reported to lessen the disease severity in tuberculosis, pneumonia, and other respiratory viral infections. Recently, our group too has reported the immunomodulatory role of *Lactobacillus rhamnosus* in inflammatory conditions [13]. Experimental evidence suggests that administration of Lactobacillus can confer a beneficial role in various respiratory diseases including respiratory tract infections (RTIs), asthma, lung cancer, cystic fibrosis (CF) and COPD, thereby modulating respiratory immunity [32–34]. Our group has also reported that LR administration skews the balance of inflammatory and anti-inflammatory cytokines and alleviates the inflammation induced bone loss [13] Strikingly, there are >34 clinical trials already underway to evaluate the efficacy of probiotics administration in COVID-19 patients (https://clinicaltrials.gov/). Together, these studies highlight the importance of probiotics in alleviating various respiratory ailments. However, the potential immunological and cellular mechanism of LR and it's metabolites in ameliorating ALI/ARDS model is still warranted. Thus, in the present study, we explored the immunomodulatory potential of LR as a potent prophylactic therapy in both pulmonary and extra-pulmonary ARDS.

Disruption of vascular integrity results in increased movement of proteins and fluid across the lung epithelium/endothelium. Moreover, there is an accumulation of protein-rich inflammatory fluid in the fluid-free alveoli resulting in increased lung weight [35]. As a consequence, there is an increase in wet/dry lung weight ratio, an indicator of pulmonary edema [35]. Our experimental data demonstrated that

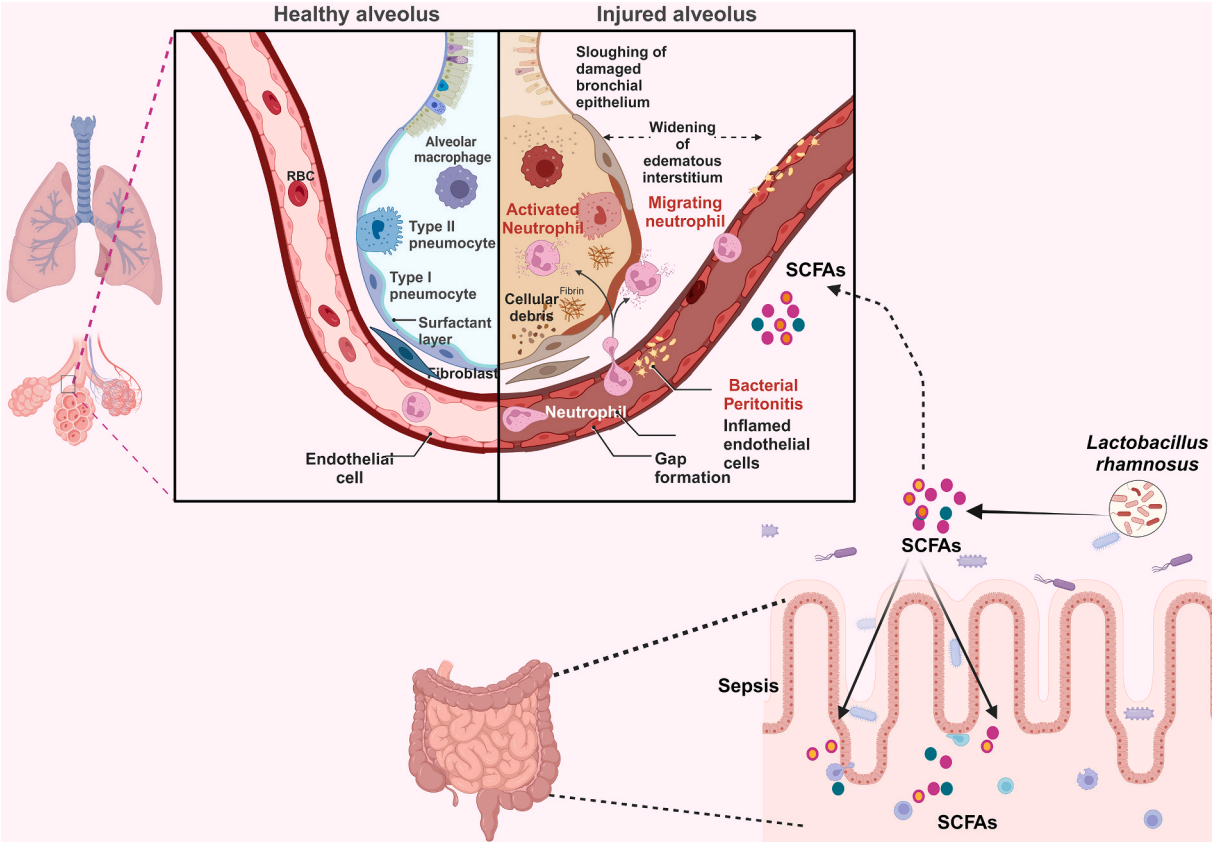

Fig. 10. Summary of the results: LR via inducing the production of short chain fatty acids mainly butyrate suppress the activation of neutrophils and thus ameliorates the histopathological lesion in the pulmonary and extrapulmonary ARDS.

pulmonary edema is drastically increased in ARDS group with respect to control group. Interestingly, pre-treatment of LR significantly decreased the pulmonary edema as evidenced by reduced lung wet/dry lung weight ratio which is further indicative of reduced fluid accumulation in lungs. The protective effect of LR against lung vascular barrier disruption was further assessed by measurement of lungs vascular permeability. Our Evans blue exclusion data clearly suggests that LR pretreatment significantly reduced the ALI induced vascular permeability in lungs. The improved vascular integrity was further supported by the significant reduction in the infiltration of immune cells in the lungs of ARDS + LR group.

Cytokines and chemotactic chemicals released by injured pulmonary tissues and inflammatory immune cells such as IL-1β, IL-6, IL-8, TNF-α are reported to induce the chemotaxis and activation of neutrophils to the site of inflammation [36]. Moreover, studies suggest that targeting these inflammatory cytokines could reduce the manifestations associated with the enhanced infiltration of neutrophils in various inflammatory diseases [21,23,37–39]. Our data clearly supports that LR pretreatment significantly reduces the expression levels of IL-1β, IL-6, IL-8, and TNF-α thereby ameliorating the clinical manifestations associated with ALI/ARDS. Specific receptors on neutrophils are responsible for identifying both endogenous and external inflammatory stimuli. This aids in the recruitment and activation of neutrophils to the site of injury. Several studies demonstrated that prolonged activation of neutrophils contributes to the observed tissue damage and lung dysfunction at inflammatory sites [19]. Of note, we too observed that LR pretreatment significantly reduced the frequencies of neutrophils in blood, lungs and BALF of pulmonary ARDS mice models. Altogether these data clearly suggest that via downregulating the expression of inflammatory mediators, LR improves the histopathological damage in the lung tissues of preclinical model of pulmonary ARDS.

Several studies reported that life-threatening sepsis-induced ARDS are also primarily caused by the excessive accumulation and activation of the neutrophils [17,40,41]. Strikingly, LR pretreatment provided efficient protection against the lung vascular barrier disruption observed in CLP mice model via significantly reducing the infiltration of neutrophils to the lungs. Moreover, we too observed that via skewing the levels of inflammatory cytokines at both transcriptomic and protein levels (such as IL-1β, IL-6, IL-17 A, IL-17F and TNF-α), in both BALF and lungs tissues, LR was able to suppress the activation and infiltration of neutrophils and thus preserved lung-vascular permeability in ARDS.

Over the past few decades, it has become apparent that LR via its gut associated metabolites viz. SCFAs may be crucial in the prevention and management of various inflammatory conditions such as gastrointestinal diseases, certain cancers, and metabolic syndromes [42–45]. A study reported that butyrate (HDAC inhibitor) reduces the mortality rate, improved the lungs pathology via suppressing inflammatory T cells [46,47]. In inflammatory bowel disease (IBD), it has been observed that butyrate suppressed the pathogenesis in IBD patients via inhibiting the NETs formation from neutrophils. The novel therapeutic potential of butyrate in the treatment of IBD is further attested by the fact that it inhibits neutrophils to produce inflammatory cytokines [48]. In consistent to this, we too observed that among all the LR associated metabolites, butyrate was found to be most efficacious in suppressing the phagocytic potential of neutrophils. NETs are a crucial part of the neutrophil's antimicrobial toolkit and a powerful way to engulf, restrain, and eliminate microbes. On the contrary, uncontrolled, or excessive NET release can harm neighboring cells and thus have detrimental role in the pathogenesis of several diseases. Interestingly, we observed that butyrate significantly reduced the formation of extracellular DNA/NETs from activated neutrophils. In CLP mice model, we further observed that butyrate treatment alleviates the alveolar damages and improve the pathophysiology of lungs along with reducing the mortality in the CLP mice via reducing the frequencies of neutrophils in the blood, lungs and BALF. Of note, the levels of inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) were also observed to be significantly reduced in the butyrate treated CLP mice.

Despite their structural differences, the gut and lungs are thought to be connected by intricate networks including their respective flora and possible anatomical interactions [49,50]. Septic ARDS has complicated and multifaceted aetiology. Systemic and local inflammatory responses can result from disruptions to the intestinal barrier function, changes to the gut microbiota, and translocation of the intestinal microbiome during the development of sepsis [51]. These events can further modify immune homeostasis in the systemic environment. Septic ARDS may be encouraged and perpetuated by disruption of immune homeostasis. In critically ill septic patients, it has been observed that failure of intestinal barrier leads to the systemic translocation of bacteria associated with multiple organ failure [52]. Tight junctional proteins such as occludin, claudins, and zonula occludens are vital for the preservation of epithelial barrier integrity [53]. In consistent to the previously reported studies, we observed that in CLP mice model the expression levels of occluding and ZO-1 were significantly reduced in comparison to the control group and both LR and butyrate treatment significantly reversed these in the colon tissues of CLP mice model.

Collectively, these investigations indicate that even a brief (2 weeks) prophylactic administration of probiotic-LR or its related metabolites, like butyrate, was successful in markedly reducing the pathophysiology of the lung in both pulmonary and extra-pulmonary ARDS. This is achieved by specifically targeting neutrophils' capacity to phagocytize and release NETs **(**Fig. 10**).** However, in the present study we only ventured onto the prophylactic role of LR and thus the therapeutic potential of LR still needs to be explored, thereby opening novel avenues for further research in the field.

Author contributions

RKS contributed to the conceptualization, formal analysis, funding acquisition, investigation, project administration, resources, supervision, validation and writing-original draft, review and editing. LS: contributed to data curation, formal analysis, methodology and writing-original draft. CS: data curation, formal analysis and methodology. SD: data curation and methodology. AS: methodology. PKM and AR: methodology and formal analysis. PKY: methodology. All authors reviewed the manuscript.

Compliance with ethical standards

All applicable institutional and/or national guidelines for the care and use of animals samples were followed.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

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