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Galectin-1 mediates interactions between polymorphonuclear leukocytes and vascular endothelial cells, and promotes their extravasation during lipopolysaccharide-induced acute lung injury

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Abstract

The lung airway epithelial surface is heavily covered with sialic acids as the terminal carbohydrate on most cell surface glycoconjugates and can be removed by microbial neuraminidases or endogenous sialidases. By desialylating the lung epithelial surface, neuraminidase acts as an important virulence factor in many mucosal pathogens, such as influenza and *S. pneumoniae*. Desialylation exposes the subterminal galactosyl moieties – the binding glycotopes for galectins, a family of carbohydrate-recognition proteins playing important roles in various aspects of immune responses. Galectin-1 and galectin-3 have been extensively studied in their roles related to host immune responses, but some questions about their role(s) in leukocyte recruitment during lung bacterial infection remain unanswered. In this study, we found that both galectin-1 and galectin-3 bind to polymorphonuclear leukocytes (PMNs) and enhance the interaction of endothelial intercellular adhesion molecule-1 (ICAM-1) with PMNs, which is further increased by PMN desialylation. In addition, we observed that *in vitro* galectin-1 mediates the binding of PMNs, particularly desialylated PMNs, onto the endothelial cells. Finally, in a murine model for LPS-mediated acute lung injury, we observed that galectin-1 modulates PMN infiltration to the

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Disclosures

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author statement

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lung without altering the expression of chemoattractant cytokines. We conclude that galectins, particularly galectin-1, may function as adhesion molecules that mediate PMN-endothelial cell interactions, and modulate PMN infiltration during acute lung injury.

Keywords

Galectin; acute lung injury; neuraminidase; desialylation; neutrophil adhesion; PMN extravasation

1. Introduction

The successful treatment of lower respiratory tract infections, especially pneumonia, remains a challenge. Even though the availability of effective antibiotics and vaccines has greatly reduced the number and mortality of these cases over the past few decades, it remains one of the leading causes of deaths from infectious diseases, accounting for 4.8% of total deaths worldwide (GBD 2013 Mortality and Causes of Death Collaborators, 2015). Respiratory viral infections are often accompanied by severe complications, such as the post-influenza bacterial superinfections (Alonso and Taha, 2003; Chen et al., 2012; Damjanovic et al., 2013), which often lead to acute lung injury (ALI) and organ failure, sepsis, and eventually, death (Arimori et al., 2013; Chertow and Memoli, 2013; Damjanovic et al., 2013; Manco et al., 2006).

The lung airway epithelial surface is heavily covered with sialic acids as the terminal carbohydrate of cell surface glycoconjugates (Nicholls et al., 2007; Ning et al., 2009). Sialic acids are widely distributed in animal tissues as gangliosides or glycoproteins on the cell surface. They are often used as the adhesion receptor by pathogens (e.g. influenza hemagglutinin) to facilitate cell entry (Luo, 2012; Nelli et al., 2010), provide nutrition for bacterial growth (e.g. pneumococcus) (Brittan et al., 2012; Siegel et al., 2014), and promote biofilm formation (Trappetti et al., 2009). Sialic acid content on the epithelial cell surface is a dynamic process modulated by two groups of enzymes: sialyltransferases, that transfer sialic acids onto the glycan as the terminal residue, and microbial neuraminidases or mammalian sialidases, that cleave the sialic acids off the glycoconjugates.

Neuraminidase is a key virulence factor in many mucosal pathogens such as influenza and pneumococcus (Brittan et al., 2012; Manco et al., 2006; McCullers and Bartmess, 2003), and is also released into the circulation during many microbial infections (Paul et al., 2011). During influenza infection, the viral neuraminidase cleaves the cell surface sialic acids to release mature virions from infected cells, a critical step for viral reinfection (Luo, 2012). As a result, airway epithelia in influenza-infected lungs are gradually desialylated (Nita-Lazar et al., 2015a; Siegel et al., 2014). In addition, primary and secondary pneumococcal infections also desialylate the airway epithelia, releasing the sialic acids as a nutrient for bacterial growth and biofilm formation (Brittan et al., 2012).

Previous studies have showed that desialylation of the cell surface alters multiple cellular functions and various aspects of the immune response, including interactions of adhesion molecules (Feng et al., 2011), cytokine production (N. M. Stamatos et al., 2010; Nicholas M. Stamatos et al., 2004), receptor signaling (Feng et al., 2012; Lillehoj et al., 2012;

Sakarya et al., 2004), neutrophil migration (Sakarya et al., 2004), wound healing (Alan S. Cross et al., 2012), and host-pathogen interactions (Lillehoj et al., 2012; N. M. Stamatos et al., 1997). Recently, we reported in a murine model that lung desialylation enhanced lipopolysaccharide (LPS)-mediated ALI (Feng et al., 2013). Experimental administration of LPS into the mouse lung induced edema, polymorphonuclear leukocyte (PMN) infiltration, and inflammatory cytokine and chemokine production, which are characteristic signs of ALI. Desialylation prior to LPS challenge increased edema and PMN infiltration, while the production of tumor necrosis factor (TNF)-α and the PMN-attracting chemokine (C-X-C motif) ligand 1 (CXCL1, KC) remained unchanged (Feng et al., 2013).

Removal of sialic acid from a glycoprotein unmasks the subterminal carbohydrate units, usually galactosyl moieties and its derivatives, which can be specifically recognized and bound by extracellular galectins (Ahmed et al., 1996; Di Lella, 2011; Feng et al., 2015; Gerardo R. Vasta et al., 2017). Galectins are a family of galactosyl-binding proteins, that mediate a multitude of biological processes, including regulation of apoptosis and inflammatory cytokine expression (Camby et al., 2006; F. T. Liu and Rabinovich, 2010). By recognizing endogenous galactosyl moieties on the surface of innate immune cells, galectins not only maintain homeostasis of inflammatory responses against infection (S. D. Liu et al., 2011), but can also promote cell adhesion and migration (Di Lella, 2011; G. R. Vasta et al., 2012). In contrast, by recognizing exogenous ligands on the surface of bacteria or enveloped viruses, galectins can function as a pattern recognition receptors (G. R. Vasta, 2009). Of the 15 galectins identified in mammals, the proto-type galectin-1 (Gal1) and chimera-type galectin-3 (Gal3) have been extensively studied in relation to host immune responses. In particular, expression of Gal1 is often up-regulated in the mouse lung during influenza or pneumococcal infection, and might have a protective role by binding to the virus envelope (Nita-Lazar et al., 2015b; Yang et al., 2011). Further, previous reports revealed that Gal3 also functions as a key factor during pneumococcal infection by promoting neutrophil adhesion to laminin *in vitro* (Kuwabara and Liu, 1996) and mediating PMN infiltration *in vivo* (Nieminen et al., 2005; Sato et al., 2002).

Both Gal1 and Gal3 share the β-galactosyl glycotope on N-linked complex-type glycans, with different preferred ligands (Stillman et al. 2006; Stowell et al. 2008) and binding avidities. The prototype Gal1 usually forms a homodimer, while chimera-type Gal3 often binds as a trimer or pentamer with high affinity repeat units of galactose and lactosamines that Gal1 does not recognize (Iwaki and Hirabayashi, 2018). In this study, we examined *in vitro* the potential roles of Gal1 in adhesion of PMNs to endothelial cells, and compared this to those mediated by Gal3. Further, we explored the roles of Gal1 in LPS-mediated PMN infiltration *in vivo* using Gal1 knockout mice. We demonstrated that both Gal1 and Gal3 bind to PMNs, enhanced the interaction of endothelial intercellular adhesion molecule-1 (ICAM-1) onto PMNs, and these effects were further increased by PMN desialylation. However, we found that on a molar basis, Gal1, more effectively than Gal3, mediated PMN binding onto endothelial cells *in vitro*, and modulated the LPS-mediated PMN infiltration *in vivo* without altering the expression of chemoattractant cytokines in the lung.

2. Materials and Methods

2.1. Reagents

LPS (*E. coli* O55:B5) and *C. perfringens* neuraminidase type X (*Cp*NA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluorescein isothiocyanate (FITC)-conjugated peanut agglutinin (PNA) was purchased from EY Laboratories (San Mateo, CA, USA); biotinylated *Maackia amurensis* lectin II (MALII) lectins were purchased from Vector Laboratories (Burlingame, CA, USA); and ICAM-1-Fc fusion proteins were purchased from R&D Systems (Minneapolis, MN, USA). Human recombinant Gal1 and Gal3 were prepared as previously reported (Nita-Lazar et al., 2015a) or purchased from R&D Systems.

2.2. Mice

C57BL/6 mice were purchased from Jackson Laboratory. Gal1 knockout mice on a C57BL/6 background were kindly provided by Dr. Noorjahan Panjwani, Tufts University School of Medicine. Mice were maintained and bred in the UMB animal facility under UMB IACUC approved protocols. The knockout mice were mated with C57BL/6 mice to generate heterozygous littermates, which were back-crossed with Gal1 knockout mice to obtain age-matched homozygous knockouts and heterozygous littermates for the experiments. The offspring were genotyped by PCR using specific primers (wildtype forward: GAC CCC ATC CCT ACA CCC CAG; knockout forward: CTA TCA GGA CAT AGC GTT GG; common reverse: AAA CTT CAG CCG GGA GAA AGG) to be identified as either knockouts or heterozygotes. Age and sex-matched mice were used in each experiment.

2.3. PMNs, endothelial cells and treatment

PMNs were prepared from the peripheral blood of healthy volunteers as previously described (Feng et al., 2011), under a protocol approved by the University of Maryland School of Medicine Institutional Review Board (Baltimore, MD, USA). Freshly isolated PMNs were treated with either *Cp*NA (10 mU/ml) or Hanks' Balanced Salt Solution (HBSS) without Ca^{2+} Mg^{2+} (untreated control) for 1 hour as previously described (Feng et al., 2011). Human lung microvascular endothelial cells (HMVEC)-Ls (Lonza, Rockland, ME) were seeded and grown on 24 well plates in DMEM medium 1 to 3 days to reach 90% confluency. In selected experiments, the HMVEC-L monolayers were pre-incubated for 1 h with either *Cp*NA (10 mU/ml) or medium alone as control and washed, after which they were incubated with PMNs for the adhesion assay.

2.4. Adhesion assay

*Cp*NA-treated or untreated PMNs were labeled with calcein AM for 30 min as previously described (Feng et al., 2012) and washed thoroughly with HBSS, before placing them onto 24-well plates grown with HMVEC-L monolayer. After 1 h of incubation, the non-adherent cells were removed with three washes of HBSS and the fluorescence intensity from each well was measured. In parallel, the fluorescent intensity from a serial dilution of labeled cells was measured to generate the regression curve (fluorescent intensity vs cell number) for calculating the percentage of the PMNs adherent to HMVEC-L cells from each sample.

2.5. Flow cytometry

For galectin binding, PMN cells were incubated with human recombinant Gal1 or Gal3 at 5 μg/ml or the indicated concentration. The binding of galectins onto PMN cells was detected by galectin-specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by FITC labelled anti-rabbit antibody (Sigma). For the binding of ICAM-1, PMN cells were incubated with ICAM-1-Fc in the presence or absence of human recombinant Gal1 or Gal3 at 1 or 5 μg/ml for 1 hour. The binding of ICAM-1 was detected by FITC labelled anti-Fc antibody (Jackson ImmunoResearch Inc., West Grove, PA, USA). Cells were then washed two times and analyzed on a MoFlo cytometer (Beckman-Coulter, Indianapolis, IN, USA).

2.6. LPS challenge

Animals were challenged intratracheally with 5 μg of LPS as previously described (Feng et al., 2013). Bronchoalveolar lavage fluid (BALF) was collected after 16 hours and the leukocyte count from each animal was determined with hemocytometer and trypan blue exclusion for viability. Total protein in BALF was quantified with Protein Assay Dye Reagent (Bio-Rad); Gal1, KC, TNF-α, and Interleukin-1-beta (IL-1β) levels in BALF were quantified by ELISA as previously described (Feng et al., 2013).

2.7. Western blot and ELISA

Proteins in the BALFs were separated on SDS-PAGE gels (4– 15%; Bio-Rad Laboratories, Hercules, CA, USA) and transferred to PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% milk in TBS and blotted with anti-Gal1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by HRP-conjugated anti-mouse IgG (Pierce, Rockford, IL, USA). Blots were developed with SuperSignal West Pico chemiluminescent substrate (Pierce). The concentration of galectin-1 in BALFs was also determined with Human Galectin-1 Quantikine ELISA Kit (R&D Systems) according to the manufacturer's recommendation.

2.8. Statistical analysis

Statistical significance was calculated using Student's t test, or two-way ANOVA by Prism Graphpad version 7, wherever it was appropriate; p values <0.05 were considered as significantly different. For flow cytometry data, the mean fluorescent intensity (MFI) of each sample and its standard deviation (SD) were obtained by Winlist (Verity Software House, Topsham, ME, USA).

3. Results

3.1. Desialylation of endothelial cells and PMNs increases galectin binding.

The subterminal galactosyl moieties exposed by desialylation of glycans are the preferred ligands for galectin binding. In a previous *in vitro* study, desialylation with *Cp*NA treatment of HMVEC-L cells or PMNs significantly increased PMN binding, with a significant synergistic effect after treatment of both HMVEC-L and PMN cell populations (Sakarya et al., 2004). This suggests that desialylation exposed binding ligands on both PMN and HMVEC-L cells to mediate the PMN adhesion onto the endothelia. Moreover, prior

desialylation enhanced PMN infiltration in LPS-mediated ALI (Feng et al., 2013). However, the potential role of galectins in these studies had not been explored.

First, we examined the effect of desialylation on the Gal1 and Gal3 binding to endothelial cells and PMNs. To evaluate the effect of desialylation of the cell surface, an HMVEC-L monolayer was treated with *Cp*NA, and stained with either PNA, that recognizes a galactosyl (β−1,3) N-acetylgalactosamine structure, or MALII, that binds sialic acid in α−2,3 linkage. Increased binding of PNA and decreased binding of MALII to the *Cp*NA-treated cells (Figure 1A) confirmed the enzymatic desialylation of the cell surface, with exposure of the subterminal galactosyl moieties. PNA bound to HMVEC-L cells even in the absence of *Cp*NA treatment which suggests that some galactose moieties were exposed without terminal sialic acids on untreated cells. As expected, both Gal1 and Gal3 bound to HMVEC-L cells even in the absence of *Cp*NA treatment (white bars) (Figure 1B, 1C). While binding of Gal1 to HMVEC-L cells was increased marginally by *Cp*NA-treatment (dark bars) ($p=0.052$), *Cp*NA-treatment had no significant effect on Gal3 binding (Figure 1B,C). Binding of both Gal1 and Gal3 to untreated and *Cp*NA-treated HMVEC-L cells was significantly decreased by addition of lactose, a specific inhibitor of galectin binding, confirming that galectin binding was glycan-dependent and specific. In contrast, binding of both Gal1 and Gal3 to *Cp*NA-treated PMNs, was significantly increased relative to the untreated (Figure 1D). As observed with HMVEC-L cells, the binding of both galectins to untreated and *Cp*NA-treated PMNs could be significantly inhibited by lactose (data not shown). These data confirmed that both Gal1 and Gal3 bind to endothelial HMVEC-L and PMN cells via pre-existing galactosyl moieties, evidenced with already high PNA binding before desialylation, and the binding is significantly enhanced by cell surface desialylation, except for the binding of Gal3 to HMVEC-L cells.

3.2. Galectins enhance ICAM-1 binding onto desialylated PMNs.

The interaction between ICAM-1 on endothelial cells and β2-integrin on PMNs is a critical step for PMN extravasation/transmigration (Diamond and Springer, 1993; Hynes, 2002). We previously demonstrated that desialylation enhances ICAM-1 binding onto PMNs (Feng et al., 2011), and as described above, also increases binding of Gal1 or Gal3 (Figure 1D). Thus, we examined the possibility that recombinant Gal1 and/or Gal3 could modulate the interaction of ICAM-1 with PMNs. While a low concentration (1 μg/ml) of Gal1 (0.067 μM) or Gal3 (0.038 μM) didn't show a significant effect on ICAM-1 binding onto untreated PMNs (white bars), the binding was significantly enhanced at higher concentration (5 μg/ml) of either Gal1 (0.333 μM) or Gal3 (0.192 μM) (Figure 2A,B). ICAM-1 binding, however, was significantly increased onto *Cp*NA-treated PMNs (dark bars), even at the low concentration of Gal1 (1 μg/ml) but not Gal3 (Figure 2A,B), with a significant interaction effect ($p<0.0001$, two-way ANOVA). The binding was inhibited by lactose, confirming the glycan-mediated, specific interaction (Figure 2C&D). These results suggested that galectin-1 and −3, enhanced by desialylation, can mediate the interaction of ICAM-1 with its ligands on PMNs, a key step for PMN extravasation.

3.3. Galectin-1 mediates PMN adhesion onto endothelial cells more effectively than Galectin-3.

As both Gal1 and Gal3 modulate the ICAM-1 binding onto PMNs, we investigated if they could enhance the PMN adhesion onto endothelial cells, which is mostly mediated by the interaction of ICAM-1 with β2-integrin (Diamond and Springer, 1993; Zhou et al., 1998). In the presence of 5 μg/ml of Gal1 (0.333 μM), the untreated PMN binding to the HMVEC-L cell monolayer was significantly increased ($p=0.0006$), which was further enhanced with *Cp*NA-treatment of PMNs ($p=0.0088$) (Figure 3A). In contrast, the presence of Gal3 (5 μg/ml = 0.192 μM) did not significantly increase the binding of untreated or *Cp*NA-treated PMNs to the HMVEC-L cell monolayer (Figure 3A). To directly compare the Gal1 and Gal3 in this process, we repeated the experiment with same molar concentration of galectins up to 1 μM (Figure 3B). Gal1 showed an optimal concentration (0.3 μM) in mediating the PMN adhesion, while Gal3 did not increase the PMN adhesion significantly up to a concentration of 1 μM. These data suggested that Gal1 could mediate PMN adhesion onto endothelial cells, the critical initial step for PMN infiltration/extravasation, while Gal3 did not, even at higher concentrations.

3.4. Endogenous galectin-1, expressed in LPS-challenged mouse lung, promotes LPS-mediated ALI.

PMN infiltration is one of the most important outcomes of LPS-mediated ALI (Feng et al., 2013; Marini and Evans, 1998; Matute-Bello et al., 2008). Because PMN adhesion to the endothelium is a critical step for PMN infiltration and Gal1 promotes PMN adhesion, we reasoned that ALI-mediated PMN infiltration would decrease in the absence of the Gal1. First, we determined if endogenous Gal1 was present in the lung and whether LPS challenge altered its expression. In BALF from the control (PBS) mice, the protein level of Gal1 was barely detectable by Western blot. However, upon LPS challenge the Gal1 protein level was increased in mouse lung (Figure 4A,B), even though no difference in mRNA level was observed (data not shown).

Next, we examined if endogenous Gal1 plays a role in LPS-mediated ALI by comparing the PMN cell count in BALF of Gal1-knockout mice and their heterozygous littermates, 16 h after LPS challenge. The BALF cell count was significantly lower in Gal1 knockout mice as compared to their control littermates (Figure 5A), suggesting the positive effect of Gal1 in PMN infiltration into the lung upon LPS challenge. In contrast, no differences were found in levels of total protein (Figure 5B), KC, IL-1β, or TNF-α in the BALF (Figure 5C), suggesting that despite the expression of PMN recruitment mediators, in the absence of Gal1, PMNs could not optimally traverse the pulmonary vascular endothelial cells into the BALF.

4. Discussion

The binding of galectins to cell surface carbohydrate ligands modulates a variety of biological functions (Gerardo R. Vasta et al., 2017). Most importantly, Gal1 maintains homeostasis in inflammatory responses against infection (S. D. Liu et al., 2011). This probably reflects a negative feedback mechanism, as it was reported that Gal1 is up-

regulated during infection, e.g. in the lungs of influenza virus-infected mice (Yang et al., 2011), and the infection-induced Gal1 reduces syncytia formation and progeny virus production (Garner et al., 2010; Garner et al., 2015; Levroney et al., 2005). Here, we observed that upon challenge with LPS, a component of gram-negative bacteria, Gal1 production was upregulated in the lung (Figure 4).

Galectins might act as pattern recognition receptors, by recognizing glycan moieties on the microbial surface, or serve as adhesion molecules for cell attachment and migration by binding to endogenous ligands (Di Lella, 2011; G. R. Vasta, 2009; G. R. Vasta et al., 2012). We have reported that Gal1 could crosslink *S. pneumonia*e to the airway epithelial surface, thereby enhancing pneumococcal adhesion (Nita-Lazar et al., 2015a). Previous reports suggested that Gal3 promoted neutrophil adhesion (Kuwabara and Liu, 1996; Sato et al., 2002) and during pneumococcal infection, mediated PMN infiltration into the lung (Nieminen et al., 2005; Sato et al., 2002). More recent reports described the role of Gal3 in enhancing neutrophil motility and extravasation into the airway during fungal infection (Snarr et al., 2020) or LPS respiratory challenge (Humphries et al., 2022; Humphries et al., 2021). In the present study we observed that although both Gal1 and Gal3 mediated the binding of ICAM-1 onto PMNs, on the basis of molar comparisons it is mostly Gal1 that may serve as the mediator of the PMN-endothelium interaction, a critical step in leukocyte infiltration/extravasation into infected or inflamed tissues. Therefore, we anticipated that increased Gal1 expression during infection or inflammation would promote the leukocyte infiltration/extravasation. Consistently, upon LPS challenge Gal1 knockout mice demonstrated less PMN infiltration than their control heterozygous littermates. Further, because there was no difference between the two groups in the expression of the chemokine KC or the inflammatory cytokines TNF-α and IL-1β, it is likely that Gal1 plays a major role in mediating PMN infiltration mainly through enhancing the PMN-endothelium interaction.

Interestingly, in this study Gal3 showed less of an effect in mediating ICAM-1-PMN and PMN-HMVEC-L interaction than did Gal1, even though it bound strongly to PMNs and HMVEC-L cells. Although both Gal1 and Gal3 share the β-galactosyl glycotope on N-linked complex-type glycans as a preferred ligand, Gal3 can also bind with high affinity a broad spectrum of glycans on the cell surface or cellular matrix, including repeat units of galactose and lactosamines which Gal1 does not recognize (Iwaki and Hirabayashi, 2018). Binding of Gal3 to these repeat glycotopes facilitates their clustering and Gal3-mediated lattice formation at the cell surface(Nabi et al., 2015). This difference apparently contributes to the distinctive biological functions of these two galectins, such as the PMN adhesion we observed. Also, unlike Gal1, Gal3 binding to HMVEC-L cells did not change after desialylation of the cell monolayer, suggesting that either Gal3 binding to certain ligands on HMVEC-L cells is not affected by the presence of terminal sialic acid, or that Gal3 binds to glycans lacking terminal sialic acid. This observation further supports previously reported observations that Gal1 and Gal3 bind to different ligands on the cell surface (Stillman et al., 2006; Stowell et al., 2008). How different ligands of Gal1 and Gal3 on PMNs and endothelial cells contribute to the differences in adhesion activity and PMN infiltration remains to be determined.

While increased PMN infiltration leads to neutrophil-mediated microbicidal responses, it may also lead to collateral lung injury, as a result of the generation of reactive oxygen species or the release of proteolytic enzymes (Lee and Downey, 2001). Therefore, reduced PMN recruitment is expected to be accompanied by a protective effect against tissue injury in ALI. To the contrary, we found no differences in the levels of lung permeability between Gal1 knockout and wild type animals, as estimated by total proteins in BALF. This observation suggests that lung damage may not be directly proportional to PMN infiltration alone. Gal1, as an immunosuppressive factor, may provide a protective action to minimize the tissue damage (Camby et al., 2006; S. D. Liu et al., 2011; Rodrigues et al., 2019). Thus, it can be speculated that without the protective effect from Gal1, PMNs in the Gal1 knockout mice may cause more harm than those in the wild type mice. Therefore, fewer infiltrated PMNs in the Gal1 knockout mice may have led to a similar degree of tissue damage as that observed in the wild type mice. Whether this interpretation is correct remains to be determined.

The desialylation of airway epithelia takes place in many respiratory pathogen infections, such as with influenza and pneumococcus (Brittan et al., 2012; Nita-Lazar et al., 2015a; Siegel et al., 2014), and often leads to altered immune responses (Feng et al., 2013; Nita-Lazar et al., 2015b). For example, we have reported that desialylation increased the LPS-mediated ALI (Feng et al., 2013). The subterminal galactosyl moieties exposed by desialylation, could serve as glycotopes for binding by galectins, which typically recognize β-galactosides. We have previously observed that desialylation enhanced Gal1 and Gal3 binding onto lung tissue and the associated intracellular signaling in pulmonary epithelial A549 cells (Nita-Lazar et al., 2015b). In our present study, we observed a similar increase in galectin binding onto PMNs after desialylation (Figure 1). Moreover, Gal1 mediated PMN adhesion increased after PMN desialylation (Figure 3). Notably, PMN simulation translocates endogenous sialidases from intracellular stores to the cell surface where it desialylates glycoconjugates (A. S. Cross and Wright, 1991; Feng et al., 2011; Sakarya et al., 2004). Upon LPS stimulation, endogenous sialidases desialyate PMNs and increase PMN adhesion onto endothelium *in vivo*, which could be inhibited by a sialidase inhibitor (Feng et al., 2011). After LPS challenge, the increase of Gal1 expression in the lung (Figure 4) and its binding to desialylated PMNs would promote the PMN adhesion. Consistently the PMN infiltration decreased significantly in the absence of Gal1 in knockout mice (Figure 5).

The role of ICAM-β2-integrin interactions in PMN adhesion onto endothelium has been firmly established (Diamond and Springer, 1993; Hynes, 2002). We have previously demonstrated that desialylation exposed an activation epitope of β2-integrin on PMNs and enhanced ICAM-1 binding onto PMNs (Feng et al., 2011). Our present results suggest that by increasing galectin binding, surface desialylation of PMNs could enhance the interaction of ICAM-1 with its ligands, presumably the β2-integrins which are recognized as strong galectin ligands (Avni et al., 1998; Dong and Hughes, 1997; Hughes, 2001). Yet, it remains possible that in addition to β2-integrins, galectins bind to other cell surface glycoproteins thereby enhancing PMN adhesion. Moreover, while it has been reported that desialylation by exogenous microbial sialidases released in the lung further increased the PMN infiltration in wildtype mice (Feng et al., 2013), the potential contribution of Gal1 in this process was

not examined. In this regard, it is noteworthy that LPS also induced *in vivo* desialylation of PMNs by endogenous sialidases (Feng et al., 2011).

Our results revealed some discrepancies from previous observations. For example, Gal3 had been proposed to promote adhesion of PMNs to the vascular endothelium, as evidenced by the enhanced binding of PMN to HUVEC (Sato et al., 2002); however, in our study Gal3 showed little effect on the PMN-HMVEC-L interaction at a concentration at which it binds strongly to PMNs and HMVEC-L separately. Moreover, it was reported that recombinant Gal1 inhibits neutrophil-HUVEC interactions in a flow chamber system *in vitro* (Cooper et al., 2008), which is inconsistent with our present results showing that Gal1 increases PMN adhesion, especially when cells are desialylated. It is possible that the observed differences are due to differences in cell behavior under shear flow conditions (Cooper et al., 2008) and the static system we used. It might also be due to the different cell surface ligands displayed by TNFα-stimulated HUVEC (Cooper et al., 2008) and our HMVEC-L cells. It has also been reported that in a non-inflammatory context Gal1 promotes human neutrophil migration, while under inflammatory conditions Gal1 inhibits it (Auvynet et al., 2013; Sundblad et al., 2017).

Our results showed reduced PMN infiltration in the LPS-challenged Gal1 knockout mice relative to the control animals expressing Gal1. This is inconsistent with a previous report in which the administration of recombinant Gal1 inhibited inflammatory cell infiltration and pro-inflammatory cytokines release, and attenuated the lung destruction in LPS-induced ALI mice (Huang et al., 2020) or IL-1β-induced peritonitis (La et al., 2003). These apparently contradictory results likely reflect functional differences between endogenous galectins and exogenous recombinant galectins, which have already been reported for Gal1 and Gal3 (Hsu and Liu, 2008; Pérez et al., 2015). Even more intriguing are the differences with the reported inhibitory effects of endogenous Gal1 on the adhesion and trafficking of neutrophils (Cooper et al., 2008) and T cells (He and Baum, 2006; Norling et al., 2008). Upon down-regulation of endogenous Gal1 expression, leukocytes exhibited increased capture and rolling on a monolayer of endothelial cells *in vitro* (Cooper et al., 2008); and increased neutrophil pulmonary infiltration was observed in Gal1 knockout mice, accompanied by increased PGE2 and less apoptosis (Rodrigues et al., 2016). The factors underlaying these apparently contradictory results remain to be determined. Some of the differences may be explained on the basis of the different inflammatory stimuli—e.g. LPS vs. virus (such as influenza) vs bacteria (such as pneumococci). It has been proposed that galectin binding to its various cell surface carbohydrate ligands may result in various outcomes, depending on the cellular and molecular networks operating in inflammatory and tissue microenvironments (Pérez et al., 2015) or the counterreceptor to which the galectin binds (Thiemann and Baum, 2016). For example, PMN infiltration was found to decrease in Gal3 null mice after *S. pneumoniae* infection but increase after *E. coli* infection (Nieminen et al., 2008), while a Gal3 inhibitor inhibited *E. coli* LPS-induced neutrophil recruitment and activation (Humphries et al., 2022). These opposite effects possibly result from different pathways downstream of the cell surface galectin-ligand interaction(s). For example, Gal1 can induce surface exposure of phosphatidylserine (PS) in activated neutrophils (but not resting cells) making these cells the targets for phagocytosis and favoring phagocytic removal (Dias-Baruffi et al., 2003; Karmakar et al., 2005; Stowell et al., 2007; Stowell

et al., 2008; Sundblad et al., 2017); while in a non-inflammatory context, Gal1 may mediate neutrophil adhesion as demonstrated by Auvynet *et al* (Auvynet et al., 2013) and as observed in this study. Thus, it becomes clear that the functional diversification of galectins observed in different experimental contexts complicates a uniform analysis of results and warrants further investigation.

Nevertheless, this study strongly suggests that under our experimental conditions Gal1 can function as adhesion molecules that mediate interactions of PMN with endothelial cells and modulate PMN infiltration during LPS-mediated acute lung injury.

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Abbreviations:

ALI	acute lung injury
BALF	Bronchoalveolar lavage fluid
***Cp*NA**	*C. perfringens* neuraminidase type X
FITC	Fluorescein isothiocyanate
Gal1	galectin-1
Gal3	galectin-3
HMVEC	Human lung microvascular endothelial cells
ICAM-1	intercellular adhesion molecule-1
IL-1β	Interleukin-1-beta
KC	chemokine (C-X-C motif) ligand 1
LPS	lipopolysaccharide
MALII	*Maackia amurensis* lectin II
MFI	mean fluorescent intensity
PMN	polymorphonuclear leukocyte
PNA	peanut agglutinin
SD	standard deviation

TNF-α	tumor necrosis factor-α

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Highlights

- Desialylation of endothelial cells and PMNs increases galectin binding.
- Galectins mediates ICAM-1 binding onto PMNs, which is increased by PMN desialylation.
- Galectin-1 mediates PMN adhesion more effectively than Galectin-3.
- Galectin-1, expressed in LPS-challenged mouse lung, promotes LPS-mediated ALI.
- Diverse galectin functions complicate the analysis and warrant further investigation

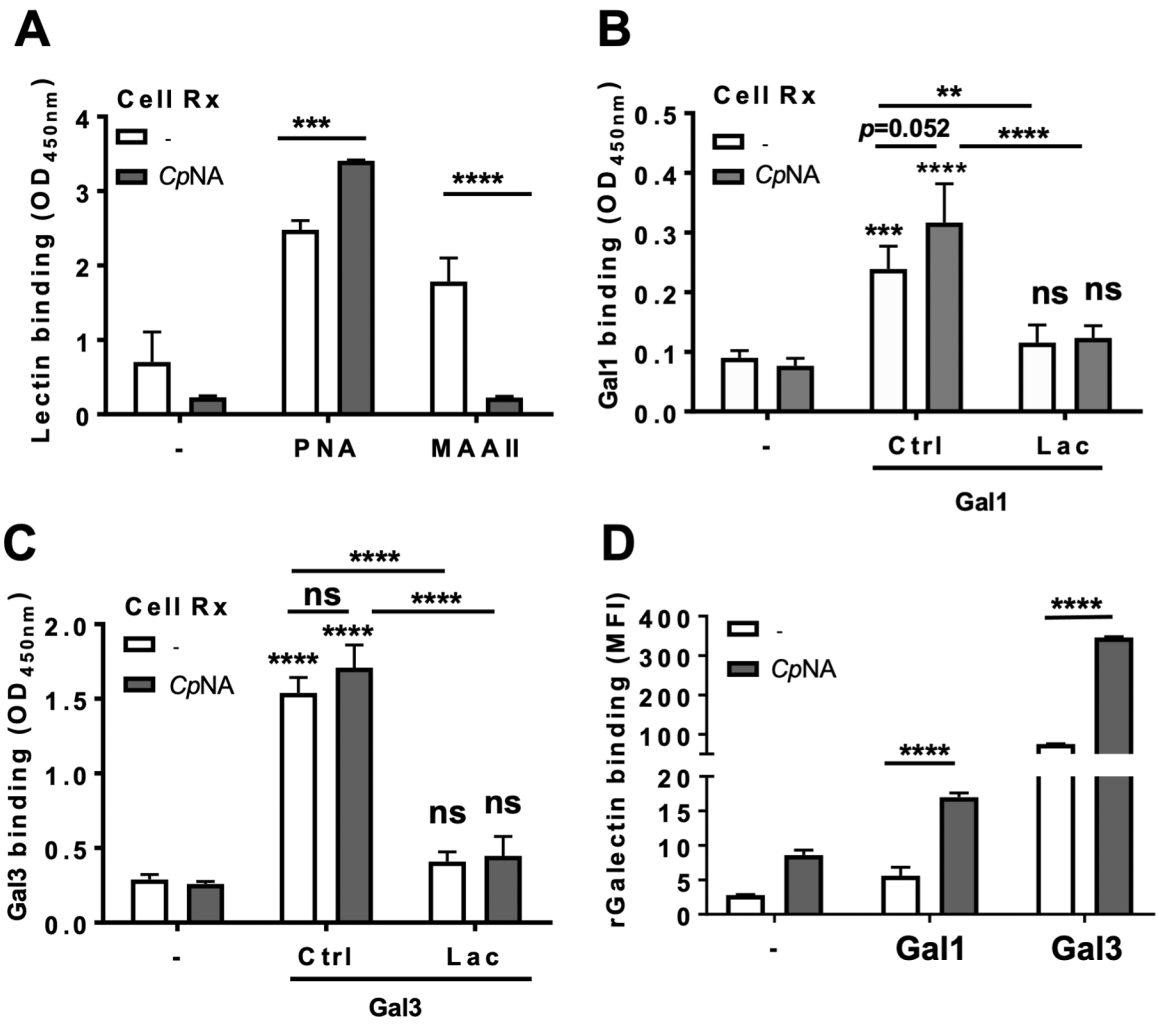

Figure 1. Desialylation enhanced galectin binding.

HMVEC-L cells grown on 96 well microplates were treated with medium (−) or 10 mU/ml of *Cp*NA, and (A) stained with biotinylated PNA or MALII, or (B&C) incubated with biotinylated recombinant human Gal1 (B) or Gal3 (C) in the absence (Ctrl) or presence of 100 mM of lactose (Lac), followed by incubation with streptavidin-HRP then developed with TMB substrate. (D) PMN cells treated with *Cp*NA or untreated (−) were incubated with 5 μg/ml of recombinant human Gal1 or Gal3 for 1 h. The galectin binding was revealed with galectin-specific antibodies followed by Cy2-conjugated secondary antibody for detection on flow cytometry. Cells incubated without galectins are included as negative control (−). Representative data from 3 independent experiments were shown. *: $p<0.05$, ***: $p<0.001$, ****: $p<0.0001$, vs negative control (−), by two-way ANOVA.

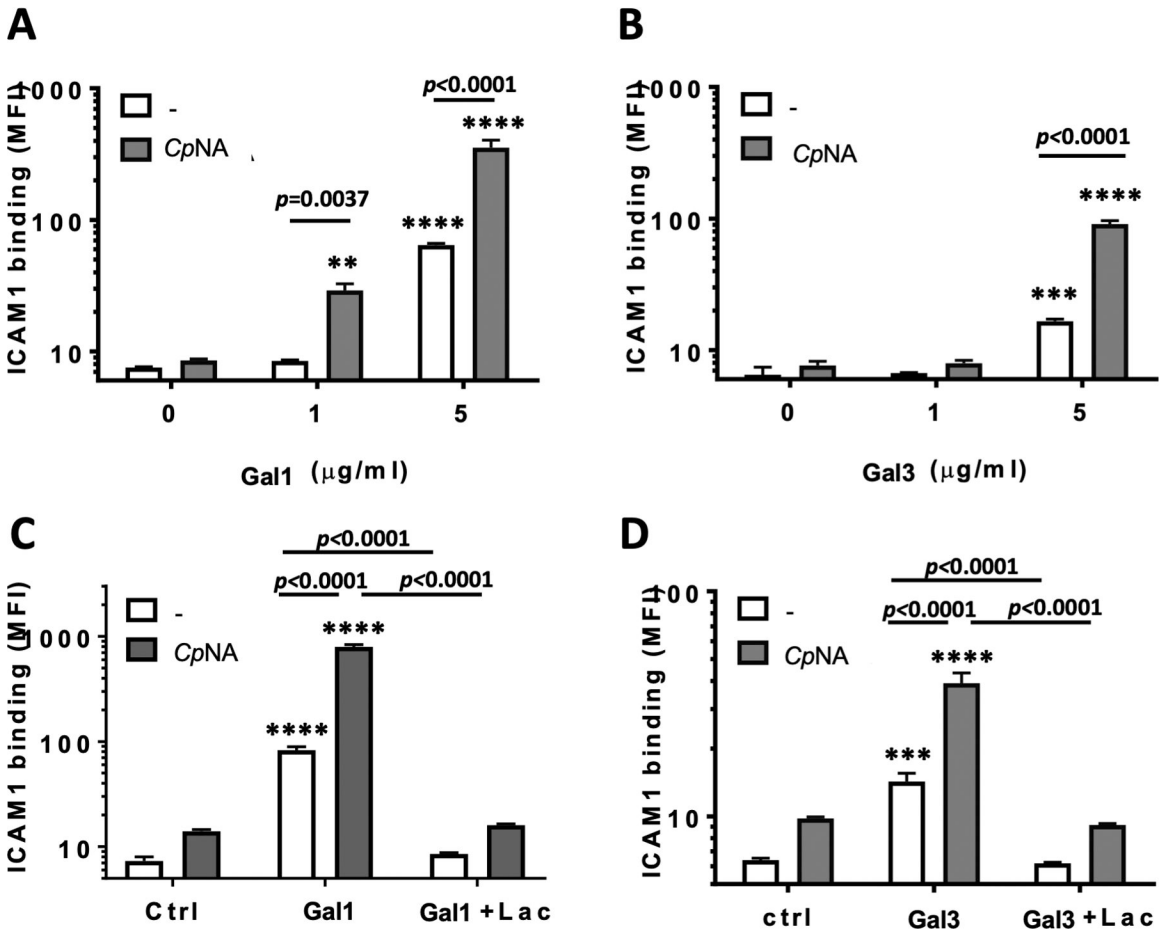

Figure 2. Galectins and desialylation enhanced ICAM-1 binding.

(A&B) Untreated (−) or *Cp*NA-treated PMNs were incubated with ICAM-1 Fc in the presence of 0–5 μg/ml of recombinant human Gal1 (A) or Gal3 (B) for 1 h followed by Cy2 conjugated anti-Fc antibody. (C&D) Untreated (−) or CPNA-treated PMNs were incubated with ICAM-1 Fc in the presence of 0 (ctrl) or 5 μg/ml of recombinant human Gal1 (C) or Gal3 (D), without (Gal1, Gal3) or with 100 mM of lactose (Gal1+Lac, Gal3+Lac) for 1 h followed by Cy2 conjugated anti-Fc antibody. The ICAM-1 binding was revealed on flow cytometry. Representative data from 3 independent experiments were shown with mean fluorescent intensity (MFI) +/− standard error. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$ vs control (0) group respectively, by two-way ANOVA.

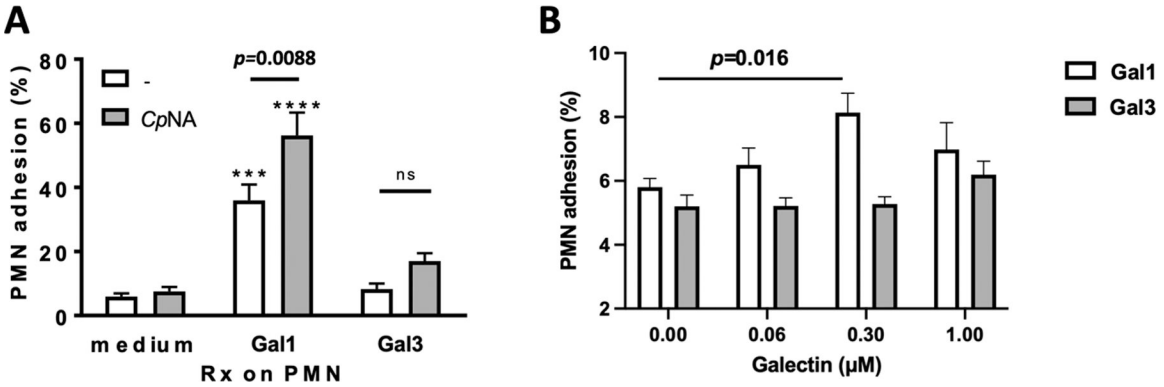

Figure 3. Galectin-1 together with cell surface desialylation synergistically enhanced PMN binding.

(A) *Cp*NA-treated or untreated (−) PMN cells were labeled with calcein AM then incubated on a HMVEC-L monolayer in the presence of 5 μg/ml of recombinant human Gal1, Gal3 or medium only (medium) for 1 h. After incubation, the non-adherent cells were washed out and the fluorescent intensity from each well was measured. The percentage of PMN adhesion was determined as described in Materials and Methods. Representative data from 3 independent experiments are shown. (B) Untreated PMN cells were treated as above in the presence of 0, 0.06, 0.3, or 1 μM of Gal1 or Gal3, and the percentage of PMN adhesion was determined. *: $p<0.05$, ***: $p<0.001$, ****: $p<0.0001$, vs control group (medium), by two-way ANOVA.

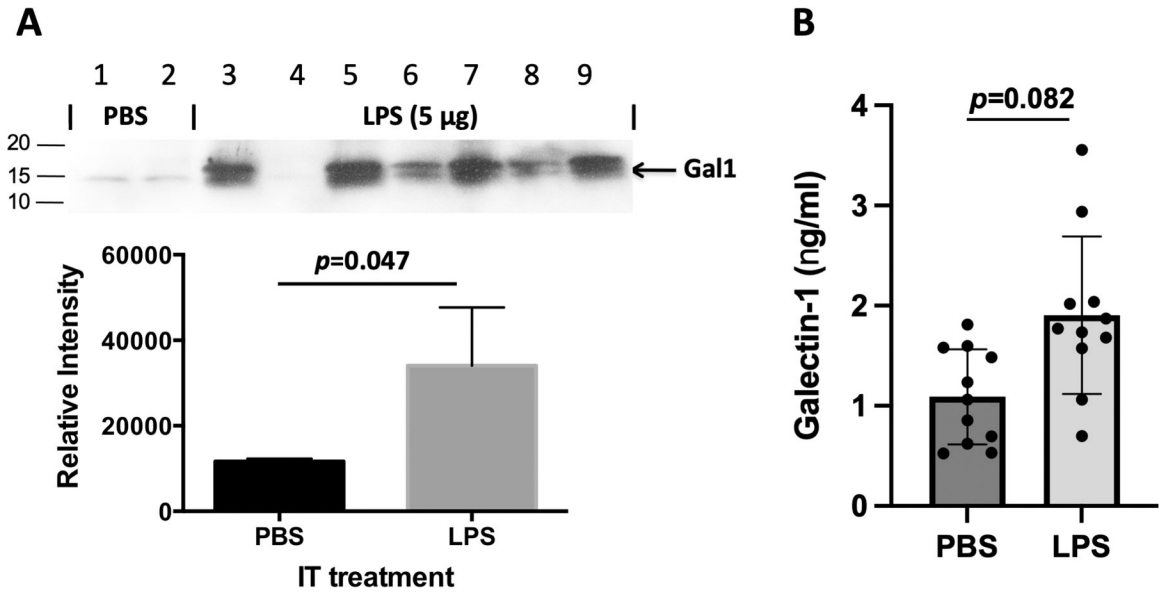

Figure 4. LPS challenge stimulates galectin-1 expression.

(A) Individual mice were challenged with 5 μg of LPS i.t. (n=7, lane 3–9) or PBS (n=2, lane 1&2) as control. BALF was collected after 3 h and analyzed on SDS-PAGE with anti-Gal1 antibody to detect protein expression. The intensity of each band was quantified by ImageJ. Representative data from 3 independent experiments are shown. (B) BALF from challenged mice was subjected to ELISA using Quantikine ELISA kit (R&D Systems) to determine the Gal1 concentration. Data from 4 independent experiments were pooled. *p* value between groups was determined with Student's t test.

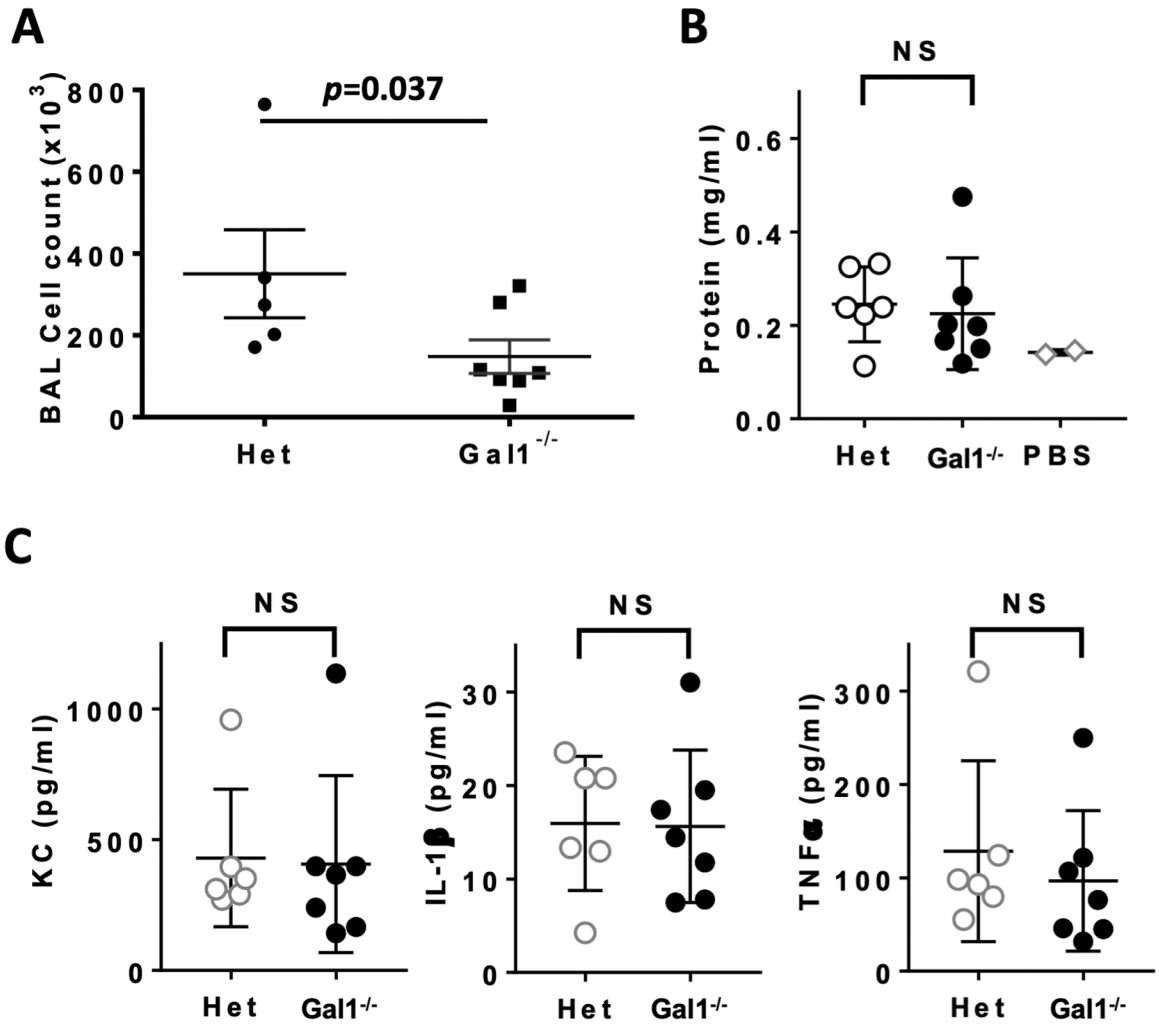

Figure 5. Galectin-1 deficiency decreased LPS-mediated PMN infiltration.

Gal1 knockout mice ($Gal1^{-/-}$, n=7) and their heterozygous littermates (Het, n=5 or 6) were challenged with 5 μg of LPS i.t. overnight and the lungs were washed and harvested BALF. The (A) cell numbers, (B) total protein, and (C) cytokine level of KC, IL-1β and TNF-α in BALF were quantified and plotted. Representative data from 3 independent experiments were shown. *p* value between groups was determined with Student's t test.