IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Galectin-3 Enhances Avian H5N1 Influenza A Virus—Induced Pulmonary Inflammation by Promoting NLRP3 Inflammasome Activation

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Highly pathogenic avian influenza A H5N1 virus causes pneumonia and acute respiratory distress syndrome in humans. Virus-induced excessive inflammatory response contributes to severe disease and high mortality rates. Galectin-3, a β -galactoside-binding protein widely distributed in immune and epithelial cells, regulates various immune functions and modulates microbial infections. Here, we describe galectin-3 up-regulation in mouse lung tissue after challenges with the H5N1 influenza virus. We investigated the effects of endogenous galectin-3 on H5N1 infection and found that survival of galectin-3 knockout (Gal-3KO) mice was comparable with wild-type (WT) mice after infections. Compared with infected WT mice, infected Gal-3KO mice exhibited less inflammation in the lungs and reduced IL-1 β levels in bronchoalveolar lavage fluid. In addition, the bone marrow–derived macrophages (BMMs) from Gal-3KO mice exhibited reduced oligomerization of apoptosis-associated speck-like proteins containing caspase-associated recruitment domains and secreted less IL-1 β compared with BMMs from WT mice. However, similar levels of the inflammasome component of nucleotide oligomerization domain–like receptor protein 3 (NLRP3) were observed in two genotypes of BMMs. Co-immunoprecipitation data indicated galectin-3 and NLRP3 interaction in BMMs infected with H5N1. An association was also observed between galectin-3 and NLRP3/apoptosis-associated speck-like proteins containing caspase-associated recruitment domain complex. Combined, our results suggest that endogenous galectin-3 enhances the effects of H5N1 infection by promoting host inflammatory responses and regulating IL-1 β production by macrophages via interaction with NLRP3. (*Am J Pathol* 2018, 188: 1031–1042; <https://doi.org/10.1016/j.ajpath.2017.12.014>)

H5N1 is a highly pathogenic avian influenza A virus (IAV) that was initially confined to poultry, but it is now recognized as a human infectious agent associated with a case-fatality rate of approximately 60%.¹ Clinical features of human H5N1 infections include typical influenza-like symptoms during early stages and rapidly progressive viral pneumonia. Complications can include acute respiratory distress syndrome and multiple organ failure.^{2,3} Lung pathologic process and progressive respiratory failure are considered the primary causes of H5N1-associated deaths.⁴ According to previous studies, virus-induced excessive acute inflammatory responses in hosts contribute to H5N1 pathogenesis.^{1,5} Those responses

include massive influxes of inflammatory cells into the lungs and aberrant production of cytokines and chemokines, resulting in pulmonary immunopathology and increased tissue injury.^{5–7} A growing body of evidence points to activated macrophages as primary contributors of elevated cytokine

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production and subsequent immunopathology induced by H5N1 infection.^{8,9}

IL-1 β is a potent proinflammatory cytokine involved in the acute lung immunopathology of IAV infections.¹⁰ Primarily produced by activated monocytes and macrophages,^{11,12} IL-1 β induces the expression of proinflammatory mediators, cytokines, and chemokines that trigger leukocyte production.¹² Respiratory epithelial cells and macrophages sense the virus during an infection, thereby triggering IL-1 β production by the activation of nucleotide oligomerization domain–like receptor protein 3 (NLRP3) signal.^{13–15} This results in the increased recruitment of inflammatory cells such as neutrophils and monocytes to infection sites. Several reports suggest that two signals are required to activate NLRP3 inflammasome (a cytoplasmic multiprotein complex). The first (priming) signal is triggered by a toll-like receptor agonist such as viral genomic RNA, resulting in the transcription of pro-IL-1 β and NLRP3 via the NF- κ B pathway.¹⁶ Secondary stress-related signals include reactive oxygen species, K⁺ efflux, and lysosomal rupture signals that promote NLRP3 inflammasome complex assembly.^{13,17} Activated NLRP3 inflammasomes act as platforms for caspase-1 activation that consequently cleaves pro-IL-1 β into mature IL-1 β .¹⁸

Galectins are a family of β -galactoside-binding animal lectins defined by a conserved carbohydrate-binding domain of approximately 135 amino acids with affinities for β -galactose-containing glycoconjugates.¹⁹ Galectin-3 (approximately 30 kDa) is the only chimera-type galectin that contains a single carbohydrate-binding domain at the C-terminal region, as well as an extended nonlectin N-terminal part that contributes to galectin-3 self-association.²⁰ Galectin-3 is widely distributed in a variety of tissues and expressed by various immune and epithelial cells.^{21,22} It is primarily detected in the cytoplasm, but it can be translocated to the cell nucleus. It is also secreted into extracellular environments by undefined secretory pathways.²³ Researchers have described multiple galectin-3 functions in a range of physiological and pathologic processes that involve intracellular and extracellular functions.^{22,24} The importance of galectin-3 in the development and regulation of immune homeostasis and responses to infections has been well demonstrated.^{24,25} A number of *in vivo* studies that used galectin-3-knockout (Gal-3KO) mice have confirmed the proinflammatory effect of endogenous galectin-3.²⁶ For example, Gal-3KO mice have been shown to exhibit attenuated inflammatory response and reduced leukocyte infiltration compared with wild-type (WT) mice in thioglycollate-induced peritoneal inflammation^{27,28} and *Streptococcus pneumoniae* infections.²⁹

Galectin-3 is up-regulated during lung injury, allergic airway inflammation, and airway infections.^{29–31} Therefore, it was hypothesized that a correlation existed between endogenous galectin-3 and H5N1-induced pulmonary immune response. During this study we observed up-regulated galectin-3 in a mouse model challenged with the H5N1

virus and more severe airway inflammation and greater IL-1 β production in WT mice than Gal-3KO mice, suggesting a promoting effect of endogenous galectin-3 on H5N1-induced inflammation. With the use of mouse macrophages for *in vitro* studies, an association was found between galectin-3 and NLRP3—specifically, galectin-3 promoted NLRP3 inflammasome assembly and activation in response to H5N1 infections. Our data demonstrate a positive role for galectin-3 in H5N1 infection–induced inflammation by NLRP3 inflammasome activation and IL-1 β production.

Materials and Methods

Cell Lines

Madin-Darby canine kidney cells (ATCC PTA-6500, Manassas, VA) were used for influenza virus propagation. Madin-Darby canine kidney, J774.1 (a mouse macrophage cell line), and HEK293T cells were maintained in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 10 U/mL penicillin (Sigma-Aldrich, St. Louis, MO), and 100 μ g/mL streptomycin sulfate (Gibco) at 37°C in a 5% CO₂ humidified incubator.

Virus

Influenza H5N1 NIBRG-14 is an attenuated reassortant strain that possesses modified HA and NA genes derived from A/Vietnam/1194/2004 (H5N1) and six internal genes from A/Puerto Rico/8/34 (H1N1).³² The NIBRG-14 virus was propagated in Madin-Darby canine kidney cells. Virus titers were determined by plaque assays and 50% tissue culture infective dose assays. All experiments that involved the handling of H5N1 NIBRG-14 viruses *in vitro* or *in vivo* were conducted in the BSL-2⁺ laboratory at the Academia Sinica Institute of Biomedical Sciences and Genomics Research Center.

Mouse Infections

Gal-3KO mice with C57BL/6 backgrounds were generated as previously described,²⁷ and WT mice with identical genetic backgrounds were used as controls. All animal procedures and experimental protocols were approved by the Institutional Animal Care and Utilization Committee of Academia Sinica and were performed according to committee guidelines.

Groups of female C57BL/6 WT and Gal-3KO mice (8 to 9 weeks old) were intranasally inoculated with 10 times the half-lethal doses (LD₅₀) of H5N1 NIBRG-14 virus (50 μ L) on day 0. Body weight, body temperature, and survival data were recorded for 14 days. Sample collection and virus challenges were performed under sodium pentobarbital anesthesia (60 to 80 mg/kg); every effort was made to minimize animal

suffering. Mice showing >25% body weight loss were considered as reaching an experimental end point and were humanely euthanized according to guidelines from the Institutional Animal Care and Use Committee.

Preparation of Mouse Bone Marrow–Derived Macrophages

Both ends of mouse femurs and tibias were cut and flushed with 5 mL of RPMI 1640 (Invitrogen, Carlsbad, CA) by using a 27-gauge $\times$ $\frac{1}{2}$ -inch needle (Terumo, Tokyo, Japan). Bone marrow cells were strained (40- μ m mesh) (BD Biosciences, San Jose, CA) and seeded in complete medium that consisted of RPMI 1640 that contained 10% fetal bovine serum, $1 \times$ non-essential amino acids (Gibco), 20 mmol/L HEPES (Gibco), and 10 ng/mL mouse granulocyte-macrophage colony-stimulating factor (PeproTech, Rocky Hill, NJ) for macrophage differentiation. One-half of culture medium was replaced every 2 days with fresh complete medium. Differentiated macrophages were harvested after 7 to 10 days with Cellstripper (Corning Cellgro, Manassas, VA) and used for virus infection experiments.

Cell Culture Infection with H5N1

Bone marrow–derived macrophages (BMMs) or J774.1 cell monolayers were cultured overnight in 6-well plates, washed two more times with phosphate-buffered saline (PBS), inoculated with H5N1 NIBRG-14 (multiplicity of infection = 1) in influenza virus growth medium, and incubated for 1 hour at 37°C. Cells were washed two additional times with PBS, and fresh cell culture medium was placed in each well before incubation at 37°C in a 5% CO₂ humidified incubator. Cell-free supernatants were collected after 6 and 24 hours for enzyme-linked immunosorbent assay (ELISA) cytokine analyses.

RNA Extraction and RT-PCR

Mice infected with H5N1 NIBRG-14 virus were sacrificed 1 to 5 days after infection. Lungs were perfused with 10 mL of PBS injected into right heart ventricles, harvested, and homogenized in 1 mL of TRIzol (Invitrogen) with a TissueLyser Adapter Set and TissueLyser II (both Qiagen, Valencia, CA). After centrifugation at $12,000 \times g$ for 15 minutes at 4°C, supernatants were collected and used for total RNA extraction. Extracted RNA quality and quantities were measured with a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Next, 1 μ g of extracted RNA was reverse-transcribed into cDNA by using iScript Reverse Transcription Supermix for quantitative real-time RT-PCR (RT-qPCR) (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

H5N1 Virus and Cytokine mRNA Quantification by Real-Time PCR

To measure H5N1 NIBRG-14 virus levels in lung tissue or cell culture supernatants, quantitative real-time PCR was performed with an ABI 7500 Real-Time PCR machine (Applied Biosystems, Foster City, CA) to amplify cDNA synthesized from extracted RNA with a FastStart Universal Probe Master (Roche Applied Science, Indianapolis, IN). For viral RNA detection, H5HA gene-specific primers (forward: 5'-AGGTCCGACTACAGCTTAGGG-3', reverse: 5'-TGATAGAACTCGAAACAACCGTTA-3') and probes designed by the Universal Probe Library Assay Design Center (Roche Applied Science) were used for real-time PCR amplification. H5N1 NIBRG-14 quantities were calculated by interpolation from standard curves constructed by the parallel running of 10-fold serial dilutions of known numbers of plasmids that contained H5HA gene segments.

Inflammasome component and cytokine mRNA levels in mouse lung tissues or BMMs were detected by real-time quantitative PCR using the primers IL-1 β (forward: 5'-AGTTGACGGACCCCAAAAG-3', reverse: 5'-AGCTG-GATGCTCTCATCAGG-3'), NLRP3 (forward: 5'-CCCT-TGGAGACACAGGACTC-3', reverse: 5'-GAGGCTGCAGT-TGTCTAATTCC-3'), tumor necrosis factor (TNF)- α (forward: 5'-CAGCCTCTTCTCCTTCCTGAT-3', reverse: 5'-GCCA-GAGGGCTGATTAGAGA-3'), and chemokine (C-C motif) ligand 2 (forward: 5'-CATCCACGTGTTGGCTCA-3', reverse: 5'-GATCATCTTGCTGGTGAATGAGT-3').

Histochemical Analysis

Three days after infection, infected mouse lungs were perfused with PBS and fixed with 1 mL of 4% paraformaldehyde (Sigma-Aldrich) by tracheas. Lungs were fixed overnight with 10% formaldehyde (Merck, Darmstadt, Germany), embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Immunohistochemical staining was performed on paraffin-embedded lung sections. Endogenous peroxidase activity and nonspecific protein binding were blocked by using Hydrogen Peroxide Block (Thermo Fisher Scientific) and Ultra V Block (Thermo Fisher Scientific), respectively. Sections were incubated with rabbit anti-galectin-3 antibodies (1:1000 dilution; made in our laboratory) or normal rabbit IgG antibodies (Abcam, Cambridge, MA) as isotype control at working concentration of 0.5 μ g/mL. Bound antibodies were detected by incubation with Histofine Simple Stain Mouse MAX PO (Nichirei, Tokyo, Japan), followed by 3, 3'-diaminobenzidine (Vector, Burlingame, CA) treatment for 5 minutes at room temperature. Slides were counterstained with hematoxylin.

ELISAs

Mouse lungs were harvested and homogenized in PBS. Bronchoalveolar lavage (BAL) fluid was collected by

injecting 1 mL of PBS into the alveolar spaces of infected mice, followed by three gentle washings via tracheas with the use of an 18-gauge $\times$ 1 $\frac{1}{2}$ -inch needle (Terumo) on day 3 after infection. Collected BAL fluid and lung homogenates were centrifuged at $14,000 \times g$ for 15 minutes at 4°C. Cytokine concentrations in the supernatants were estimated by using Ready-Set-Go! ELISA Sets (eBioscience, San Diego, CA). Cytokine levels in cell-free supernatants from virus-infected BMMs were also measured by using ELISA kits. Absorbance at 450 nm was measured with an Infinite 200 PRO spectrophotometer (Tecan, Research Triangle Park, NC).

Immunoblot Analysis

Cells were lysed in RIPA lysis buffer on ice. Total proteins (20 μ g) were used for SDS-PAGE analyses. Separated proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). Membranes were blocked overnight at 4°C for nonspecific protein binding in blocking buffer with 5% milk (BD Biosciences) in 0.1% Tween 20 in PBS before incubation with anti-galectin-3, anti-caspase-1 (p20) (gifts from Genentech, San Francisco, CA), anti-IL-1 β (R&D, Minneapolis, MN), anti-NLRP3 (Adipogen, San Diego, CA), anti-apoptosis-associated speck-like proteins containing caspase-associated recruitment domain (ASC; Adipogen), and anti- α -tubulin antibodies (Epitomics, Burlingame, CA). Membranes were washed with 0.1% Tween 20 in PBS to remove unbound primary antibodies and incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 hour. After another washing with 0.1% Tween 20 in PBS, membranes were developed with WesternBright ECL (enhanced chemiluminescence) HRP (horseradish peroxidase) Substrate (Advansta, Menlo Park, CA). Hybridized protein bands were visualized with a BioSpectrum Auto Imaging System (UVP, Upland, CA) and quantified with ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA).

NLRP3 Inflammasome Complex Reconstitution

HEK293T cells were transfected with plasmids encoding pro-IL-1 β (200 ng), flag-tagged ASC (50 ng), pro-caspase-1 (30 ng), and NLRP3 (75 ng) according to Effectene Transfection Reagent protocols as described by Qiagen. Proteins in supernatants were precipitated with trichloroacetic acid (Sigma-Aldrich), and mature IL-1 β was detected by Western blot analysis.

ASC Pyroptosome Cross-Link Assays

BMMs were harvested 15 hours after infection and lysed in buffer that contained 20 mmol/L HEPES-KOH (pH 7.5), 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L EGTA, 320 mmol/L sucrose, and protease inhibitor cocktail (Calbiochem, San Diego, CA) (15 passes on ice with the use of a 27-gauge syringe). Lysates were centrifuged at $520 \times g$ for 8

minutes to remove cell nuclei. Supernatants were diluted with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) buffer (20 mmol/L HEPES-KOH, pH 7.5, 5 mmol/L MgCl₂, 0.5 mmol/L EGTA, 0.1% CHAPS) plus 0.1 mmol/L fresh phenylmethane sulfonyl fluoride and protease inhibitor cocktail, and they were centrifuged at $4000 \times g$ for 8 minutes. Pellets were resuspended in CHAPS buffer with 2 mmol/L disuccinimidyl suberate cross-linker (Thermo Fisher Scientific) and held at room temperature for 30 minutes. After centrifugation at $4000 \times g$ for 10 minutes, pellets were resuspended with $1 \times$ IAV sample buffer and fractionated on 12% SDS polyacrylamide gels, followed by immunoblotting with anti-ASC antibodies.

Immunofluorescence

293T cells were seeded on poly-L-lysine-coated glass coverslips (Sigma-Aldrich) and transfected with plasmids that encoded galectin-3, NLRP3, and ASC for 48 hours. Cells were fixed with 4% paraformaldehyde and were permeabilized with 0.05% saponin (Sigma-Aldrich). After blocking with buffer, cells were stained with anti-galectin-3, anti-NLRP3 (Adipogen), and anti-ASC (Adipogen) antibodies. Fluorescence-conjugated secondary antibodies were then used to detect primary antibodies. Cells were mounted on glass slides with Fluoromount-G (Southern Biotech, Birmingham, AL). Immunofluorescent images were acquired with a Zeiss LSM700 confocal microscope (Carl Zeiss, Thornwood, NY) and quantified with Metamorph image analysis software (Molecular Device).

Immunoprecipitation

J774.1 cells were infected with H5N1 NIBRG-14 virus (multiplicity of infection = 1). At 15 hours after infection, cells were washed with PBS and treated with 8 μ g/mL dithiobis succinimidyl propionate cross-linker (Sigma-Aldrich) for 30 minutes at room temperature. Cross-linking was stopped by the addition of one-tenth volume of 1 mol/L Tris-HCl (pH 7.5), followed by incubation for 15 minutes at room temperature. Next, cells were lysed in NP-40 buffer (150 mmol/L NaCl, 25 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EDTA, 0.5% NP-40, 5% glycerol) with protease inhibitor cocktail and passed through a 27-gauge syringe 15 times at 4°C. Lysates were centrifuged at $550 \times g$ for 8 minutes. Anti-galectin-3 or anti-NLRP3 antibodies (1 mg each) were mixed with protein A/G-Sepharose beads (Merck) overnight at 4°C. After forming pellets, beads were washed with NP-40 buffer. Total proteins (100 μ g) were incubated with antibody-conjugated beads overnight at 4°C. After thorough washes with NP-40 buffer, beads were covered with 50 μ L of $2 \times$ SDS sample buffer and boiled for 10 minutes. Supernatants with precipitated proteins were collected and separated on 12% SDS polyacrylamide gels, followed by immunoblotting with anti-galectin-3 and anti-NLRP3 antibodies.

LDH and Protein Concentrations

Lactate dehydrogenase (LDH) release in BAL collected from infected mice was measured with CytoTox 96 Non-Radioactive Cytotoxicity Assays (Promega, Madison, WI) according to the manufacturer's instructions. Bradford Protein Assays (Bio-Rad) were used to measure total protein concentrations in the BAL samples.

Statistical Analysis

Statistical differences between experimental groups were analyzed by *t*-tests (GraphPad Prism 6 software; GraphPad Inc., San Diego, CA), with statistical significance established as $P < 0.05$. The mouse survival data were analyzed by log-rank (Mantel-Cox) test with GraphPad Prism 6 software (GraphPad Inc.).

Results

Galectin-3 Is Up-Regulated in Mouse Lungs during H5N1 Infections

The correlation between galectin-3 expression and H5N1 infection was first determined. Lungs collected from H5N1-infected and PBS-treated WT mice were homogenized, and galectin-3 levels in lung homogenates were quantified by real-time quantitative PCR and Western blot analysis. Results indicated significant up-regulation of both galectin-3 mRNA (Figure 1A) and protein (Figure 1B) levels after H5N1 infection, suggesting a positive role for galectin-3. Sources of increased galectin-3 in infected lungs were verified. Immunohistochemical staining indicated enhanced galectin-3 staining in H5N1-infected lungs compared with controls treated with PBS (Figure 1C). The observed increase in galectin-3 expression was primarily due to infiltrating cells and to a lesser degree to lung epithelial cells.

Survival of Gal-3KO Mice and WT Mice after H5N1 Infections Is Comparable and the Viral Copies in Lungs Are Also Similar

Both WT and Gal-3KO mice were used to determine endogenous galectin-3 involvement in H5N1 pathogenesis *in vivo*. A survival rate of approximately 36% was observed for Gal-3KO mice compared with 0% for WT mice (Figure 2A); however, no statistically significant difference was found. Although all mice lost body weight after challenges with the H5N1 virus, some of the Gal-3KO mice regained weight, starting on day 7 after infection, whereas all WT mice died by day 8 (Figure 2B). Body weight changes were similar between the two genotypes during the first 3 days (Figure 2C).

To address whether galectin-3 affected H5N1 replication *in vivo*, viral loads in the lungs of both WT and Gal-3KO mice were determined by real-time quantitative PCR between days 1 and 5 after infection. Results indicated the presence of

Figure 1 Increased galectin-3 (Gal-3) expression in mouse lungs during H5N1 infections. Wild-type mice were intranasally inoculated with phosphate-buffered saline (PBS) as a control or $10 \times \text{LD}_{50}$ of the H5N1 NIBRG-14 virus. **A:** Lungs were harvested and homogenized on day 5 after infection. Real-time quantitative PCR was performed to quantify Gal-3 mRNA levels in lung homogenates. **B:** Gal-3 protein levels in lung homogenates were measured by Western blot analysis. Relative levels of PBS-treated versus H5N1-infected tissues were 1.00, 1.19 versus 1.19, 1.69, 1.22, 1.35. **C:** Results from immunohistochemical staining that used IgG control and anti-Gal-3 antibodies with lung sections from control (PBS-treated) and H5N1-infected mice on day 3 after infection. Brown indicates positive reactivity. Data are expressed as means $\pm$ SEM from two independent experiments. $n = 6$ per group. $*P < 0.05$. Original magnification, $\times 400$. KO, knockout.

similar viral copies in the two genotypes at different times after H5N1 challenges (Figure 2D), indicating the absence of any significant effect from galectin-3 on viral loads.

H5N1-Infected Gal-3KO Mice Exhibit Reduced Lung Inflammation with Less IL-1 β Secretion in Airways Compared with H5N1-Infected WT Mice

Lung inflammation was next examined in H5N1-infected mice. Hematoxylin and eosin staining indicated lower amounts of inflammatory cells, such as neutrophils, in lung tissue samples from H5N1-infected Gal-3KO mice compared with samples taken from WT mice (Figure 3A). Because excessive inflammation is a known cause of tissue injury, total protein concentrations and LDH levels (two markers of injured lungs) were quantified in BAL fluid collected from both types of mice. Results indicated total protein (Figure 3B) and LDH (Figure 3C) levels in BAL fluids from H5N1-infected Gal-3KO were comparable with WT mice.

Figure 2 Post-H5N1 infection death, weight loss, and viral burden data. Wild-type (WT) and galectin-3 knockout (Gal-3KO) mice were intranasally inoculated with $10 \times \text{LD}_{50}$ of the H5N1 NIBRG-14 virus or phosphate-buffered saline as a control. **A:** Mouse survival data for the 14 days after infection (p.i.). **B:** Body weight change data for individual mice. Weights recorded for 14 days p.i. are expressed as percentages of body weight on day 0. **C:** Mean changes in body weights for the first 3 days p.i., when all mice in both groups were still alive. **D:** Lungs were harvested and homogenized in TRIzol before total RNA extraction between days 1 and 5 p.i. Viral loads were determined at different time points by using real-time quantitative PCR for influenza hemagglutinin proteins. Data are expressed as means $\pm$ SEM. $n = 11$ mice per group from 3 independent experiments (**A–C**); $n = 3$ mice per group from 3 independent experiments (**D**).

H5N1 has been described as a significant inducer of cytokine response,⁵ and cytokine storms caused by excessive immune responses are known contributors to H5N1 immunopathogenesis.^{6,7} Data from an examination of H5N1-induced cytokine production in WT and Gal-3KO mice showed that levels of IL-6 (Figure 3D), TNF- α (Figure 3E), monocyte chemoattractant protein-1 (Figure 3F), and IL-1 β levels (Figure 3G) in the lung homogenates of H5N1-infected Gal-3KO mice were comparable with those in WT mice on day 3 after infection. However, the IL-1 β levels in BAL fluid from H5N1-infected Gal-3KO mice were significantly lower ($P < 0.05$) compared with those from H5N1-infected WT mice (Figure 3H). This indicated that galectin-3 up-regulated H5N1-induced IL-1 β production. Combined, these results suggested that galectin-3 may enhance H5N1 effects by regulating IL-1 β production and inflammatory responses.

Gal-3KO BMMs Secrete Less IL-1 β than WT BMMs in Response to H5N1 Infection while Expressing Similar NLRP3 Inflammasome Component Levels

In addition to serving as H5N1 infection targets in lung tissue, macrophages are key producers of proinflammatory cytokines such as IL-1 β and TNF- α .^{8,33} Therefore, BMMs were used to study the role of galectin-3 in IL-1 β production after H5N1 challenges *in vitro*. Results indicated that BMMs from Gal-3KO mice produced lower quantities of IL-1 β 18 hours after infection ($P < 0.05$) compared with BMMs from WT mice (Figure 4A). Influenza viruses are known inducers of IL-1 β production by NLRP3 inflammasome activation.^{13,14} This activation required two signals: a toll-like receptor–NF- κ B signaling pathway and NLRP3 inflammasome assembly.¹⁷ To verify the role of galectin-3 in NLRP3 inflammasome activation, pro-IL-1 β and NLRP3 mRNA levels were examined in H5N1-infected WT and Gal-3KO BMMs. Similar levels of the two mRNAs (Figure 4B) were found, indicating a lack of galectin-3 involvement in H5N1 infection–induced NF- κ B pathways. In contrast, Western blot data showed that at 15 hours after infection, Gal-3KO BMMs secreted smaller amounts

of mature IL-1 β into supernatant compared with WT BMMs (Figure 4C). Similarities were also found in NLRP3 inflammasome component expression at the protein level (ie, NLRP3, ASC, pro-IL-1 β , and pro-caspase-1) (Figure 4C). These results suggested that the effects of galectin-3 on IL-1 β production in macrophages during H5N1 infections were not associated with regulation of the toll-like receptor–NF- κ B signaling pathway.

Galectin-3 Promotes ASC Pyroptosome Formation and NLRP3 Inflammasome Assembly and Is Associated with NLRP3 and ASC Speck

NLRP3 activation has been shown to induce the formation of ASC oligomers or dimers (ASC pyroptosomes), resulting in the recruitment and activation of caspase-1.³⁴ Accordingly, ASC oligomerization served as an indicator of inflammasome activation. To determine whether galectin-3 regulated ASC pyroptosome formation during H5N1 infections, WT and Gal-3KO BMMs were used to perform ASC pyroptosome cross-linking assays. Results indicated lower levels of ASC oligomers in Gal-3KO BMMs compared with WT BMMs at 24 hours after infection (Figure 5A), suggesting that galectin-3 enhances NLRP3 inflammasome assembly and ASC oligomerization. To verify galectin-3 regulation of NLRP3 inflammasome assembly, NLRP3 inflammasome complex was reconstituted in HEK293T cells by transfecting specific inflammasome components, resulting in the bypassing of the NF- κ B pathway and in autonomous IL-1 β secretion. Immunoblotting data showed that galectin-3 co-transfection dose-dependently enhanced the secretion of mature IL-1 β 48 hours after transfection (Figure 5B). This indicated that galectin-3 promoted NLRP3 inflammasome assembly and activation in addition to IL-1 β processing and secretion. Combined, these results suggested that during H5N1 infections galectin-3 regulated IL-1 β production by promoting NLRP3 inflammasome assembly and ASC pyroptosome formation.

Figure 3 Galectin-3 knockout (Gal-3KO) mice exhibit reduced lung inflammation, neutrophil infiltration, and lung injuries, and they express lower levels of IL-1 β compared with wild-type (WT) mice during H5N1 infections. WT and Gal-3KO mice were intranasally inoculated with $10 \times \text{LD}_{50}$ of the H5N1 NIBRG-14 virus or phosphate-buffered saline (PBS) as control and sacrificed on day 3 after infection. **A**: Lung tissues were fixed, paraffin embedded, and sectioned for hematoxylin and eosin staining. **Insets**: Higher magnification of the boxed areas in main images. **Arrows** indicate neutrophils. **B** and **C**: Levels of total protein ($P = 0.06$; **B**) and lactate dehydrogenase (LDH) ($P = 0.31$; **C**) in bronchoalveolar lavage (BAL) fluid were measured by using Bradford protein and LDH cytotoxicity assays, respectively. **D–G**: Concentrations of IL-6 (**D**), tumor necrosis factor- α (TNF- α ; **E**), monocyte chemoattractant protein-1 (MCP-1; **F**), and IL-1 β ($P = 0.09$; **G**) in the supernatants of lung homogenates, as measured by enzyme-linked immunosorbent assays (ELISAs) and normalized to total protein concentrations. No statistically significant differences were noted for **B** through **G**. **H**: IL-1 β concentrations in BAL fluid were measured by ELISAs. Black bars, WT; white bars, Gal-3KO (**B–H**). Data are expressed as means $\pm$ SEM. $n = 4$ mice per group from 3 independent experiments. * $P < 0.05$. Original magnification: $\times 200$ (**A**, main images); $\times 600$ (**A**, insets). Conc, concentration.

To determine whether galectin-3 regulated NLRP3 inflammasome assembly through direct interaction with inflammasome components, immunofluorescence assays were used to examine galectin-3 and NLRP3 inflammasome localization in transfected HEK293T cells. The presence of galectin-3 enhanced ASC speck formation and ASC-NLRP3 association (Figure 5, C and D). In addition, associations of galectin-3 were observed with both ASC

speck and NLRP3 inflammasome complex (Figure 5). Co-immunoprecipitation assays were performed to determine the association between galectin-3 and NLRP3 during H5N1 infections. NLRP3 was pulled down when galectin-3 was immunoprecipitated in an H5N1-infected mouse macrophage J774A.1 cell line (Figure 5E), suggesting galectin-3 interaction with NLRP3 during H5N1 infections.

Figure 4 Galectin-3 knockout (Gal-3KO) bone marrow–derived macrophages (BMMs) produce lower levels of IL-1 β compared with wild-type (WT) BMMs in response to H5N1 infections, but nucleotide oligomerization domain–like receptor protein 3 (NLRP3) component expression is similar. BMMs from WT and Gal-3KO mice were infected with H5N1. **A:** IL-1 β levels in cell-free supernatants were measured by enzyme-linked immunosorbent assays. **B:** Cells were lysed for RNA extraction at different time points; pro-IL-1 β and NLRP3 mRNA levels were measured by real-time quantitative PCR. **C:** Mature IL-1 β in cell-free supernatant (Sup) was precipitated with trichloroacetic acid and measured by Western blot analysis. Protein levels of NLRP3, pro-caspase-1 (pro-Casp1), pro-IL-1 β , apoptosis-associated speck-like protein containing a caspase-associated recruitment domain (ASC), and galectin-3 in cell lysates were measured by Western blot analysis; α -tubulin was used as an internal control. IL-1 β level in supernatant from Gal-3KO was 53% that from WT. Data are expressed as means $\pm$ SEM. $n = 3$ samples from 2 independent experiments (**A** and **B**); $n = 3$ separate experiments (**C**). * $P < 0.05$. Conc., concentration; p.i., after infection.

Discussion

Galectin-3 is involved in a number of infectious and lung-centered diseases.^{29–31,35} However, its role in pulmonary influenza virus–induced host response has not been fully evaluated previously. We found evidence to indicate that endogenous galectin-3 enhances H5N1-induced inflammation and IL-1 β production by functioning intracellularly and promoting NLRP3 inflammasome complex formation.

After treatment with large doses (10 LD₅₀) of H5N1 NIBRG-14, significantly lower IL-1 β levels were observed in the airways of Gal-3KO mice and moderate lung inflammation 3 days after infection (Figure 3). No statistically significant differences between Gal-3KO and WT mice were noted in terms of body weight loss, death, or viral loads (Figure 2). The observations for Gal-3KO mice challenged with large doses of H5N1 with regard to lung inflammation were similar to previously reported outcomes for IL-1R–deficient mice,^{10,36} suggesting that the effects of galectin-3 on IAV-induced inflammation may be primarily due to IL-1 β production regulation. However, unlike that resulting from IL-1R deficiency, survival of Gal-3KO mice was comparable with WT mice. Thus, galectin-3 may affect certain pathologic responses to IAV infections by other mechanisms besides IL-1 β production control.

Several efforts have been made to use lung macrophages or BMMs to evaluate avian IAV-induced immune responses and cytokine production.^{37,38} Their data indicate that lung-based

macrophage populations are heterogeneous, consisting of alveolar macrophages within airways plus interstitial and exudate-derived macrophages. Steady-state alveolar macrophages have been described as alternatively activated, with an M2-like anti-inflammatory phenotype thought to maintain lung homeostasis.^{37–41} We surmised that classically activated M1-like BMMs induced by granulocyte-macrophage colony-stimulating factor with a proinflammatory phenotype might be more suitable for identifying the role of galectin-3 H5N1-induced proinflammatory response, especially inflammasome activation.

Researchers have described the contributions of stronger and prolonged viral replication, differences in cell tropism, broader tissue dissemination of viruses, and aberrant host immune responses to severe human H5N1 pathogenesis.^{4,6} Further, lung pathology and progressive respiratory failure are considered primary factors that contribute to human H5N1-related death.⁴ Although researchers have shown that innate immune responses are rapidly induced to control viral replication,⁴² they have also demonstrated that the infiltration of excessive neutrophils into airways are associated with immunopathology during infections with highly virulent influenza.⁴³ Neutrophils have been reported as key contributors to acute respiratory distress syndrome.⁴⁴ Gal-3KO mice exhibited less neutrophil infiltration and pulmonary inflammation compared with WT mice (Figure 3A), suggesting that galectin-3 promotes H5N1 disease-associated inflammation.

For the past 20 years, H5N1 influenza has evolved extensively and been classified according to their HA gene sequences. Evidence indicates their divergence into at least 40 clades,⁴⁵ with only four currently circulating globally (clades 2.2.1.2; 2.3.2.1a; 2.3.2.1c; and 2.3.4.4.6).^{45–47} For this study H5N1 vaccine strain A/Vietnam/1203/04 was used, which belongs to clade 1. Because a single H5N1 vaccine strain was used to evaluate the effects of galectin-3

on H5N1 infection, additional studies are required to determine whether the promoting effect of the galectin-3 is generalizable to all H5N1 clades.

Hypercytokinemia is a major feature of human H5N1 immunopathogenesis.^{5–7} Severe lung inflammation can lead to more obvious clinical symptoms and potentially lethal lung injuries.^{48,49} Data from the comparison of cytokine expression in lung homogenates from H5N1-infected mice indicate significantly lower IL-1 β levels in BAL fluid collected from Gal-3KO mice compared with WT mice (Figure 3H). These findings suggest that galectin-3 enhances proinflammatory environments during H5N1 infection. Note that the difference in IL-1 β levels in lung homogenates from infected WT and Gal-3KO mice were not statistically significant (Figure 3G). Because ELISAs detect both pro- and mature forms of IL-1 β in lung homogenates, IL-1 β secretions in BAL fluid may be more representative of mature IL-1 β levels.

Several efforts have been made to clarify the role of endogenous galectin-3 in infection-induced inflammatory response during microbial infections. One research team found that Gal-3KO mice subjected to pulmonary infections that involved *Francisella novicida* (a Gram-negative pathogen that causes severe sepsis) exhibited reductions in neutrophil infiltration, vascular injury, and inflammatory cytokine production, including IL-1 β , TNF- α , and interferon- γ .⁵⁰ These mice experienced improved survival with bacterial burdens similar to a group of WT mice, suggesting that galectin-3 plays a pathogenic role in sepsis development by increasing inflammatory response during *F. novicida* infections.⁵⁰ These findings are consistent with our conclusion that galectin-3 enhances H5N1 pathogenic effects by promoting IL-1 β production and pulmonary inflammation, with little change in viral load. However, in a study that involved *Rhodococcus equi* (an intracellular macrophage bacterium), Gal-3KO mice developed delayed but

Figure 5 Galectin-3 (Gal-3) enhances apoptosis-associated speck-like protein containing a caspase-associated recruitment domain (ASC) oligomerization and nucleotide oligomerization domain–like receptor protein (NLRP3) inflammasome activation, and associates with both NLRP3 and ASC speck. **A:** Wild-type (WT) and Gal-3 knockout (Gal-3KO) bone marrow–derived macrophages were infected with H5N1 at the multiplicity of infection (MOI) of 1. ASC oligomers in cells were cross-linked with dextran sulfate sodium and measured by Western blot analysis. **B:** HEK293T cells were transfected with plasmids by using Effectene as indicated. Supernatants (Sup) and cell extracts were collected after 48 hours. Levels of IL-1 β in supernatants and levels of pro-IL-1 β , NLRP3, ASC, and Gal-3 in cell extracts were measured by Western blot analysis. **C:** Immunofluorescence microscopy images of Gal-3, NLRP3, and ASC in transfected HEK293T cells 48 hours after transfection (p.i.). **Insets:** Higher magnification of the boxed areas in main images. **D:** Quantitative results for **C** ASC specks and ASC-NLRP3 associations per cell. **E:** J774.1 cells were infected with H5N1 (MOI = 0.5 or 1). Cells were cross-linked with dithiobis-succinimidylpropionate and harvested for immunoprecipitation (IP) assays 15 hours p.i. Gal-3 was immune-precipitated with anti-Gal-3 antibodies. Co-precipitated NLRP3 was detected by immunoblot (IB) analysis. Rabbit IgG was used as an isotype control (ISO). All results are representative of independently conducted experiments. Data are expressed as means $\pm$ SEM. $n = 2$ independent experiments. $*P < 0.05$. Original magnification: $\times 400$ (**C**, main images); $\times 920$ (**C**, insets). Casp1, caspase-1.

stronger inflammatory reactions with higher levels of IL-12, interferon- γ , and IL-1 β .⁵¹ These findings suggest that galectin-3 alters mouse sensitivity to *R. equi* by reducing IL-1 β production from macrophages,⁵¹ which conflicts with our finding that galectin-3 promotes IL-1 β production. These contradictory results may be due to the inducement of different IL-1 β production and/or galectin-3–associated immune system regulation mechanisms. It may be that the roles of galectin-3 in infection-induced immune responses differ depending on the pathogens and organs involved.

The cell-based data indicate an association between galectin-3 and NLRP3 (Figure 5D) that affects ASC oligomerization (Figure 5A) and IL-1 β production. No effect was observed on NF- κ B signaling, as indicated by similar levels of pro-IL-1 β and NLRP3 transcripts and proteins in macrophages collected from H5N1-infected WT and Gal-3KO mice (Figure 4, B and C). In the NLRP3 inflammasome reconstitution system, galectin-3 promoted NLRP3-ASC inflammasome assembly (Figure 5, C and D). A possible explanation is that binding with galectin-3 triggers conformational changes in NLRP3, which in turn activates NLRP3 and induces ASC oligomerization. In summary, our data support the idea that galectin-3 serves as a bridge for NLRP3-ASC interaction and complex formation.

The conclusion that galectin-3 promotes NLRP3-ASC inflammasome assembly and IL-1 β processing, suggests that the positive effect of galectin-3 on NLRP3 inflammasome complex formation may not be restricted to H5N1 infections. Pro-IL-1 β levels in the lysates of cells transfected with pro-IL-1 β plasmids were much lower than in cells transfected with other inflammasome components (Figure 5B). According to this observation, it is likely that the presence and assembly of NLRP3 inflammasome components may stabilize pro-IL-1 β in cells. The presence of a basal amount of ASC speck was noted in ASC-expressing 293T cells (Figure 5C), perhaps because of protein overproduction.

Other researchers have described the role of galectin-3 in NLRP3-mediated IL-1 β production. In studies that involved dextran sulfate sodium–induced colitis and primary biliary cholangitis, Gal-3KO mice exhibited reduced disease severity, decreases in the production of IL-1 β and other proinflammatory cytokines, and lower NLRP3 expression compared with WT mice.^{52,53} These studies suggest a positive role for galectin-3 in the NF- κ B pathway, which contrasts with our finding that galectin-3 specifically acts on NLRP3 inflammasome assembly. It may be that galectin-3 regulatory mechanisms linked with IL-1 β production differ, depending on the disease model being used. During IAV infections, various virus components sensed by cells are known to trigger different downstream inflammatory signals—examples include the IAV proteins M2, NS1, and PB1 and virus RNA, all of which have been described as triggering or inhibiting NLRP3 inflammasome activation.^{13,54–56} Researchers may be interested in investigating the potential involvement of galectin-3 in these processes.

Conclusions

Our results indicate that after H5N1 infection i) endogenous galectin-3 enhances IL-1 β production and NLRP3 inflammasome assembly but not NF- κ B pathways in macrophages and ii) galectin-3 and NLRP3 interaction increases NLRP3-ASC inflammasome assembly and ASC oligomerization. From our finding that endogenous galectin-3 plays a key role in H5N1-induced inflammation, targeting galectin-3 may hold therapeutic potential for H5N1-induced pneumonia and lung injuries by attenuating inflammatory responses. We also accumulated additional evidence about the effects of galectin-3 in H5N1 infections that involve potent cytokine storms and lung inflammation.^{5,6} Further study is required to determine whether the role of galectin-3 is restricted to H5N1 or is common among IAV strains.

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