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Optimizing Lectin Staining Methodology to Assess Glycocalyx Composition of *Legionella*-Infected Cells

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Abstract

Introduction: *Legionella* is a gram-negative bacterium that replicates intracellularly within macrophages. *Legionella* utilizes effector proteins to hijack ER-Golgi vesicle trafficking to sustain proliferation in its intracellular niche. *Legionella* has a considerable influence on O-glycosylation but not N-glycosylation events in the Golgi of infected cells. This research aims to optimize the use of fluorescent lectins, which are proteins that bind carbohydrates, to effectively label host-cell glycocalyx during *Legionella* infection.

Methods: Epifluorescence imaging or flow cytometry were used to optimize the lectin staining methodology. We noted that the most effective conditions for lectin-labeling were when live HeLa cells were incubated with lectins diluted in Hank's balanced salt solution (HBSS) with 3% Bovine serum albumin (BSA) for 10-30 minutes at 4 °C.

Results: Incubating suspended cells with lectins necessitated smaller lectin concentrations, whereas lectin labeling of adherent cells required considerably larger concentrations. Wheat germ agglutinin (WGA) lectin mean fluorescence intensity (MFI) was concentration-dependent, but Concanavalin A (ConA) and Maclura pomifera (MPA) MFIs did not alter substantially with increasing lectin concentrations.

Discussion: The optimal lectin concentration required was lectin-specific and based on whether the lectin fluorescence was assessed using flow cytometry or epifluorescence. Furthermore, the use of phosphate-buffered saline (PBS) for lectin dilution, cell permeabilization for intracellular labelling, and incubation of lectins in fixed cells reduced productive labelling of lectins on cell surfaces because it inhibited the lectin's ability to effectively bind the associated carbohydrate structure.

Conclusion: Further research using diverse lectins on U937 macrophages is necessary to reach a definitive conclusion on the effect of *Legionella* on the overall host-cell glycocalyx composition during infection of these relevant immune cells.

Keywords: *Legionella*; macrophage; lectin; glycocalyx; N-linked glycosylation; O-linked glycosylation

Introduction

Legionnaires disease, a type of respiratory pneumonia, accounts for 2-15% of community-acquired pneumonia in North America [1]. *Legionella pneumophila* is a gram-negative intracellular bacterium that spreads through respiratory aerosols [2]. During infection, the pathogen is internalized by a macrophage through the process of phagocytosis. The internalization of *L. pneumophila* creates a phagosome which is referred to as the *Legionella*-containing vacuole (LCV) [3]. The LCV has been shown to hijack endoplasmic reticulum (ER)-derived vesicles through effector proteins produced, enabling it to remain undetected inside the host cell and proliferate intracellularly [4, 5]. Additionally, during *L. pneumophila* infection, Golgi fragmentation is not observed [6]. In fact, experimentally inducing Golgi fragmentation negatively impacts bacterial growth in the LCV [6].

The Golgi cisternae are divided into three compartments: cis, medial, and trans. ER-vesicles migrate along the secretory pathway, passing via the cis, medial, and trans-Golgi, allowing for post-translational protein modifications. O-glycosylated proteins are produced exclusively in the Golgi by the addition of N-acetylgalactosamine (GalNac) sugar to serine/threonine residues, whereas N-glycosylation of proteins is initiated in the ER by the addition of N-acetylglucosamine (GlcNac) to asparagine residues, and the proteoglycans are further modified in the Golgi [7,8]. Additionally, O-linked, and N-linked glycans can be categorized further depending on the branching composition of their internal core structures that are grafted onto the primary GalNac or GlcNac monomer. O-linked glycans are classified into four unique core structures, with cores 1 and 2 being the most prevalent in mammalian physiology (Figure 1) [8]. N-glycans, on the other hand, are found branching in complex, hybrid, or oligomannose configurations, each with distinct properties (Figure 1) [9]. As they pass through the Golgi or ER, O-linked and N-linked glycans are modified further by the

incorporation of various sugars extending onto their core structures [8,9]. Interestingly, during *L. pneumophila* infection, the LCV reduces O-glycosylation events in the Golgi but has no effect on N-glycosylation events [6].

Glycosylated carbohydrates contribute to the glycocalyx of the cell plasma membrane. Glycoproteins, proteoglycans, and glycolipids are only a few examples of the sugar units that are present in their totality on the plasma membrane and are referred to collectively as the "glycocalyx" [10]. It serves a variety of functions, including defining cell shape, and defending the cell from invading pathogens [10]. The present study aims to optimize the application of lectins in assessing glycocalyx composition in *L. pneumophila* -infected cells. Lectins are proteins that may be used as probes to recognize specific glycan types [11]. The presence of N-linked and O-linked glycans will be examined using fluorescein isothiocyanate (FITC)-conjugated or Alexa Fluor™488-conjugated lectins, both of which have green fluorescence. We will use Amaranthus caudatus (ACA), Maclura pomifera (MPA), and Artocarpus integrifolia (AIA) to locate O-glycans in the cell's glycocalyx. MPA and AIA lectins recognize core 1 and core 3 configurations of O-glycans, whereas ACA recognizes core 1 and core 2 (Figure 1) [12] Concanavalin A (ConA) lectin will be utilized to identify N-glycans since it can bind the oligomannose core of N-glycans (Figure 1) [12]. Wheat germ agglutinin (WGA) will be employed to establish optimal fluorescence intensity for lectin staining methodologies owing to its capability to bind a variety of different sugars [12]. We want to optimize our current lectin staining methodology to allow for a reliable assessment of their fluorescence intensity using epifluorescence or flow cytometry.

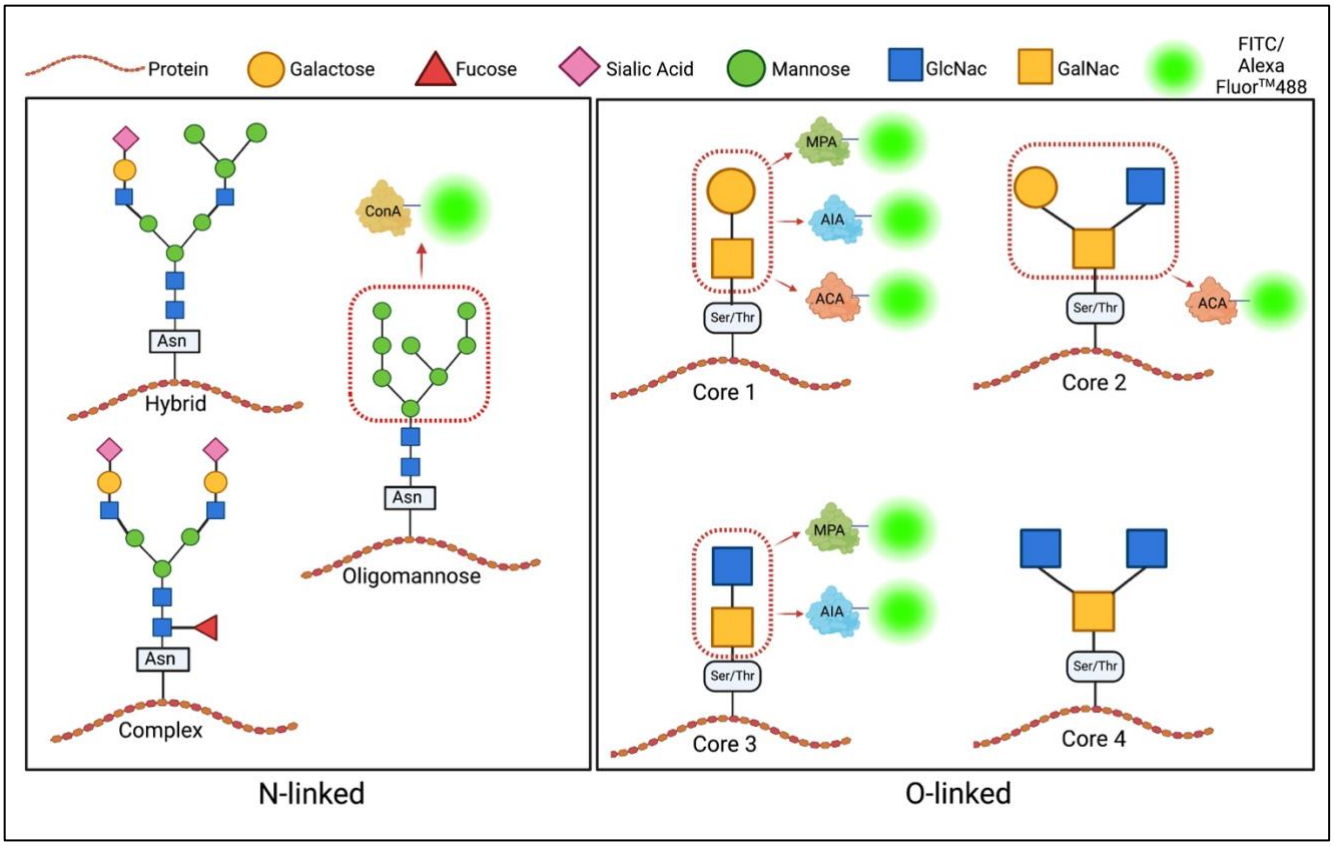

Figure 1. N-linked and O-linked glycan core structure configurations. Red boxes highlight lectin binding sites within the glycans. Note that the image illustrates core structures only. Mature glycans are extended beyond these structures. Image was constructed using BioRender.com.

Methods

Reagents

U937 human monocytic cells (CRL-1593.2), HeLa epithelial cells (CRM-CCL-2), and Trypsin-EDTA were supplied by American Type Culture Collection (ATCC). RPMI 1640, Dulbecco's minimal essential medium (DMEM), Fetal bovine serum (FBS), and Hanks' Balanced Salt Solution (HBSS) were supplied from Gibco (Gaithersburg, MD, USA). Bovine serum albumin (BSA) was obtained from Bioshop. Dr. Mauricio Terebiznik's laboratory (University of Toronto Scarborough, Scarborough, ON, Canada) provided *L. pneumophila* strains

Lp02 (wild type) and the rabbit polyclonal anti-*Legionella* antibody. Cy5-conjugated secondary antibody was from Cedarlane. Phorbol-12-myristate-13-acetate (PMA) was obtained from Sigma-Aldrich (Oakville, ON, Canada). Paraformaldehyde (PFA) was supplied by Electron Microscopy Sciences (Hatfield, PA, USA). Artocarpus integrifolia (AIA)-FITC (fluorescein), Amaranthus caudatus (ACA)-FITC (fluorescein), and Maclura pomifera (MPA)-FITC (fluorescein) lectins were from GlycoMatrix/Cedarlane. Alexa Fluor™488 conjugated Wheat germ agglutinin (WGA) and Concanavalin A (ConA) were from Life Technologies (Carlsbad, CA, USA).

Cell Cultures

HeLa cell monolayers in T75 flasks were maintained at 37 °C in an incubator with 5% CO_2 in DMEM media with 10% FBS and passed every 2 or 3 days to preserve cell confluence between 70% and 80%. For plating and subculturing, HeLa cells were lifted with 0.25% trypsin - 0.53mM EDTA.

Suspended U937 cells in T75 flasks were maintained at 37 °C in an incubator with 5% CO_2 in RPMI-1640 media with 10% FBS and passed every 2 or 3 days to preserve cell density between $1x10^5$ and $2x10^6$ cells/mL. For infection assays, suspended U937 cells were cultured for 24 hrs prior to infection in RPMI with 1uM PMA and kept at 37 °C with 5% CO_2 to differentiate them into macrophages.

Cell Infections with *L. pneumophila*

Bacteria from frozen glycerol stocks were streaked over ACES-containing buffered charcoal yeast extract (BCYE) agar plates. Bacteria were scraped from BCYE plates after 3 days of growth and cultivated in BYE broth for 21 hrs at 35°C with shaking at 100 rpm, yielding a final OD600 of 2.0 for Lp02.

Lp02 with an OD600 of 2.0 was introduced onto U937 macrophages with a multiplicity of infection (MOI) of 100. Plates were spun at 300g for 5 minutes at 4 °C then cells were incubated at 37 °C for 30 minutes to allow for bacterial infection. The cells were washed and then fresh RPMI media containing 10% FBS was added. Infections were allowed to progress at 37 °C and cells were fixed with 4% PFA after 6 hrs of infection.

Fixed Cell Lectin Surface Staining

Fixed U937 macrophages after infection with wild-type Lp02 strain were blocked in PBS with 5% FBS for 15 minutes to prevent non-specific binding. Cells were then washed with PBS and incubated for 1 hr with 1-20 ug/ml of lectin (WGA, ConA, MPA, AIA, or ACA) diluted in PBS with 2% FBS or HBSS with 3% BSA. Following lectin cell-surface binding, cells were washed with 1X PBS and permeabilized for 20 minutes in 0.1% Triton X-100/PBS containing 100 mM glycine. The cells were then washed and blocked for 1 hr with 5% FBS in PBS. To label *Legionella*, cells were treated for 1 hr with 1:3000 rabbit polyclonal anti-*Legionella* antibody diluted in PBS containing 1% FBS. After washing the cells with PBS, they were treated for 1 hr with a 1:500 Cy5-conjugated secondary antibody. To label the nucleus, cells were washed twice with double distilled water and incubated for 10 minutes with 1 ug/mL DAPI. Dako Fluorescent Mounting Media was used to mount cells for epi-fluorescence microscopy imaging. The preceding incubation procedures were all completed at room temperature. A similar procedure was used for fixed uninfected HeLa cells. HeLa cells were blocked, lectin-treated, permeabilized for DAPI staining, and mounted. HeLa cells were immediately mounted on coverslips following lectin labeling in certain experiments.

Live Cell Lectin Surface Staining

Uninfected HeLa cells were rinsed with cold PBS (stored at 4°C). Cells were then incubated for 1 hr or 10 minutes at 4°C with 1 ug/ml, 2 ug/ml, 10 ug/ml, or 20 ug/ml lectin (WGA, ConA, MPA, AIA, or ACA) diluted in PBS with 5% FBS or HBSS with 3% BSA. Cells were then rinsed three times with cold PBS before being fixed in 4% PFA for 15 minutes at 4°C. Fixed cells were then washed and mounted, or DAPI staining was performed using a similar approach as described in the fixed cell lectin surface staining protocol.

Flow Cytometry Live Cell Lectin Stain and Cell Preparation

Uninfected HeLa cells on 6-well plates were lifted using 0.25% Trypsin-0.53mM EDTA before being collected. The cells were aliquoted and pelleted in microcentrifuge tubes to be resuspended in FACS buffer (2% FBS in PBS). Then the cells were pelted and resuspended in 100ul of FACS buffer with lectin (1-20 ug/ml of WGA, ConA, MPA, AIA, or ACA) at 4 °C for an hour or 30 minutes. To wash unbound lectin, cells were washed twice in the FACS buffer. After the final wash, cells were resuspended in 500ul of 4% PFA for 15 minutes in the dark at 4°C. Cells were spun at 400g for 5 minutes and resuspended in 1 ml of FACS buffer at least twice more. To retain the initial cell concentration, cells were resuspended in 500ul FACS during the final wash. Microcentrifuge tubes were kept at 4°C in the dark until flow cytometry analysis.

Flow Cytometry and Statistical Analysis

BD FACSDiva Software was used to collect and analyze all flow cytometry data. The FITC channel was used to identify the presence of FITC/Alexa Fluor™488-conjugated lectins. All test conditions were subjected to a live cell gate. Negative HeLa cell controls were gated to exclude autofluorescence in uninfected conditions, and this gate was used in all instances where uninfected HeLa cells were treated with lectins. For each condition, histogram overlays of FITC/Alexa Fluor™488 mean fluorescence

intensity (MFI) were produced. The geometric MFI of FITC/Alexa Fluor™488, standard deviation, and cell count were measured for each treatment and entered into prism. Prism was then used to perform a two-tailed, unpaired t-test between the negative controls and the lectin test condition. The P-value of 0.05 was used to evaluate whether there was a statistically significant difference between the control and each test condition.

Results

Experiment 1: Macrophage Infection with *L. pneumophila* Strain Lp02 Followed by Fixed Cell Lectin Staining

We began our studies using U937 macrophage cells after differentiation, acutely infected with *Legionella*. These U937 macrophages had a low cell confluence and hence a lower number of infected macrophages (Figure 2). Moreover, FITC/Alexa Fluor™488 fluorescent lectin (WGA, ConA, MPA, AIA, and ACA) labelling after cell fixation showed low intensity with considerable background fluorescence in all treatments (Figure 2). Under infected conditions, the Lp02 secondary antibody (Cy5) similarly showed increased background fluorescence and reduced effectual labeling of Lp02 bacteria. In both infected and uninfected conditions, non-specific binding of the Cy5 secondary antibody was detected (Figure 2). In some situations, lectin artifacts, recognized as a bright green fluorescence in empty space, were also found (Figure 2).

Experiment 2: Fixed Cell Lectin Staining of Uninfected HeLa Cells

We turned to HeLa cells to troubleshoot the lectin staining procedure after experiencing challenges with infected U937 macrophages. Because *Legionella* infection is a time-consuming process, we opted to optimize the approach on uninfected HeLa cells for the time being. In contrast to U937 macrophages, HeLa cells had a greater cell confluence (Figure 3). WGA and ConA lectin fluorescence at the surface of HeLa cells was considerably elevated compared to MPA, AIA, and ACA (Figure 3). Non-specific lectin binding was recognized as increased nuclear lectin staining relative to surface lectin fluorescence in all lectin conditions (WGA, ConA, MPA, AIA, and ACA).

Experiments 3 & 4: Live Cell Lectin Staining of Uninfected HeLa Cells

Qualitative Measure: Epi-fluorescence Microscopy Data

We used live cell staining to enhance labelling of HeLa cells with fluorescent lectins and decrease background staining observed with fixed cell staining. Live HeLa cells were treated for 1 hour at 4°C with lectin (1 or 2 ug/ml) (Figure 4a). Additional treatment parameters, such as the following cell permeabilization process for DAPI labelling and the solution in which the lectins were diluted, remained unchanged. We noticed that the live cell labelling approach significantly reduced HeLa cell confluence (Figure 4a). With the exception of WGA lectin, the fluorescence intensity of FITC/Alexa Fluor™488-conjugated lectins was essentially non-existent in most conditions. Nevertheless, the WGA fluorescence was only found in the HeLa nucleus and not on the cell surface (Figure 4a).

To optimize previous findings and recover cell confluence, we abbreviated the lectin incubation duration at 4°C, changed the solution in which the lectins were diluted, raised lectin concentration by a factor of 10, and eliminated cell permeabilization and subsequent DAPI labelling. We also decided to test the new conditions on fixed HeLa cells to see which technique yielded the most favorable results. In live cells treated with lectin (10 ug/ml or 20 ug/ml) for 10 minutes at 4°C, HeLa cell confluence and FITC/Alexa Fluor™488-conjugated lectin fluorescence was considerably greater than in other experiments (Figure 4b). There was no lectin nuclear labeling observed in any conditions. Fixed HeLa treated with identical lectin concentrations and incubated for 1 hr at room temperature yielded comparable results. In comparison to the fixed cell staining approach, the live cell staining method demonstrated a stronger overall FITC/Alexa Fluor™488 lectin fluorescence intensity (WGA, ConA, MPA, ACA, and AIA) (Figure 4b).

Quantitative Measure: Flow Cytometry Data

Epifluorescence allowed us to examine the effectiveness of lectin staining and visualize lectin localization on HeLa cells. To quantify lectin fluorescence in HeLa cells, we turned to flow cytometry. Live HeLa cells were first incubated in 1 ug/ml of lectins diluted in PBS with 5% FBS (Figure 5a). Lectin incubation was done in suspended cells at 4°C for 1 hour. This incubation period was shortened to 30 minutes when lectins were diluted in HBSS with 3% BSA and lectin concentrations were increased to 10 or 20 ug/ml (Figure 5b). We noted that the MFI was significantly higher in all lectin treatments (WGA, ConA, AIA, MPA, ACA) compared to the negative control, regardless of lectin concentration (1 ug/ml, 10 ug/ml, or 20 ug/ml). WGA lectin curves at 1ug/ml or 10 ug/ml produced the largest peaks in comparison to other lectin MFI curves, correlating to a higher abundance of WGA-labeled HeLa cells and a higher MFI at both concentrations (Figure 5a, b). MFI was significantly higher at 10 ug/ml of WGA than at 1 ug/ml of WGA (Figure 5c). ConA, MPA, AIA, and ACA at 1 ug/ml and 20 ug/ml showed comparable MFI relative to each other, indicating similarity in the abundances of lectin-labeled HeLa cell populations (Figure 5c).

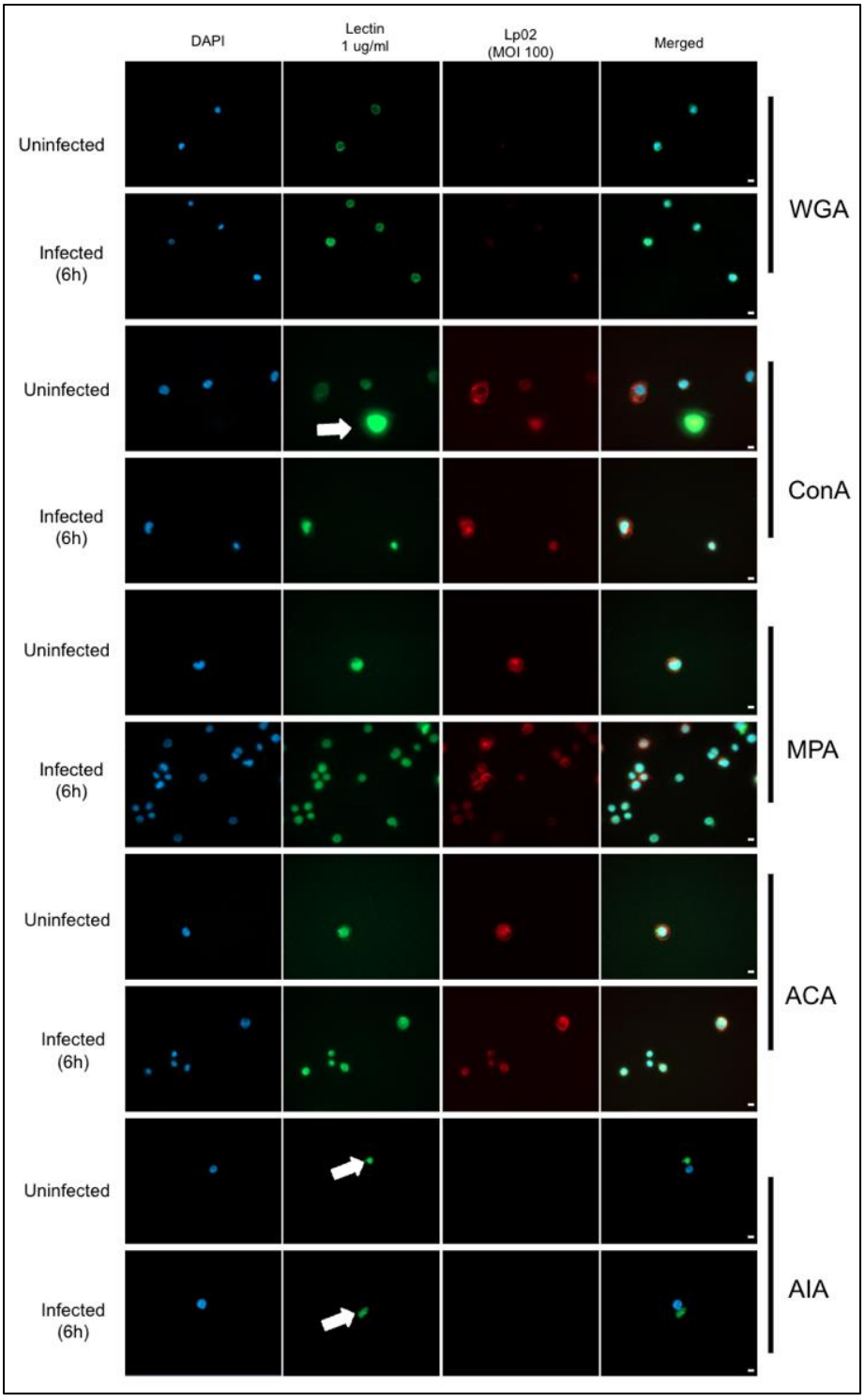

Figure 2. U937 macrophages were infected with *L. pneumophila* (strain Lp02) at a MOI of 100. Cells were fixed 6hr post-infection and stained with lectins (green) WGA, ConA, MPA, AIA, or ACA to label N-linked or O-linked glycans at the cell surface. Lectins were diluted in PBS containing 5% FBS to a concentration of 1ug/ml (1:500 dilution). Cy5 (red) was used to visualize *L. pneumophila*, while DAPI (blue) was utilized to show the nucleus of U937 macrophages. The white arrow indicates lectin artifacts, which are characterized as intense green fluorescence in empty space. Scale bars = 10 µm. Cell images were taken using ZEISS microscopy software. Image was constructed using Microsoft PowerPoint.

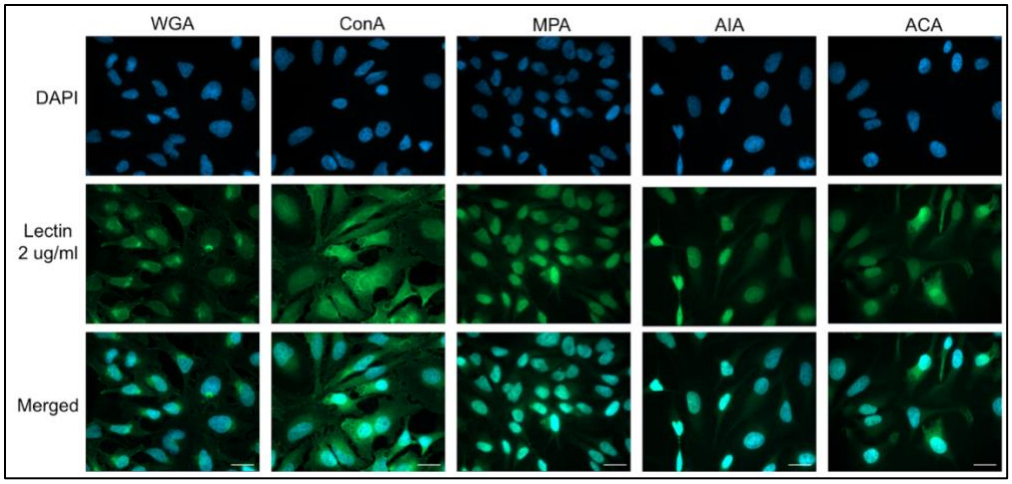

Figure 3. HeLa cells fixed and labelled with fluorescent lectins. Fixed HeLa cells were stained with lectin (green) to identify N-linked or O-linked glycans. DAPI was used to identify the nucleus of HeLa cells (blue). Lectins were diluted in PBS containing 5% FBS to a concentration of 2 ug/ml (1:250 dilution). The HeLa cell surface and nucleus were both labeled with lectin. Scale bars = 10 µm. Cell images were taken using ZEISS microscopy software. Image was constructed using Microsoft PowerPoint.

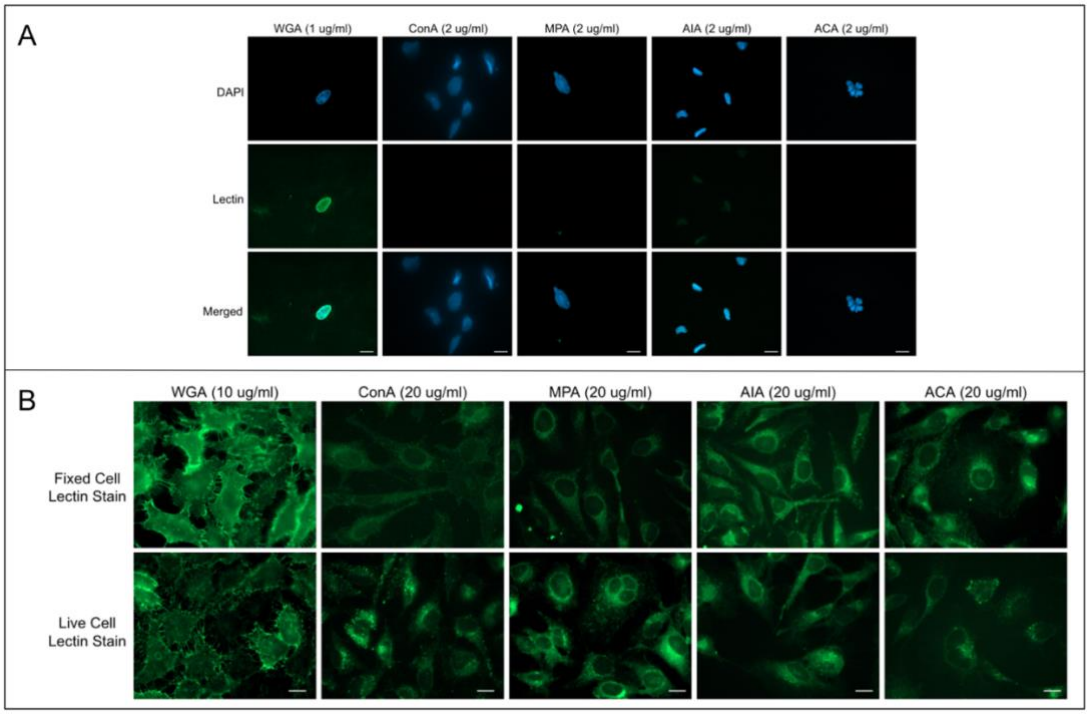

Figure 4. (A) Live Hela cells were incubated with lectins followed by cell permeabilization. To label N-linked or O-linked glycans, live HeLa cells were treated with lectins (green) for 1 hr at 4°C. Lectins were diluted in PBS containing 5% FBS at a concentration of 2ug/ml (1:250 dilution) for ConA, MPA, AIA, and ACA, and 1ug/l (1:500 dilution) for WGA. After fixation, cells were permeabilized and the nucleus of the HeLa cell was stained with DAPI (blue). **(B)** Optimized lectin incubation conditions without cell permeabilization were compared between live and fixed HeLa cells. To label N-linked or O-linked glycans, live HeLa cells (bottom row) were treated with lectins (green) for 10 minutes at 4°C. Lectins were diluted in HBSS containing 3% BSA at a concentration of 20 ug/ml (1:50 dilution) for ConA, MPA, AIA, and ACA, and 10 ug/l (1:100 dilution) for WGA. Using the same approach as for live cell lectin staining, fixed HeLa cells (top row) were treated with lectin (green) for 1 hr at room temperature. There was no cell permeabilization or DAPI staining in either the fixed or

live cell staining procedures. Scale bars = 10 µm. Cell images were taken using ZEISS microscopy software. Image was constructed using Microsoft PowerPoint.

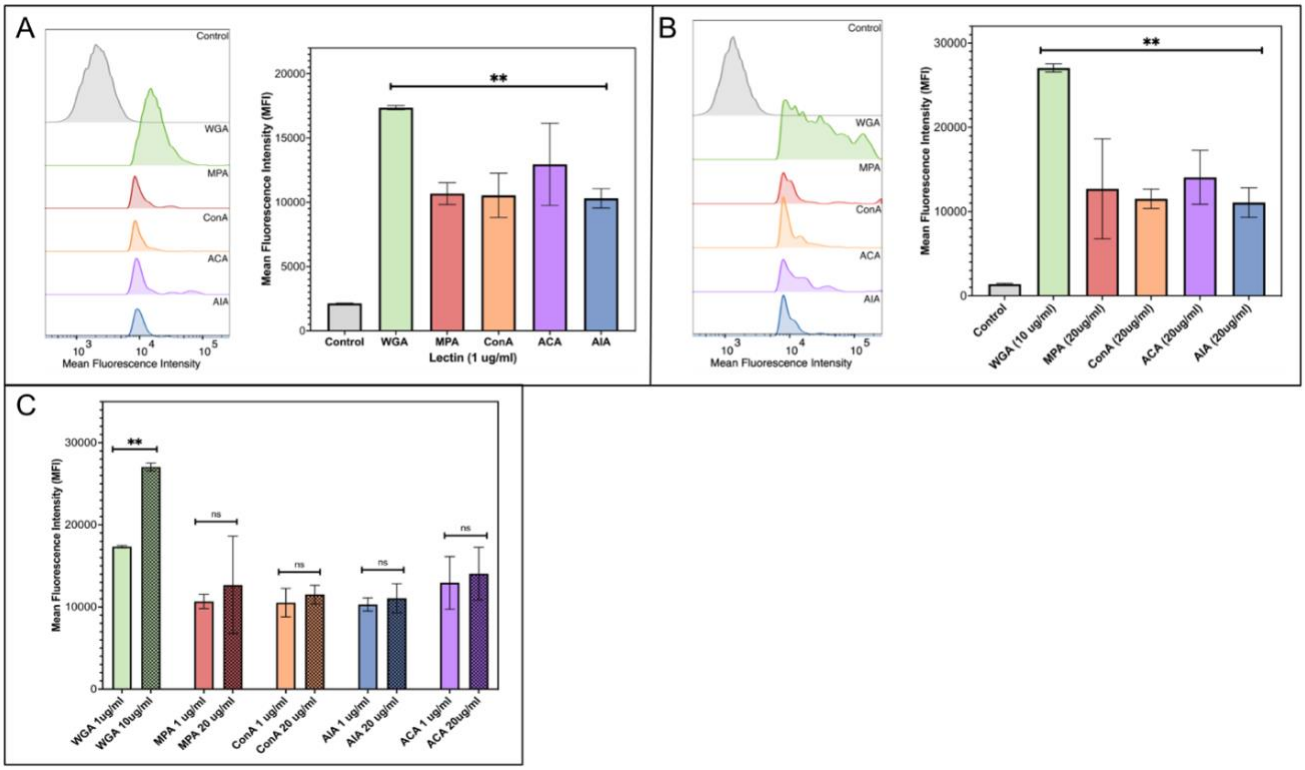

Figure 5. (A) Live HeLa cells were incubated with FITC/Alexa Fluor™488-conjugated lectins in suspension at 4°C for 1 hr. 1 ug/ml of each lectin was diluted in PBS with 5%FBS. **(B)** Live HeLa cells were incubated with FITC/Alexa Fluor™488-conjugated lectins in suspension at 4°C for 30 mins. 10 ug/ml of WGA and 20 ug/ml of ConA, MPA, AIA, and ACA were diluted in HBSS with 3% BSA. **(C)** The MFI for low and high lectin concentrations is represented as a bar graph, with lower concentrations highlighted by solid-colored bars and larger concentrations highlighted by patterned-bars. For all graphs, green is WGA, red is MPA, orange is ConA, purple is ACA, blue is AIA, and gray is control (no lectin). Half offset histograms represent MFI of FITC/Alexa Fluor™488 on the x-axis while the y-axis is normalized to mode (at 10,000). Bar graphs represent MFI of FITC/Alexa Fluor™488 on the y-axis for each corresponding condition on the x-axis while the error bars represent mean ± S.E.M. (** = $p < 0.05$, ns = non-significant, two-tailed, unpaired T-test). All graphs were made using Prism and FlowJo Softwares.

Discussion

The pathophysiology of infectious diseases has been linked to cell-surface glycans; hence, glycan profiling could potentially be a valuable diagnostic approach [13]. Due to their versatility, cost-effectiveness, and widespread commercial availability, the use of lectin fluorescence probes has become the predominant technique for examining glycans [14]. The purpose of this research was to optimize the use of lectins in analyzing the glycocalyx composition of *Legionella*-infected cells. We report that FITC/Alexa FluorTM488-conjugated lectins exhibit enhanced fluorescence intensity when diluted in HBSS with 3% BSA and incubated with live cell cultures at 4°C for 10 minutes for adherent cells or 30 minutes for suspended cells. Ineffective lectin labelling was seen when lectins were diluted in PBS with 5% FBS, most likely due to the presence of phosphate ions in the buffer solution, which impedes lectin-binding sites [15]. Moreover, live-cell lectin labelling at 4°C allowed cells to lower metabolic activity, endocytosis, and retain native glycan structures, all of which contributed to enhanced lectin visualization and quantification. The longer incubation duration (1hr) of HeLa cells at 4°C, on the other hand, considerably reduced both lectin visualization and cell confluence because the lower temperature likely caused the cells to lift and be washed away during the staining procedure. Consequently, with adherent HeLa cells, we noticed the best outcomes were when incubation times were reduced to 10 minutes. However, when HeLa cells are incubated with lectins in suspension, as done with the flow cytometry protocol, cell

lifting is not a concern, so longer incubation periods of 30 minutes can be employed. We also noted that the live cell lectin labelling methodology outperformed the fixed cell lectin labelling protocol, possibly because the carbohydrate moieties became secluded/denatured due to fixation [15].

Additionally, using HeLa cells rather than U937 macrophages allowed us to deal with an inherently adherent cell population, eliminating the heterogeneity of seeding cell density found in U937 cells, which require additional treatments to differentiate into adherent monolayer macrophages. HeLa cells are considerably larger and flatter than U937 cells, which helped with lectin imaging as well. Epi-fluorescence imaging required greater lectin concentrations (10 ug/ml or 20 ug/ml) for adequate fluorescence visualization, whereas flow cytometry functioned effectively with lower concentrations (1 ug/ml or 2 ug/ml). ConA, MPA, AIA, and ACA displayed concentration-independent MFI, but WGA had considerably larger MFI as concentration increased. Additionally, removing the cell permeabilization step and subsequent DAPI staining decreased aberrant nuclear lectin labelling and maintained lectins confined to the cell surface, allowing for efficient and specific glycocalyx analysis.

Conclusions

Our ultimate goal was to see if *Legionella* infection impacted the glycocalyx. To adequately characterize the glycocalyx composition of *Legionella*-infected macrophages, lectin staining under optimal conditions should be replicated in U937 macrophages with the inclusion of several different lectins in the future. This research sheds light on lectin analysis as a method of glycan profiling, which is becoming a recognized in-vitro approach for assessing carbohydrates on the cell surface. The methodologies mentioned in this research might be employed to investigate the interaction between host-cell glycan modification and bacterial pathogenesis in the pursuit of developing therapeutic interventions for bacterial infections.

List of Abbreviations Used

HBSS: Hank's balanced salt solution (HBSS)
BSA: bovine serum albumin
WGA: wheat germ agglutinin
ConA: Concanavalin A
MPA: Maclura pomifera
ACA: Amaranthus caudatus
AIA: Artocarpus integrifolia
MFI: mean fluorescence intensity
LCV*: Legionella*-containing vacuole
GalNac: N-acetylgalactosamine
GlcNac: N-acetylglucosamine
FITC: fluorescein isothiocyanate
ATCC: American Type Culture Collection
DMEM: Dulbecco's minimal essential medium
FBS: fetal bovine serum
PMA: phorbol-12-myristate-13-acetate
PFA: paraformaldehyde
BCYE: buffered charcoal yeast extract
ACES: N-(2-Acetamido)-2-aminoethanesulfonic acid
OD: optical density
Rpm: revolution per minute
BYE: buffered yeast extract
MPO: multiplicity of infection
RPMI: Roswell Park Memorial Institute
PBS: phosphate buffered saline
DAPI: 4′,6-diamidino-2-phenylindole
EDTA: ethylenediaminetetraacetic acid
FACS: Flow Cytometry Staining Buffer
ER: endoplasmic reticulum

Conflicts of Interest

The authors declare that they have no conflict of interests.

Ethics Approval and/or Participant Consent

This study did not require ethics approval as it was not done on human/animal subjects.

Authors' Contributions

SSK: made contributions to the design of the study, the collection of data as well as interpretation and analysis of the data, revised and drafted the manuscript critically, and gave final approval of the version to be published.
REH: made contributions to the design of the study, the collection of data as well as interpretation and analysis of the data, edited the manuscript, and gave final approval of the version to be published.
REH: made contributions to the design of the study and planning, assisted with analysis of data, edited the manuscript, and gave final approval of the version to be published.

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