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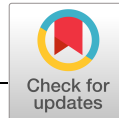
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## ARTICLE

# High- and low-molecular-weight chitosan act as adjuvants during single-dose influenza A virus protein vaccination through distinct mechanisms

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**Abstract**

The investigation of new adjuvants is essential for the development of efficacious vaccines. Chitosan (CS), a derivative of chitin, has been shown to act as an adjuvant, improving vaccine-induced immune responses. However, the effect of CS molecular weight (MW) on this adjuvanticity has not been investigated, despite MW having been shown to impact CS biological properties. Here, two MW variants of CS were investigated for their ability to enhance vaccine-elicited immune responses in vitro and in vivo, using a single-dose influenza A virus (IAV) protein vaccine model. Both low-molecular-weight (LMW) and high-molecular-weight (HMW) CS-induced interferon regulatory factor pathway signaling, antigen-presenting cell activation, and cytokine messenger RNA (mRNA) production, with LMW inducing higher mRNA levels at 24 h and HMW elevating mRNA responses at 48 h. LMW and HMW CS also induced adaptive immune responses after vaccination, indicated by enhanced immunoglobulin G production in mice receiving LMW CS and increased CD4 interleukin 4 (IL-4) and IL-2 production in mice receiving HMW CS. Importantly, both LMW and HMW CS adjuvantation reduced morbidity following homologous IAV challenge. Taken together, these results support that LMW and HMW CS can act as adjuvants, although this protection may be mediated through distinct mechanisms based on CS MW.

**KEYWORDS**

adaptive immunity, adjuvants, chitosan, innate immunity, IRF signaling

## 1 | INTRODUCTION

Adjuvants have been used for over 85 years to improve the immunogenicity of vaccines (Coffman et al., 2010; Di Pasquale et al., 2015; McKee et al., 2010). Adjuvants are used to enhance innate and adaptive immune responses induced during vaccination, allowing for

the development of effective memory responses and protection against infection (Coffman et al., 2010; Di Pasquale et al., 2015; McKee et al., 2010). In protein vaccines, adjuvants are particularly important, as protein vaccines lack natural pathogen-associated molecular patterns that initiate innate immune responses through activation of pattern recognition receptors (Bergmann-Leitner &

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Leitner, 2014; Di Pasquale et al., 2015; Modlin, 2012). Innate immune responses are required to activate adaptive T-cell responses, which enhance cellular and humoral immunity. Currently, there exists only a small number of adjuvants that are used in vaccines that are approved for human use in the US. Adjuvants used in approved human vaccines include aluminum salts (alum), oil-in-water emulsions (such as MF-59), monophosphoryl lipid A (MPLA), saponins (QS-21), and unmethylated CpG oligodeoxynucleotides (Centers for Disease Control and Prevention, 2016). Each adjuvant induces unique immune responses, allowing for tailoring of the vaccine-induced response to the target pathogen (Awate et al., 2013; Ciabattini et al., 2016; Coffman et al., 2010; Di Pasquale et al., 2015; Ho et al., 2018; Kwissa et al., 2012; Lee & Nguyen, 2015; McKee et al., 2010, 2007; McKee & Marrack, 2017). However, the small number of adjuvants used in approved human vaccines limits the ability to finetune the vaccine-induced immune response. Continued investigation of new materials that can act as vaccine adjuvants will increase the pool of available adjuvants that can be utilized during the generation of vaccines.

Chitosan (CS), an immunostimulatory biomaterial, has been investigated as a possible adjuvant (Carroll et al., 2016; Chang et al., 2010; Ghendon et al., 2008, 2009; Heffernan et al., 2011; Scherließ et al., 2013; Sui, Chen, Fang, et al., 2010; Sui, Chen, Wu, et al., 2010; Wang et al., 2012; Westerink et al., 2002; Xie et al., 2007). CS is a positively charged, linear polysaccharide, which is a partially deacetylated derivative of the natural compound chitin derived from crustaceans (Ravi Kumar, 2000). CS is biodegradable, biocompatible, and nontoxic and has been investigated for many applications, including tissue engineering and delivery of genes, drugs, and DNA vaccines (Farris et al., 2017; Mohebbi et al., 2019; Ravi Kumar, 2000). Despite being considered nonimmunogenic (Dragostin et al., 2016; Mohebbi et al., 2019; Ravi Kumar, 2000), CS and CS particles have been shown to act as immunostimulants *in vitro* and *in vivo* (Bueter et al., 2011; Carroll et al., 2016; Chang et al., 2010; Ghendon et al., 2008, 2009; Heffernan et al., 2011; Lin et al., 2014; Mori et al., 2012; Scherließ et al., 2013; Sui, Chen, Fang, et al., 2010; Sui, Chen, Wu, et al., 2010; Wang et al., 2012; Westerink et al., 2002; Xie et al., 2007).

As an adjuvant, CS has been shown to improve protection against lethal infection, antibody responses, and interferon (IFN)- $\gamma$  production by T cells in mouse models using a variety of vaccination routes and antigens (Carroll et al., 2016; Chang et al., 2010; Ghendon et al., 2008, 2009; Heffernan et al., 2011; Scherließ et al., 2013; Sui, Chen, Fang, et al., 2010; Sui, Chen, Wu, et al., 2010; Wang et al., 2012; Westerink et al., 2002; Xie et al., 2007). However CS is a polymer, and, thus, its properties are heavily influenced by parameters including molecular weight (MW) (Aggarwal & Matthew, 2009; Huang et al., 2005, 2004; Kiang et al., 2004; Maurstad et al., 2007; Ravi Kumar, 2000; Shukla et al., 2013). Previous studies examining the adjuvanticity of CS have investigated single and very broad MW ranges of CS (150–400 kDa [Carroll et al., 2016], 50–1000 kDa [Scherließ et al., 2013], 200–600 kDa [Heffernan et al., 2011], 190–310 kDa [Westerink et al., 2002], and the mixture of 300 and 10 kDa [Yuri Ghendon et al., 2009]), but many published

studies do not define the properties of the CS used, making it difficult to draw conclusions about the effect of MW on adjuvanticity of CS (Chang et al., 2010; Ghendon et al., 2008; Sui, Chen, Fang, et al., 2010; Sui, Chen, Wu, et al., 2010; Wang et al., 2012; Xie et al., 2007). Given that CS MW has been shown to impact its interactions with proteins and phospholipid bilayers (Bekale et al., 2015; Fang et al., 2001), MW has the potential to alter how CS interacts with host cells, which could affect CS adjuvanticity during vaccination.

In this report, the effect of MW on the adjuvant properties of CS was investigated. Given that many previous investigations of CS have used MW ranging from 50 to 1000 kDa, a low-molecular-weight (LMW) CS variant examining the lower range and a high-molecular-weight (HMW) CS variant examining the middle range were selected for this investigation. In addition, an influenza A virus (IAV) model was used to determine CS adjuvanticity, given the need for improvements to the IAV vaccine strategy. IAV is an RNA virus and during replication, point mutations can be incorporated in the outer coat proteins in a process known as antigenic drift. Antigenic drift variants escape antibody-mediated neutralization; thus, vaccines have to be reformulated each year according to the seasonal IAV strains currently circulating in the population. For example, early data from the 2019–2020 influenza season estimate the vaccine was only 45% effective in preventing laboratory-confirmed influenza (Dawood et al., 2020). One method to improve IAV vaccine efficacy is to incorporate adjuvants, which are thought to boost the immunogenicity of IAV proteins in an attempt to induce broader, more universal protection against seasonal strains that undergo antigenic drift (Tregoning et al., 2018). Here, we investigate the ability of LMW CS, averaging 50–190 kDa, and HMW CS, averaging 310–375 kDa, to act as adjuvants in an IAV protein vaccine by enhancing antigen-presenting cell (APC) function, increasing antibody production, and providing protection against influenza challenge.

## 2 | MATERIALS AND METHODS

### 2.1 | Ethics statement

Experimental procedures using mice were conducted in accordance with the US Animal Welfare Act and approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln. IAVs are Biosafety Level (BSL)-2 pathogens and were used in accordance with guidelines set forth in Biosafety in Microbiological and Biomedical Laboratories, Centers for Disease Control, and National Institutes of Health. All personnel was trained in BSL-2 safety and protocols were approved by the University of Nebraska-Lincoln Institutional Biosafety Committee (Protocol #112).

### 2.2 | Mice

Female BALB/cByJ mice were purchased from Jackson Laboratories (Bar Harbor). Male OT-II T-cell receptor transgenic mice (B6.Cg-Tg

(TcraTcrb)425Cbn/J) on the C57BL/6 background were also used in select experiments. Breeding pairs for the OT-II mice were purchased from Jackson Laboratories. OT-II mice were used for the generation of bone marrow-derived dendritic cells (BMDCs) that were used for flow cytometric analysis of cellular activation and viability. Three- to six-month-old mice were used in all experiments.

### 2.3 | BMDC culture and CS treatment

BMDCs were generated as previously described (Lampe et al., 2020). Bone marrow was collected by washing the femurs and tibiae of mice with phosphate-buffered saline (PBS) using a 3 ml syringe with a 26 G needle. Red blood cells in the cellular suspension were lysed using ammonium-chloride-potassium (ACK) buffer, remaining cells were collected, washed, and resuspended at  $2 \times 10^6$  cells/ml. After resuspension, 1 ml of cells were cultured in six-well plates in a total volume of 5 ml of complete Roswell Park Memorial Institute (RPMI) media for 7 days in the presence 5 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF). BMDCs were cultured in RPMI 1640 media (Thermo Fisher Scientific) containing 2-mM L-glutamine (Invitrogen), 100 IU penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen), 10 mM HEPES (Sigma-Aldrich), 50 µM 2-mercaptoethanol (Sigma-Aldrich), and 7% fetal bovine serum (FBS) (Hyclone). On Days 3 and 5 after initiation of culture, half of the media was removed and replaced with fresh media containing 5 ng/ml GM-CSF (Lampe et al., 2020).

On Day 7 after initiation of culture, BMDCs were collected from six-well plates by gentle washing, counted, and replated in 12-well plates at  $2 \times 10^6$  cells in 1 ml total complete RPMI media containing 5 ng/ml GM-CSF for treatment. LMW and HMW CS were separately added to the media at the indicated concentrations on day seven and cells were incubated at 37°C for the indicated time. A range of CS doses spanning 0.01–10 µg/ml was chosen to determine the doses at which of CS-induced detectable responses. Both LMW and HMW CS were obtained from Sigma-Aldrich. Both LMW and HMW CS were suspended at 5 mg/ml in 1% acetic acid solution before dilution to the indicated treatment dose in cell media. Nonparticle forms of CS were used throughout the report. As indicated by the supplier, LMW CS averaged 50–190 kDa with 75%–85% deacetylation. HMW CS averaged 310–375 kDa with greater than 75% deacetylation (Table S1). LMW and HMW CS were tested for endotoxin impurities using a LAL Endochrome-K Kit (Charles River) and found to be endotoxin-free (Table S1). Cells were also treated with 0.01 µg/ml MPLA a known agonist of TLR4 (Invivogen) as a positive control.

### 2.4 | Extraction of RNA and real-time quantitative reverse transcription-polymerase chain reaction analysis

After designated CS treatment duration, BMDCs were harvested and resuspended in 500 µl TRIzol reagent (Ambion) and placed

at –80°C. From the TRIzol reagent, RNA was isolated using either a RiboPure kit (Ambion™ #AM1924) or without a kit as described briefly here. After thawing, 200 µl chloroform was added to cells in TRIzol. Samples were then vortexed and centrifuged at 9200g for 15 min at 4°C. The aqueous layer was collected after centrifugation and the RNA was precipitated with isopropanol for 15 min at room temperature. The RNA pellet was washed with 75% ethanol, samples were centrifuged, and the supernatant was discarded. Samples were air-dried at 37°C and resuspended in 50 µl RNase-free double-distilled water (ddH<sub>2</sub>O). Complementary DNA (cDNA) was obtained using Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Following cDNA generation, quantitative reverse transcription-polymerase chain reaction (qRT-PCR; Step One Plus; Applied Biosystems Fisher Scientific) was used for amplification and quantification of select genes. Primers were purchased from Applied Biosystems, *Il6* (Mm00446190\_m1), *Cxcl10* (Mm00445235\_m1), and *Ifnb1* (Mm00439552\_s1).

### 2.5 | Flow cytometry of BMDC activation and viability

To assess BMDC activation status and viability after CS treatment, BMDCs were treated for 24 h. After treatment, cells were harvested and stained for CD11b BV421 (BioLegend, clone: M1/70), CD11c APC Fire 750 (BioLegend, clone: N418), I-A/I-E major histocompatibility complex (MHC) Class II V500 (BD Biosciences, clone: M5/114.15.2), CD40 PE-Cy7 (BioLegend, clone: 3/23), CD80 PE (BD Pharmingen, clone: 16-10-A1), CD86 APC (eBiosciences, clone: GL1), Annexin V PE (BD Biosciences), and 7-aminoactinomycin D (7AAD; BD Biosciences). Samples were analyzed on a Cytex DxP10 (Cytex Biosciences, Inc.) flow at the University of Nebraska-Lincoln Flow Cytometry Service Center. Data were analyzed using FlowJo software (Becton, Dickinson and Company).

### 2.6 | Assessment of nuclear factor-κB and interferon regulatory factor pathway activation

J774-Dual™ Cells (Invivogen) were grown in high-glucose Dulbecco's modified Eagle's medium (Thermo Fisher Scientific), 1.5 g/L sodium bicarbonate (Thermo Fisher Scientific), 1.0 mM sodium pyruvate (Thermo Fisher Scientific), 10% FBS (Thermo Fisher Scientific), 100 µg/ml Normocin™ (Invivogen), 100 U/ml penicillin (Thermo Fisher Scientific), and 100 µg/ml streptomycin (Thermo Fisher Scientific). J774-Dual™ Cells were treated with LMW or HMW CS at the indicated concentrations and for the indicated duration. Cells were also treated with 0.01 µg/ml MPLA (Invivogen) as a positive control. After treatment, secreted alkaline phosphatase (SEAP) and Lucia luciferase expression were measured using a protocol provided by Invivogen. SEAP expression was measured using an Epoch Microplate Spectrophotometer

(Agilent Technologies) and luciferase expression measured using a Veritas™ Microplate Luminometer (Turner BioSystems). Results were normalized to total protein, measured via bicinchoninic acid assay using an Epoch Microplate Spectrophotometer (Agilent Technologies).

## 2.7 | Immunizations

All immunizations were performed under anesthesia using an isoflurane vaporizer. Immunizations were administered intramuscularly (i.m.) in 50 µl total volume. All immunizations contained either 5 µg EndoFit ovalbumin (OVA) protein (Invivogen) or 1 µg hemagglutinin (HA) recombinant protein from A/California/07/2009 H1N1 (pdm09) (International Reagent Resource), or 1 µg HA protein from A/Puerto Rico/8/34 (Sino Biological Inc.), as indicated. In addition to antigen, mice received LMW or HMW CS at 4 or 40 µg. Low dose, 1 µg antigen per mouse vaccinations was chosen to allow for modest CS MW effects to be observed, as well as to investigate potential antigen dose sparing effects of CS adjuvantation (Lampe et al., 2020). As a negative control, mice were immunized with antigen protein alone. Low dose PR8 immunizations were delivered intranasally (i.n.) at 500 egg infective dose (EID)<sub>50</sub> in 30 µl PBS as a positive control for protection against viral challenge and antibody production. Antigen combined with 20 µg MPLA delivered i.m. was also used as a positive control for antibody production (Lampe et al., 2020). Mice were weighed for up to 7 days after immunization to assess adverse effects caused by adjuvantation.

## 2.8 | Antibody production after immunization

Three and four weeks after immunization, blood was collected from mice and serum separated by centrifugation at 4°C for 15 min at 16,300g. Serum was used to perform an enzyme-linked immunosorbent assay (ELISA) to assess antibody titers. ELISAs were performed using a Clear Flat-Bottom Immuno 96-well plate (Thermo Fisher Scientific) that had been coated overnight at 4°C with the immunizing antigen at 10 µg/ml diluted in PBS. After coating, plates were washed with PBS and blocked for 1 h with PBS containing 2% FBS and 10 mM HEPES (Sigma-Aldrich) (blocking buffer). Serum was diluted to 1:20 in blocking buffer and serially diluted twofold. Plates were then incubated at room temperature for 2–3 h before alkaline phosphatase-conjugated secondary antibodies against immunoglobulin G (IgG), IgG2a, or IgG1 (Southern Biotech) were added for 1 h at room temperature. After incubation with secondary antibodies, plates were developed in the dark using p-nitrophenyl phosphate for 30 min. Plates were then read at an optical density (OD) of 405 nm on a BioTek ELx808 (BioTek). OD was used to calculate endpoint titers by determining the reciprocal of the dilution at which the sample OD fell below two times the background.

## 2.9 | Influenza virus challenge

Mice were anesthetized with an isoflurane vaporizer and challenged with a homologous IAV, pdm09 (H1N1) or PR8 (H1N1), based on immunizing antigen. Pdm09 was diluted in PBS in 30 µl total and administered i.n. at  $6.3 \times 10^5$  chicken embryo infectious dose (CEID)<sub>50</sub>. PR8 was diluted in sterile PBS in 30 µl and administered i.n. at 5000 EID<sub>50</sub>. After a viral challenge, mouse weight and survival were monitored daily for up to 30 days. IAV, A/California/07/2009 (H1N1) pdm09, FR-201, was obtained through the Influenza Reagent Resource, Influenza Division, WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention.

## 2.10 | T-cells examination by flow cytometry

Mice were administered an IAV challenge (PR8) as described above 4.5 weeks after immunization. Two and 5 days after the challenge, mice were killed and draining lymph node (DLN, a pool of cervical and mediastinal lymph node [LN]) and lungs were examined. DLN samples were prepared as single cell suspensions by dissociation through a 70 µm screen. Lungs were perfused before removal, chopped into a slurry, and incubated with collagenase-D (Sigma-Aldrich; 5 µg/ml final concentration) and DNase (Invitrogen; 100 U/ml final concentration) for 1 h at 37°C before being filtered through a 70 µm screen. Lung samples were then treated with ACK buffer. Single cell suspensions were counted, and viability analyzed using a TC10™ Automated Cell Counter (Bio-Rad Hercules). For intracellular cytokine staining (interleukin [IL]-2, IFN-γ, and IL-4), single cell suspensions were restimulated with PR8 HA peptides alone (DLN) or PR8 HA peptide pulsed LB27.4 cells (ATCC) (lung). IAV peptides used for restimulation included HA peptide 126–138 (HNTNGVTAACSHE), HA peptide 518–526 (IYSTVAASL), and HA peptide 126–140 (SSFERFEIFPKESW). Cells were restimulated with peptide for a total of 6 h with 10 µg/ml Brefeldin A (Sigma-Aldrich) addition for the final 2 h. Single cell suspensions were stained for: CD3 FITC (BioLegend, clone: 145-2C11), CD4 BV421 (BioLegend, clone: GK1.5), CD8 APC/Fire 750 (BioLegend, clone: 53-6.7), CD44 PE-Cy7 (eBiosciences, clone: IM7), CD69 PE-Cy7 (eBiosciences, clone: H1.2F3), CD103 PerCP-Cy5.5 (BioLegend, clone: 2E7), IL-2 FITC (eBiosciences, clone: JES6-5H4), IFN-γ APC (eBiosciences, clone: XMG1.2), and IL-4 PE (BioLegend, clone: 11B11). Samples were analyzed on a Cytex DxP10 (Cytex Biosciences, Inc.) at the University of Nebraska-Lincoln Flow Cytometry Service Center. Data were analyzed using FlowJo software (FlowJo, LLC, a subsidiary of Becton, Dickinson and Company).

## 2.11 | Statistical analysis

All in vitro results are indicative of 2–17 replicates done within 1–3 individual experiments, as indicated in the figure legend. All in vivo

results are indicative of an experiment with an  $n$  of 5. Statistical analyses were completed using one-way analysis of variance with Sidak's multiple comparisons test or Tukey's multiple comparisons test post hoc analysis, as indicated in the figure legend. Statistical analyses performed using Prism software (GraphPad Prism 5).

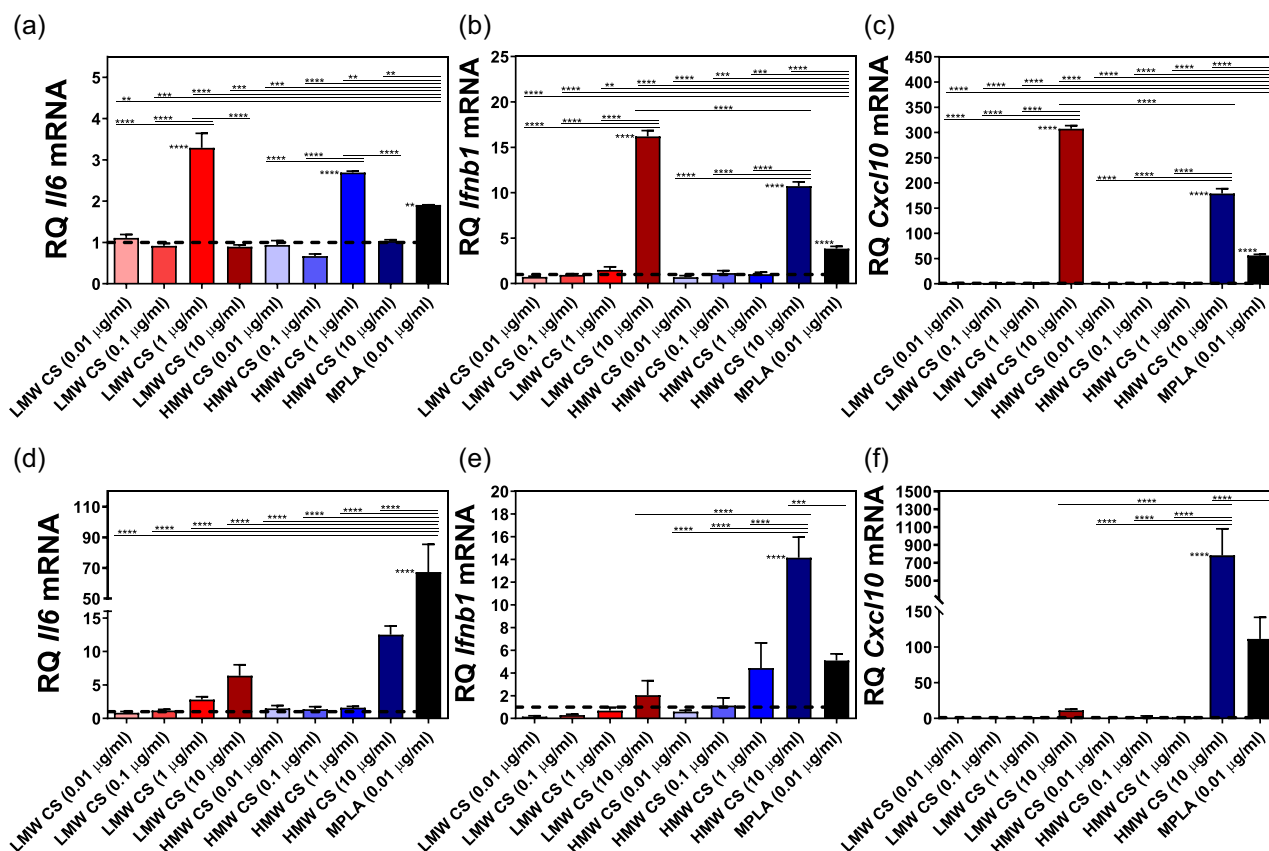
### 3 | RESULTS

#### 3.1 | LMW and HMW CS induce cytokine production by BMDCs in vitro

To assess the immune-modulatory effects of CS on APCs in vitro, BMDCs were treated with LMW (50–190 kDa) or HMW (310–375 kDa) CS at doses ranging from 0.01 to 10  $\mu\text{g}/\text{ml}$  for 24 or 48 h before assessment of *Il6*, *Ifnb1*, and *Cxcl10* messenger RNA (mRNA) levels. Examination of cytokine and chemokine mRNA expression was used as an initial indicator of dendritic cell activation, with elevated mRNA expression indicating enhanced APC potential (Lampe et al., 2020). At 24

and 48 h after treatment, BMDCs were harvested and RNA isolated for gene expression analysis by qRT-PCR. Gene expression was normalized to untreated cells (media alone), which is set to 1 and indicated by the dotted line of respective graphs. Cells treated with 0.01  $\mu\text{g}/\text{ml}$  MPLA served as a positive control for cytokine gene expression.

At 24 h after treatment, both LMW and HMW CS at 1  $\mu\text{g}/\text{ml}$ -induced elevated *Il6* mRNA levels compared to untreated cells and cells treated with MPLA (Figure 1a). Treatment with CS at 0.01, 0.1, or 10  $\mu\text{g}/\text{ml}$ -induced mRNA levels that were not significantly different from untreated cells, but these levels were significantly lower than cells treated with MPLA (Figure 1a). No significant differences in *Il6* mRNA levels were observed between BMDCs treated with the same doses of LMW and HMW CS (Figure 1a). In contrast to *Il6* mRNA levels, 10  $\mu\text{g}/\text{ml}$  treatment induced significantly higher levels of *Ifnb1* and *Cxcl10* mRNA for both LMW and HMW CS compared to all lower treatment doses and MPLA treatment (Figure 1b,c). No significant increases in *Ifnb1* or *Cxcl10* mRNA levels over untreated cells were observed with either LMW or HMW CS treatment at 0.01, 0.1, or 1  $\mu\text{g}/\text{ml}$ , while 10  $\mu\text{g}/\text{ml}$  of both LMW and



**FIGURE 1** LMW and HMW CS significantly enhances cytokine production by BMDCs 24 h after treatment. BMDCs ( $n = 3$ ) were treated with varying doses of two varieties of CS or 0.01  $\mu\text{g}/\text{ml}$  MPLA as a positive control. BMDCs were treated for 24 (a–c) or 48 h (d–f) before cells were harvested, placed in TRIzol reagent, and RNA was isolated. qRT-PCR was performed on complementary DNA generated from the isolated RNA. qRT-PCR was performed for *Il6* (a, d), *Ifnb1* (b, e), and *Cxcl10* (c, f). \* $p \leq .05$ , \*\* $p \leq .01$ , \*\*\* $p \leq .001$ , \*\*\*\* $p \leq .0001$  by one-way ANOVA with Sidak's multiple comparisons test compared to untreated BMDCs or indicated comparison. The dotted line indicates untreated BMDC (media alone) reference control set to 1. Error bars represent SEM. ANOVA, analysis of variance; BMDC, bone marrow-derived dendritic cell; CS, chitosan; HMW, high molecular weight; LMW, low molecular weight; MPLA, monophosphoryl lipid A; mRNA, messenger RNA; qRT-PCR, quantitative reverse transcription-polymerase chain reaction [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]



HMW CS significantly elevated *Ifnb1* and *Cxcl10* mRNA levels over MPLA-treated cells (Figure 1b,c). When comparing LMW CS treatment to HMW CS treatment, significantly higher *Ifnb1* and *Cxcl10* mRNA levels were observed after treatment with 10 µg/ml LMW CS compared to treatment with 10 µg/ml HMW CS.

In contrast to the 24 h results, no significant changes in *Il6* mRNA were observed after 48 h in LMW or HMW treated cells compared to the untreated control, and all CS treated cells displayed significantly lower *Il6* mRNA levels compared to MPLA-treated cells (Figure 1d). In addition, the increases in *Ifnb1* and *Cxcl10* mRNA levels that were induced by LMW CS treatment at 24 h were not sustained through 48 h, with no significant changes observed in *Ifnb1* or *Cxcl10* mRNA levels after LMW CS treatment at any dose compared to untreated or MPLA-treated BMDCs (Figure 1e,f). However, HMW CS treatment at 10 µg/ml did significantly increase *Ifnb1* and *Cxcl10* mRNA levels over untreated and MPLA-treated cells at 48 h (Figure 1e,f). Treatment with 10 µg/ml HMW significantly increased *Ifnb1* and *Cxcl10* mRNA levels over BMDCs treated with the same dose of LMW CS at 48 h (Figure 1e,f). Taken together, these results suggest that both LMW and HMW CS treatments induce cytokine and chemokine mRNA expression in BMDCs. LMW and HMW CS treatment resulted in similar *Il6* mRNA levels at 24 and 48 h. In contrast, after 24 h treatment, LMW CS treatment induced significantly higher levels of *Ifnb1* and *Cxcl10* mRNA compared to HMW CS treatment. However, after 48 h treatment, HMW CS resulted in significantly higher *Ifnb1* and *Cxcl10* mRNA levels over LMW CS, suggesting a difference in the kinetics of cytokine mRNA induction after LMW and HMW CS treatment.

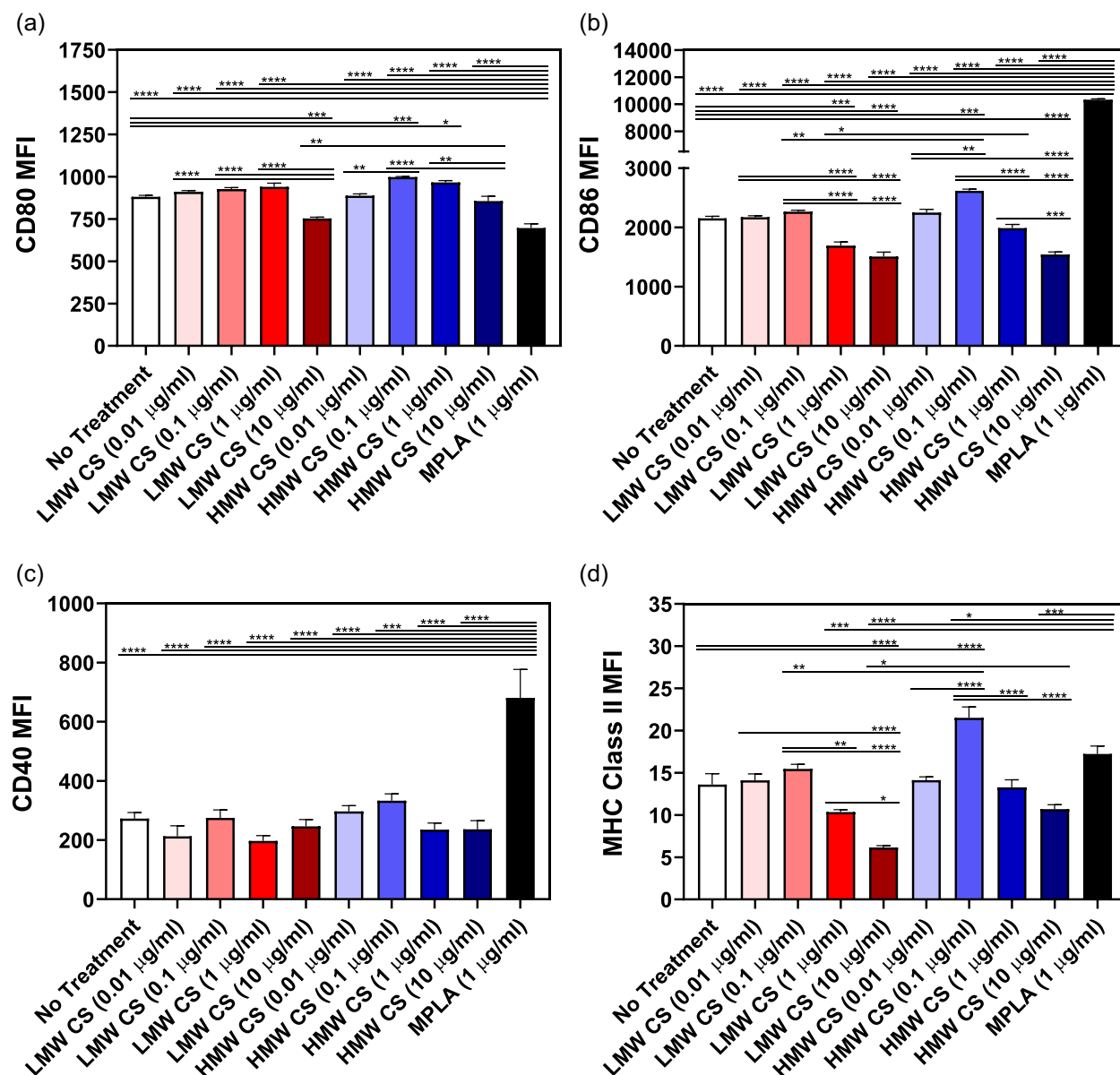
### 3.2 | HMW CS activates BMDCs in vitro

After observing increased cytokine and chemokine mRNA levels in BMDCs that had been treated with LMW or HMW CS relative to untreated cells, BMDC activation status after CS treatment was investigated. BMDC activation and maturation, including upregulation of costimulatory and MHC class II molecule expression, is essential for effective activation of naïve T cells (Lanzavecchia & Sallusto, 2001). MHC class II is required for the presentation of antigen to CD4 T cells, while CD80, CD86, and CD40 serve as costimulatory molecules that provide secondary signals during T-cell activation, which impacts T-cell function (Hubo et al., 2013). To assess BMDC activation after CS treatment, BMDCs were treated with varying concentrations of LMW or HMW CS for 24 h. Again, cells treated with MPLA served as a positive control (Goff et al., 2015; Mesa et al., 2004). After treatment, cells were analyzed using flow cytometry for the expression level of surface markers: CD80, CD86, CD40, and MHC class II (Figure 2). Mean fluorescence intensity (MFI), a measure of staining intensity, was used to compare the relative expression levels of these surface markers between treatment groups.

All treatment groups, besides LMW CS at 10 µg/ml, resulted in significantly elevated CD80 expression compared to MPLA-treated BMDCs (Figure 2a). MPLA treatment did not result in elevated CD80

expression compared to untreated controls after 24 h (Figure 2a), which may indicate that 1 µg/ml MPLA at this time point is not an optimal treatment condition to observe optimal MPLA effects on CD80 expression in BMDCs. BMDCs treated with LMW CS at any dose did not display increased CD80 expression compared to untreated BMDCs, with high-dose (10 µg/ml) LMW resulting in decreased CD80 expression compared to untreated BMDCs (Figure 2a). When BMDCs were treated with HMW CS at 0.1 and 1 µg/ml significantly increased CD80 expression was observed compared to untreated BMDCs (Figure 2a). No significant differences were observed between untreated BMDCs and those treated with 0.01 and 10 µg/ml HMW CS (Figure 2a). Cells treated with 0.1 µg/ml HMW CS displayed the highest expression of CD80 compared to other HMW CS doses (Figure 2a). HMW CS treatment at 10 µg/ml resulted in significantly higher CD80 expression compared to cells treated with LMW at the same dose (Figure 2a). LMW CS treatment resulted in no significant increases from untreated cells, with LMW CS treatment at 1 and 10 µg/ml inducing significantly lower CD86 MFIs compared to untreated cells (Figure 2b). When BMDCs were treated with HMW CS at 0.01, 1, or 10 µg/ml, no significant increases were observed in CD86 MFI compared to untreated BMDCs, while HMW CS treatment at 0.1 µg/ml induced significantly higher CD86 MFI compared to untreated cells (Figure 2b). HMW CS treatment at both 0.1 and 1 µg/ml resulted in significantly higher CD86 levels over treatment with LMW CS at the same doses (Figure 2b). Examination of CD40 expression revealed no significant differences between untreated cells and those treated with LMW or HMW CS at any dose (Figure 2c). As expected, MPLA treatment resulted in significantly higher CD40 MFI levels compared to untreated cells and all treatment groups receiving LMW or HMW CS at any dose (Figure 2c).

MPLA-treated BMDCs expressed significantly elevated MHC class II expression over cells treated with LMW CS at 1 and 10 µg/ml and HMW CS at 10 µg/ml, while HMW CS treatment at 0.1 µg/ml induced significantly higher MHC class II expression compared to MPLA treatment (Figure 2d). MHC class II examination showed that treatment with LMW CS at any dose did not induce increases in expression over untreated cells (Figure 2d). LMW CS treatment of 10 µg/ml induced the lowest MHC class II expression levels compared to all other LMW CS treatment doses (Figure 2d). MHC class II MFIs in BMDCs treated with HMW CS at 0.01, 1, and 10 µg/ml were not significantly changed from untreated cells; however, 0.1 µg/ml HMW CS treatment resulted in significantly elevated MHC class II levels compared to untreated cells and cells treated with other doses of HMW CS (Figure 2d). In addition, HMW CS treatment at 0.1 and 10 µg/ml resulted in elevated MHC class II MFIs compared to LMW CS treatment at the same doses (Figure 2d). In addition to inducing cytokine and chemokine mRNA production (Figure 1) and increasing activation markers on BMDCs (Figure 2), LMW and HMW CS treatment at 10 µg/ml significantly increased BMDC Annexin V staining over untreated cells, but no CS treatment increased 7AAD or Annexin V<sup>+</sup>/7AAD<sup>+</sup> double-positive staining compared to untreated cells (Figure S1), suggesting LMW and HMW CS treatment may induce low levels of apoptosis.



**FIGURE 2** HMW CS treatment significantly increases the activation status of BMDCs. BMDCs ( $n = 3$ ) were treated with varying doses of two varieties of CS for 24 h or with MPLA at 1  $\mu\text{g}$  as a positive control for activation. Untreated cells (media alone) were used as negative control. After treatment, BMDCs were harvested and CD11b<sup>+</sup>/CD11c<sup>+</sup> cells were analyzed for CD80 (a), CD86 (b), CD40 (c), and I-A/I-E major histocompatibility complex (MHC) class II (d) using flow cytometry. Cells were examined for the level of expression using mean fluorescent intensity (MFI). \* $p \leq .05$ , \*\* $p \leq .01$ , \*\*\* $p \leq .001$ , \*\*\*\* $p \leq .0001$  between indicated comparisons by one-way ANOVA with Sidak's multiple comparisons test. Error bars represent SEM. ANOVA, analysis of variance; BMDC, bone marrow-derived dendritic cell; CS, chitosan; HMW, high molecular weight; LMW, low molecular weight; MPLA, monophosphoryl lipid A [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

Analysis of costimulatory molecules and MHC class II as indicators of BMDC activation revealed that LMW and HMW CS result in different activation marker profiles. HMW CS-induced higher expression of CD80, CD86, and MHC class II than LMW CS treatment at corresponding doses. Neither LMW nor HMW CS treatment induced upregulation of the costimulatory molecule CD40 compared to untreated cells. Furthermore, the BMDC activation profiles observed after LMW and HMW CS treatment, indicated by cytokine/chemokine expression and surface markers, do not appear to be induced by high levels of CS-associated cell death, as evidenced by

7AAD or Annexin V<sup>+</sup>/7AAD<sup>+</sup> double-positive staining compared to untreated cells.

### 3.3 | CS induces interferon regulatory factor, but not nuclear factor- $\kappa$ B, pathway signaling

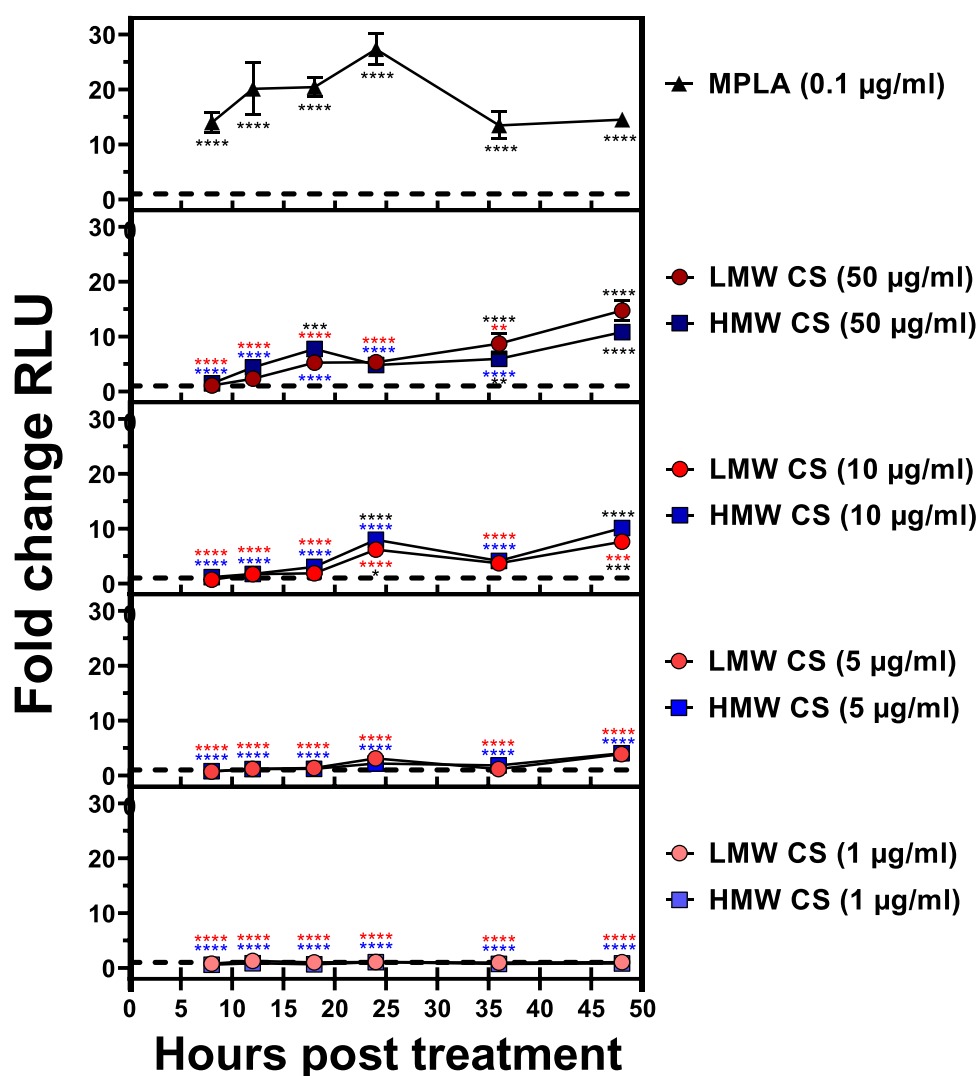
To examine how CS treatment may induce signaling through canonical innate immune response transcription factor pathways, CS was used to treat J774-Dual™ Cells, a mouse macrophage-like cell line



engineered to report changes in interferon regulatory factor (IRF) and nuclear factor (NF)- $\kappa$ B pathway activation via Lucia luciferase and SEAP reporters, respectively (Lampe et al., 2020). J774-Dual™ Cells were treated with varying doses of LMW and HMW CS for 8, 12, 18, 24, 36, and 48 h. IRF activation was normalized to untreated cells, which is set to 1 and indicated by the dashed line. MPLA at 0.1  $\mu$ g/ml treatment was used as a positive control for IRF pathway activation (Mata-Haro et al., 2007), resulting in significant IRF pathway activation over untreated cells at all time points (Figure 3).

No significant IRF pathway activation compared to untreated cells was observed after treatment with LMW or HMW CS at 0.01, 0.1, 1, or 5  $\mu$ g/ml (Figure 3 and data not shown). In addition, no significant IRF pathway activation was observed over untreated cells after

8 or 12 h with either LMW or HMW CS treatment (Figure 3). Significant IRF pathway activation was observed after 18 h in 50  $\mu$ g/ml HMW CS treated cells compared to untreated cells (Figure 3). At 24 h, HMW CS at 10  $\mu$ g/ml-induced significant IRF pathway activation compared to untreated cells (Figure 3). By 36 h, both LMW and HMW CS at 50- $\mu$ g/ml-induced significant IRF pathway activation over untreated cells (Figure 3). After 48 h treatment with both LMW and HMW CS at 10- and 50- $\mu$ g/ml-induced significant IRF pathway activation over untreated cells (Figure 3). MPLA treatment induced greater IRF pathway activation compared to LMW and HMW CS treated cells at all doses for 8, 12, 18, 24, and 36 h treatments (Figure 3). However, no significant differences in IRF pathway activation was observed at 48 h between MPLA-treated cells and LMW



**FIGURE 3** LMW and HMW CS induce IRF pathway signaling. J774-Dual™ cells ( $n = 12-17$ ) were treated with two varieties of CS at the indicated dose. J774 dual cells use the Lucia luciferase gene to report IRF activity. Treatment durations included 8, 12, 18, 24, 36, and 48 h. Reporter output normalized to total protein and fold change in relative light units (RLU) calculated over untreated cells (media alone). \* $p \leq .05$ , \*\* $p \leq .01$ , \*\*\* $p \leq .001$ , \*\*\*\* $p \leq .0001$  compared to MPLA-treated (red and blue asterisk) and untreated (black asterisk) cells by two-way ANOVA with Tukey's multiple comparisons test. Error bars represent SEM. ANOVA, analysis of variance; CS, chitosan; HMW, high molecular weight; IRF, interferon regulatory factor; LMW, low molecular weight; MPLA, monophosphoryl lipid A [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

CS at 50 µg/ml and HMW CS at both 50 and 10 µg/ml (Figure 3). No significant differences in IRF pathway activation were observed at any time point between LMW CS and HMW CS treated cells receiving the same dose (Figure 3).

In contrast to the IRF pathway activation, no significant NF-κB activation over untreated cells was observed with CS treatment at any time point investigated (Figure S2). Taken together, examination of IRF and NF-κB pathway activation suggests that both LMW and HMW CS at 10 and 50 µg/ml are able to activate the IRF pathway to a similar degree, despite seeing differential effects of LMW and HMW CS on BMDC cytokine production and surface activation marker expression. However, IRF pathway activation in response to LMW and HMW CS treatment is delayed compared to MPLA treatment.

### 3.4 | LMW CS adjuvantation enhances IgG production after vaccination

After observing innate immune activation induced by both LMW and HMW CS treatment *in vitro*, LMW and HMW CS were investigated for their ability to act as adjuvants during protein vaccination in a mouse model. The effect of LMW and HMW CS on antibody production was examined. To do this, mice were first vaccinated *i.m.* with 5 µg of a model antigen OVA protein in combination with LMW or HMW CS at 4 or 40 µg per mouse. Two doses of CS were examined to determine the effective dose that was able to enhance antibody production. The 40 µg per mouse dose was similar to the dose investigated by other groups (Da Silva et al., 2009; Sui, Chen, Fang, et al., 2010; Sui, Chen, Wu, et al., 2010; Wang et al., 2012), and is 4–40-fold higher than the optimum *in vitro* dose for cytokine gene expression and IRF pathway signaling (Figures 1 and 3). We chose an order of magnitude higher dose for *in vivo* experiments based on our previously published report (Lampe et al., 2020), and the presumed dilution of CS within the injection site, which can reduce the accessibility of CS to APC in the muscle. We also used a 10-fold lower dose (4 µg per mouse) to remain within the range of our *in vitro* studies and to investigate if either LMW or HMW CS was effective at lower doses. As a positive control, mice were vaccinated with OVA antigen combined with 20 µg MPLA per mouse (Ko et al., 2017; Lampe et al., 2020). At 3 and 4 weeks after vaccination, serum was collected from the immunized mice analyzed for anti-OVA IgG via ELISA.

At 3 weeks postvaccination, all mice vaccinated with OVA + MPLA had significantly elevated anti-OVA IgG levels compared to OVA alone and all treatment groups receiving LMW or HMW CS, except HMW CS at 40 µg (Figure 4a). Enhanced anti-OVA IgG titers were also observed three weeks after vaccination in mice receiving CS, LMW, or HMW, at 40 µg compared to mice receiving OVA alone, although these differences were not significant (Figure 4a). Four weeks after vaccination, mice receiving MPLA had significantly elevated anti-OVA IgG levels compared to OVA alone and mice receiving LMW or HMW CS at both doses (Figure 4b). Mice receiving

LMW or HMW CS at 40 µg showed increased levels of serum anti-OVA IgG compared to mice receiving OVA protein alone; however, this increase was only significant in the 40 µg LMW CS group, suggesting that LMW, but not HMW CS is able to significantly increase anti-OVA IgG antibody titers after vaccination (Figure 4b).

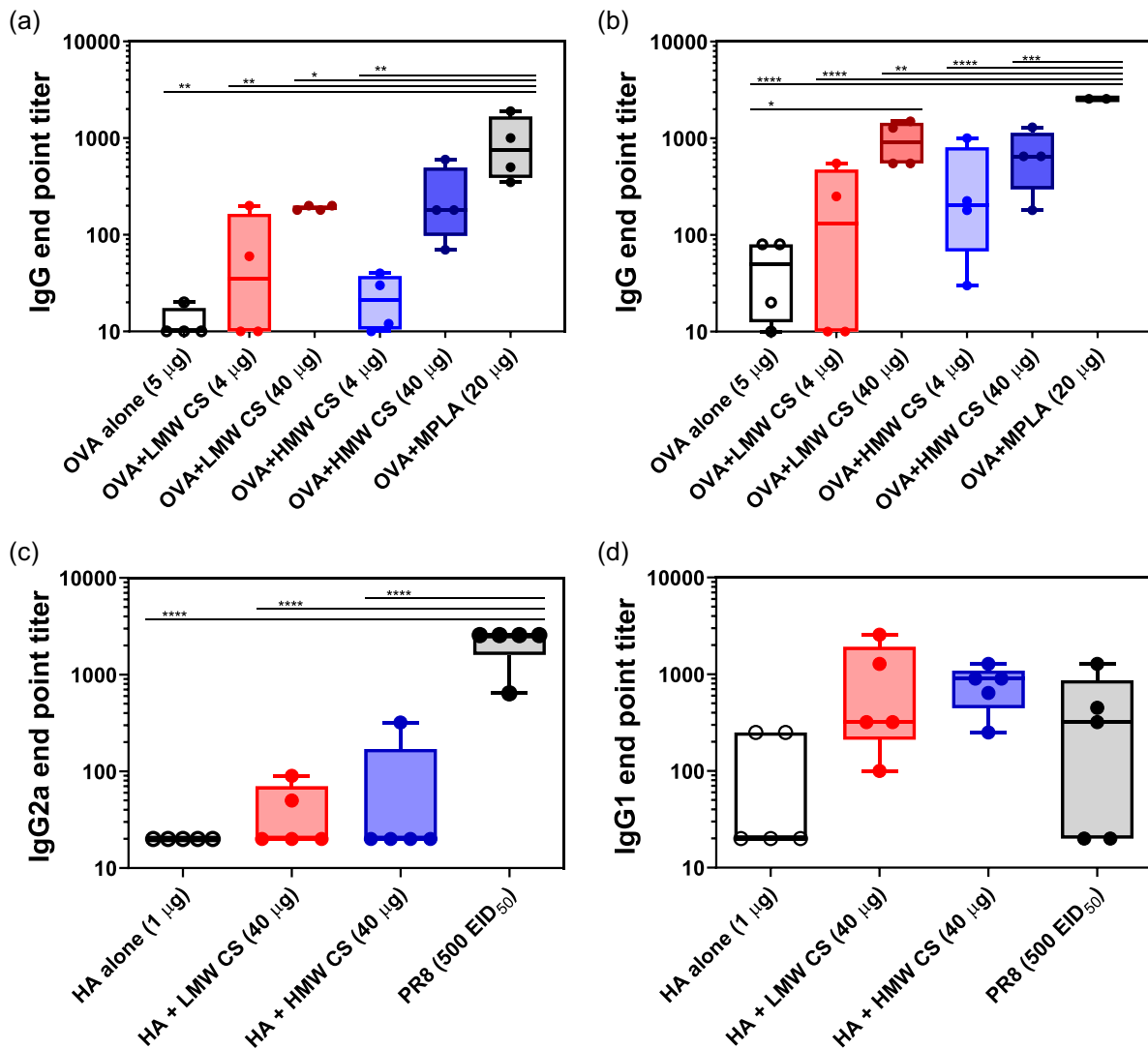
A separate experiment was conducted to determine if LMW or HMW CS adjuvantation could allow for antigen dose sparing effects. A low-dose vaccination of 1 µg HA protein from pdm09 H1N1 was chosen to allow for modest CS MW effects to be observed and to examine potential antigen dose sparing effects of CS adjuvantation using a relevant IAV antigen. Mice were vaccinated with a single low dose of HA protein alone or combined with LMW or HMW CS at 40 µg. The 40-µg dose was chosen based on the elevated IgG titers after vaccination with 40-µg LMW CS. As a positive control, mice were infected with a low-dose (500 EID<sub>50</sub>) PR8 virus at the time of immunization. Serum was collected 4 weeks after vaccination and examined for HA-specific IgG subtype, IgG2a and IgG1, antibodies using ELISA (Figure 4c,d).

Mice previously infected with the PR8 virus displayed significantly higher IgG2a endpoint titers compared to mice receiving HA alone or HA combined with LMW or HMW CS (Figure 4c). No significant differences in IgG2a and IgG1 titers were observed between mice receiving HA protein alone and those receiving LMW or HMW CS (Figure 4c,d). However, LMW and HMW CS mice displayed IgG1 endpoint titers greater than or equal to 100 (Figure 4d). Together, results examining antibody production after LMW and HMW CS vaccination suggest that LMW CS, but not HMW CS, as an adjuvant is able to significantly increase IgG production after immunization over unadjuvanted vaccines.

### 3.5 | LMW and HMW CS improve protection against homologous IAV challenge compared to unadjuvanted vaccines

After observing significantly enhanced IgG antibody production in mice receiving LMW CS, but not HMW CS, during vaccination compared to mice receiving antigen alone, the ability of both LMW and HMW CS adjuvantation to improve protection against IAV challenge was investigated. Mice were immunized *i.m.* with 1 µg HA protein from pdm09 virus alone or in combination with LMW or HMW CS. LMW and HMW CS were administered at 40 µg per mouse. As a positive control for protection against IAV infection, a group of mice was immunized with a low dose (500 EID<sub>50</sub>) of PR8 virus. Seven weeks after vaccination, mice were administered a homologous viral challenge of 6.3e5 CEID<sub>50</sub> pdm09 virus and weighed daily as an indication of infection-induced morbidity (Figure 5a).

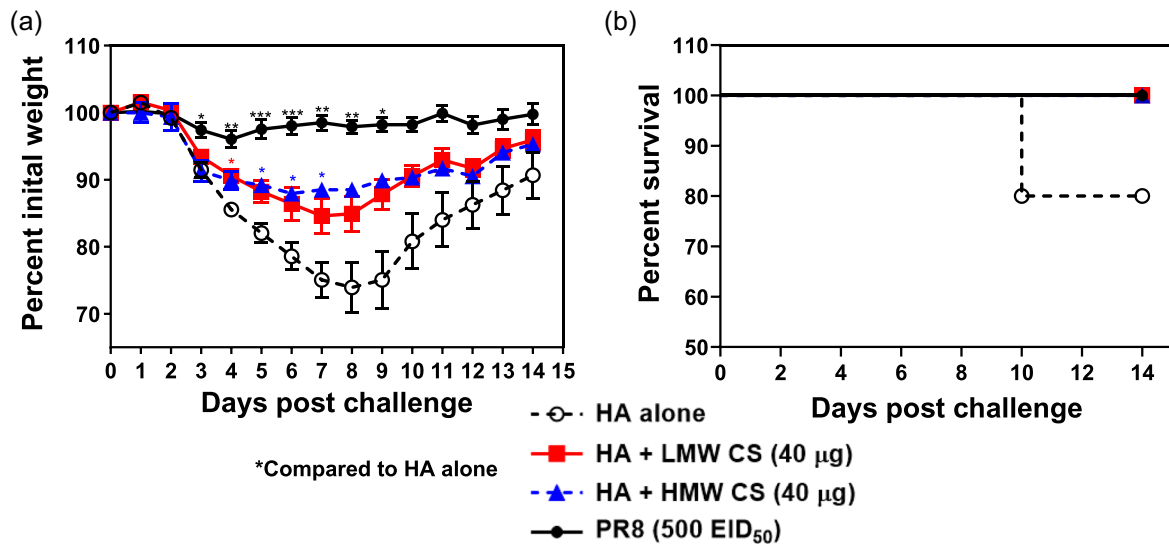
As expected, mice previously infected with the PR8 virus did not lose weight in response to the pdm09 viral challenge, displaying significantly less weight loss compared to mice receiving HA alone on Days 3–9 (Figure 5a). Mice vaccinated with HA protein alone experienced severe weight loss that peaked around Day 8 after



**FIGURE 4** Mice vaccinated with LMW CS as an adjuvant produce significantly more anti-OVA IgG compared to mice receiving unadjuvanted vaccines. Mice ( $n = 4$ – $5$ ) were vaccinated i.m. with  $5 \mu\text{g}$  OVA protein (a, b) or  $1 \mu\text{g}$  pdm09 HA (c, d)  $\pm$  CS as an adjuvant at the indicated dose. Vaccination with antigen alone served as the negative control. As positive control mice were vaccinated with antigen with MPLA (a, b) or mice were infected with a low-dose ( $500 \text{ EID}_{50}$ ) PR8 virus (c, d). At 3 (a) and 4 weeks (b–d), serum was collected from the mice and ELISA performed to assess levels of anti-OVA (a, b) or anti-PR8 (c, d) antibodies induced by vaccination. Antigen-specific IgG (a, b), IgG2a (c), and IgG1 (d) subtypes were investigated. \* $p \leq .05$ , \*\* $p \leq .01$ , \*\*\* $p \leq .001$ , \*\*\*\* $p \leq .0001$  between indicated comparisons by one-way ANOVA with Sidak's multiple comparisons test (a, b). \* $p \leq .05$ , \*\* $p \leq .01$ , \*\*\* $p \leq .001$ , \*\*\*\* $p \leq .0001$  between indicated comparisons by one-way ANOVA with Tukey's multiple comparisons test (c, d). ANOVA, analysis of variance; CS, chitosan; EID, egg infective dose; ELISA, enzyme-linked immunosorbent assay; HA, hemagglutinin; HMW, high molecular weight; IgG, immunoglobulin G; i.m., intramuscularly; LMW, low molecular weight; MPLA, monophosphoryl lipid A; OVA, ovalbumin [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

infection, with an average of about 26% loss of initial body weight (Figure 5a). In contrast, mice that were vaccinated with formulations that contained either LMW or HMW CS experienced significantly less weight loss compared to mice receiving HA protein alone (LMW Day 4, HMW Days 5–7) (Figure 5a). Mice receiving LMW CS lost an average of about 15% of their initial weight by Day 8 after the challenge, while mice receiving HMW CS lost an average of about 11.5% initial weight. There were no significant differences in weight loss between groups of mice receiving LMW and HMW CS. Although

displaying less weight loss after viral challenge, mice receiving LMW or HMW CS as adjuvants did not experience significantly increased survival over mice receiving unadjuvanted vaccines (Figure 5b). Mice receiving HA protein alone experienced 80% survival, with all other groups experiencing 100% survival (Figure 5b). Decreased weight loss after homologous challenge suggests that both LMW and HMW CS can increase protection against morbidity to a similar degree, despite observing differences *in vitro* in innate activation and *in vivo* in antibody production between LMW and HMW CS.

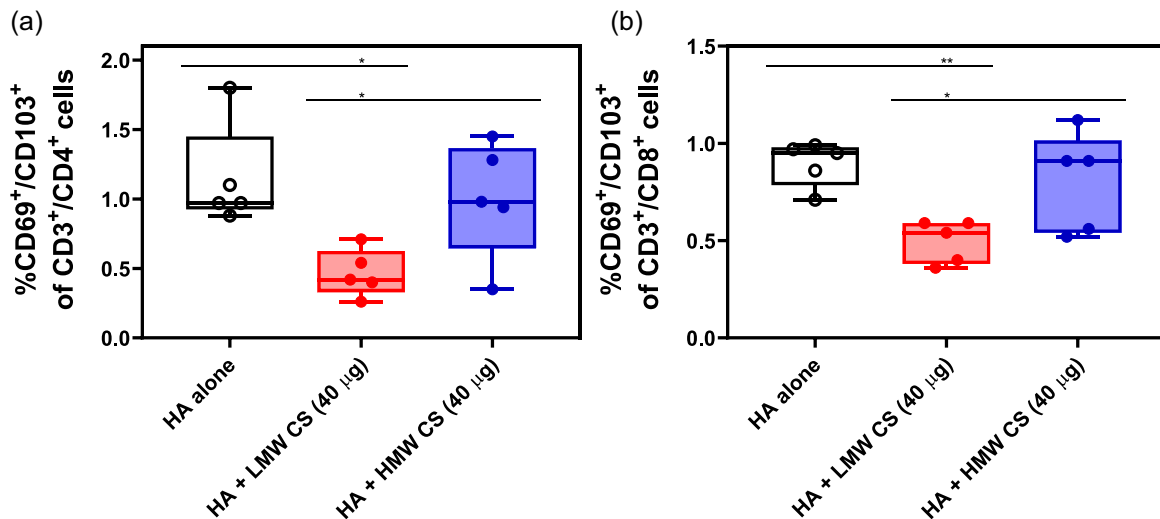


**FIGURE 5** Mice vaccinated with formulations that contain CS as an adjuvant experience significantly less weight loss after homologous infection compared to mice receiving unadjuvanted vaccines. Mice ( $n = 5$ ) were vaccinated i.m. with 1 µg H1N1 pdm09 HA  $\pm$  40 µg CS as an adjuvant. Four weeks after vaccination mice were challenged with 6.3e5 CEID<sub>50</sub> H1N1 pdm09 virus. Following the viral challenge, weight loss (a) and survival (b) were recorded daily. Five mice were included in each treatment group.  $*p \leq .05$  by mixed-effects analysis using Tukey's multiple comparisons test compared to HA alone. Error bars represent SEM. CEID, chicken embryo infectious dose; CS, chitosan; EID, egg infective dose; HA, hemagglutinin; HMW, high molecular weight; i.m., intramuscularly; LMW, low molecular weight [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

### 3.6 | LMW and HMW CS induce distinct CD69<sup>+</sup>/CD103<sup>+</sup> CD4 and CD8 T-cell populations in the lung after homologous challenge

Although mice receiving LMW and HMW CS during vaccination experienced significantly less weight loss than mice receiving antigen

alone, these mice did experience an average of 15% and 11.5% loss of initial body weight, respectively (Figure 5a). The observed weight loss suggests that the LMW and HMW CS-induced protection is not mediated by IgG facilitated sterilizing immunity, and, therefore, cellular immune responses may be contributing. Thus, the T-cell populations present in the lung 2 and 5 days after viral challenge were



**FIGURE 6** HMW CS as an adjuvant increases the frequency of CD4 and CD8 lung T<sub>RM</sub> 5 days after challenge compared to LMW CS. Mice ( $n = 5$ ) were vaccinated i.m. with 1 µg H1N1 PR8 HA  $\pm$  40 µg CS as an adjuvant. Four weeks after vaccination, mice were challenged with 5000 EID<sub>50</sub> PR8 viruses. At 5 days after the challenge, mice were killed, and lung T-cell populations examined using flow cytometry. CD4 (a) and CD8 (b) T cells were CD69/CD103 expressing populations.  $*p \leq .05$ ,  $**p \leq .01$ ,  $***p \leq .001$ ,  $****p \leq .0001$  by one-way ANOVA with Tukey's multiple comparisons test. ANOVA, analysis of variance; CS, chitosan; EID, egg infective dose; HA, hemagglutinin; HMW, high molecular weight; i.m., intramuscularly; LMW, low molecular weight; T<sub>RM</sub>, T resident memory cell [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

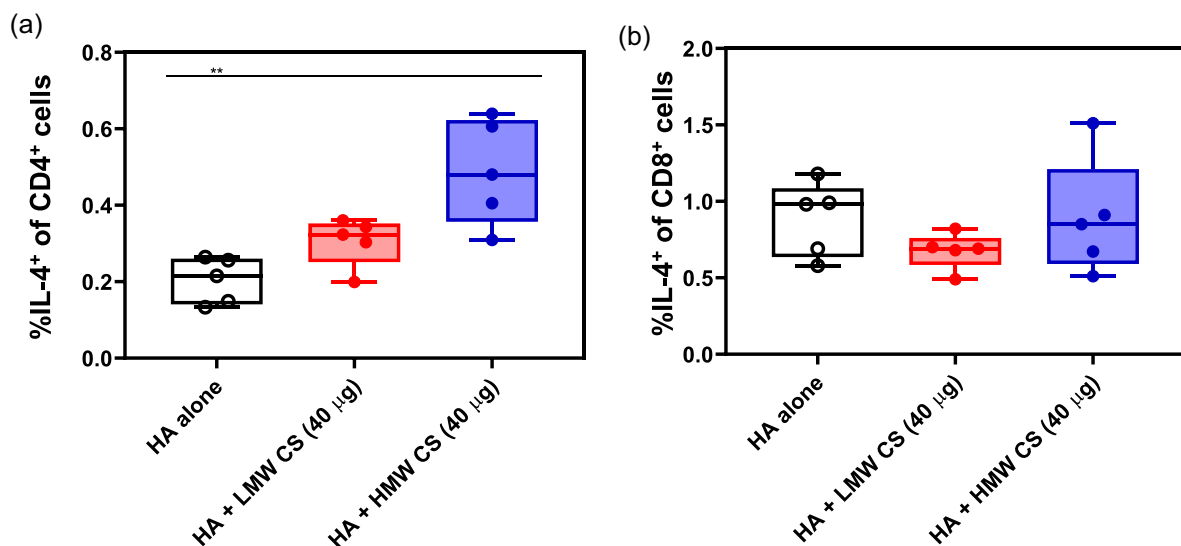
examined to investigate if cellular immunity at the site of infection was mediating the enhanced protection observed. To examine these populations, mice were vaccinated i.m. with 1- $\mu$ g HA protein from PR8 with or without LMW or HMW CS at 40  $\mu$ g. Four weeks after vaccination, mice were challenged i.n. with a lethal dose of PR8 virus (5000 EID<sub>50</sub>). Two and 5 days after the homologous challenge, mice were killed, and lungs harvested for T-cell population analysis using flow cytometry. CD4 and CD8 T cells were examined for CD69 and CD103 expression to indicate lung T resident memory cells (T<sub>RM</sub>) populations.

Two days after infection, no significant differences were observed between any vaccination groups in the frequency of CD69<sup>+</sup>/CD103<sup>+</sup> CD4 or CD8 T cells (Figure S3). However, at Day 5, mice receiving HA alone and mice receiving HA with HMW CS displayed significantly higher CD69<sup>+</sup>/CD103<sup>+</sup> CD4 T-cell frequencies compared to mice receiving HA combined with LMW CS (Figure 6a). No significant differences were observed between mice receiving HA alone and HA plus HMW CS (Figure 6a). Similarly, to the CD69<sup>+</sup>/CD103<sup>+</sup> CD4 T-cell populations, 5 days after vaccination, mice receiving HA alone or HA with HMW CS displayed significantly higher CD69<sup>+</sup>/CD103<sup>+</sup> CD8 T-cell populations compared to mice receiving HA plus LMW CS (Figure 6b). Examination of CD4 and CD8 T<sub>RM</sub> populations in the lung indicates HMW CS adjuvantation induces CD69<sup>+</sup>/CD103<sup>+</sup> CD4 and CD8 T-cell responses that do not significantly differ from HA alone, while LMW CS induces significantly lower CD69<sup>+</sup>/CD103<sup>+</sup> CD4 and CD8 T-cell populations compared to HA alone and HMW CS. The CD69<sup>+</sup>/CD103<sup>+</sup> CD4 and CD8 T-cell responses observed following homologous challenge suggest that HMW CS-induced significantly elevated CD69<sup>+</sup>/CD103<sup>+</sup> CD4 and CD8 T-cell populations compared to LMW CS.

### 3.7 | HMW CS induces enhanced IL-4 by CD4 T cells in the lung 5 days after homologous challenge

After observing distinct CD69<sup>+</sup>/CD103<sup>+</sup> CD4 and CD8 T-cell responses in the lung of LMW and HMW CS vaccinated mice following homologous infection, an investigation of the cytokines produced by CD4 and CD8 T cells in the lung 5 days after infection was conducted. Mice were vaccinated with a single i.m. dose of 1- $\mu$ g recombinant HA protein from PR8 alone, or in combination with LMW or HMW CS at 40  $\mu$ g. Four weeks after vaccination, mice were challenged with a lethal dose of 5000 EID<sub>50</sub> PR8 viruses. Five days after the challenge, mice were killed, and lungs were harvested for the examination of IFN- $\gamma$  and IL-4 production by CD4 and CD8 T cells.

No significant differences in IFN- $\gamma$  production by CD4 or CD8 T cells were observed between LMW or HMW CS vaccinated mice and mice receiving HA protein alone (Figure S4). In contrast to IFN- $\gamma$ , mice that had received immunizations containing HMW CS displayed significantly elevated frequencies of IL-4 producing CD4 T cells compared to mice receiving HA protein alone (Figure 7a). No significant differences in CD4 IL-4 production were observed between mice receiving LMW CS and those receiving HA alone or HA plus HMW CS (Figure 7a). No differences were observed between mice receiving LMW or HMW CS and those receiving HA alone in the frequency of IL-4<sup>+</sup> CD8 T cells (Figure 7b). Investigation of T-cell cytokine production in the lung following homologous challenge suggests that HMW CS, but not LMW CS, increases CD4 IL-4 production in the lung 5 days after homologous viral challenge. Therefore, elevated IL-4 production by CD4 T cells in the lungs of mice



**FIGURE 7** HMW CS as an adjuvant increases IL-4 production by CD4 T cells in the lung 5 days after challenge. Mice ( $n = 5$ ) were vaccinated i.m. with 1  $\mu$ g H1N1 PR8 HA  $\pm$  40  $\mu$ g CS as an adjuvant. Four weeks after vaccination mice were challenged with 5000 EID<sub>50</sub> H1N1 PR8 virus. Five days after the challenge, mice were killed, and lung T-cell cytokine production was examined using flow cytometry. CD4 (a) and CD8 (b) T cells were examined for IL-4 production. \* $p \leq .05$ , \*\* $p \leq .01$ , \*\*\* $p \leq .001$ , \*\*\*\* $p \leq .0001$  by one-way ANOVA with Tukey's multiple comparisons test. ANOVA, analysis of variance; CS, chitosan; EID, egg infective dose; HA, hemagglutinin; HMW, high molecular weight; IL-4, interleukin 4; i.m., intramuscularly; LMW, low molecular weight [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

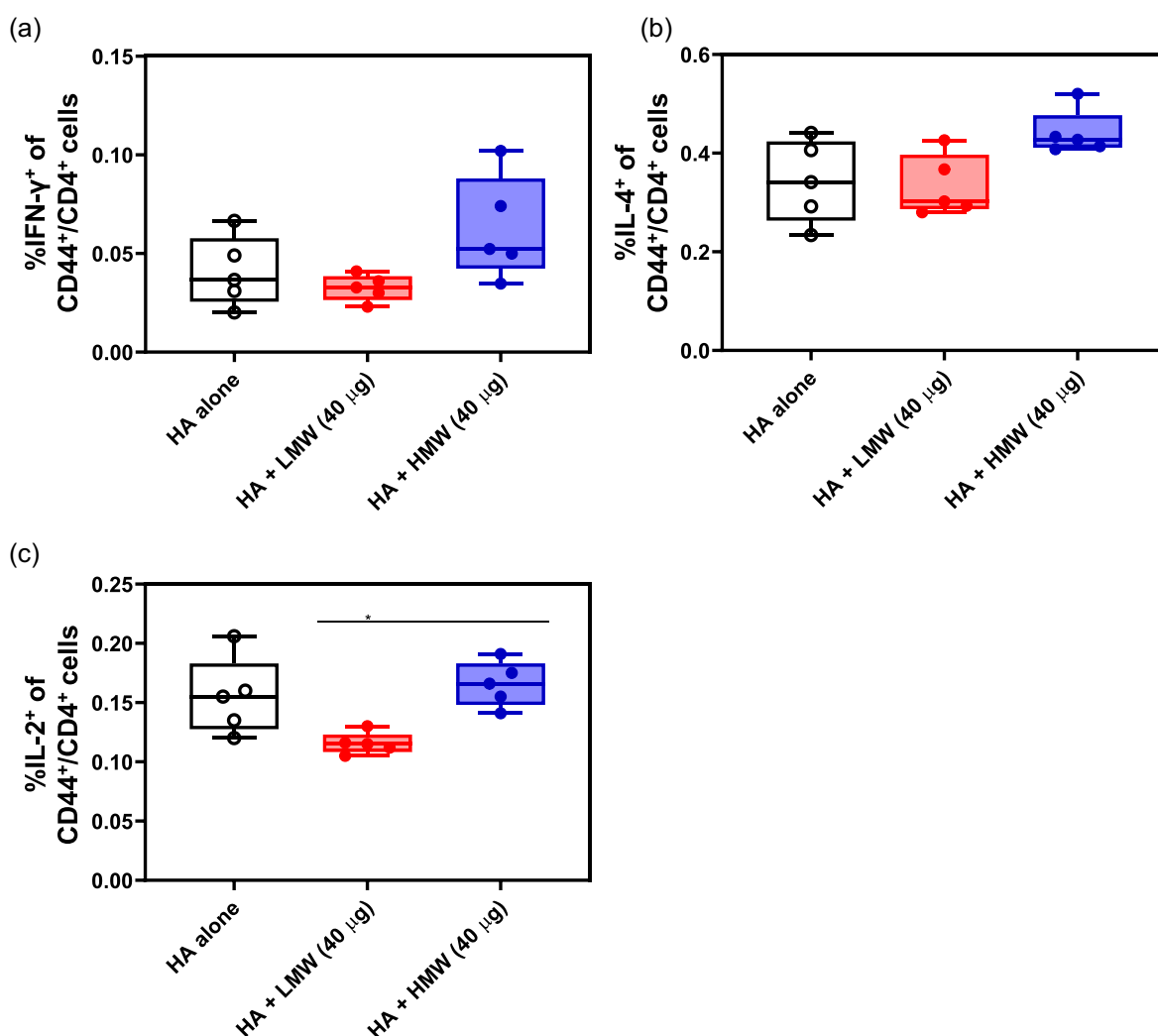
vaccinated with HMW CS may be contributing to the enhanced protection observed with HMW CS adjuvantation. However, IL-4 is not elevated with LMW CS adjuvantation, suggesting that LMW and HMW CS may be inducing enhanced protection through distinct mechanisms.

### 3.8 | HMW CS induces elevated IL-2 producing CD44<sup>+</sup>/CD4<sup>+</sup> T cells in the DLN 2 days after homologous challenge compared to LMW CS

T-cell cytokine production in the DLN early after the viral challenge was also investigated to determine if T-cell function in the DLN during the response to challenge was contributing to decreased

morbidity observed in LMW and HMW CS vaccinated mice compared to those receiving antigen alone. Mice were vaccinated as above, i.m. with 1  $\mu$ g HA protein from PR8 with or without LMW or HMW CS at 40  $\mu$ g. Four weeks after vaccination, mice were challenged i.n. with a lethal dose of PR8 virus (5000 EID<sub>50</sub>). Two days after the challenge, mice were killed for the examination of DLN CD44<sup>+</sup>/CD4<sup>+</sup> T-cell production of IFN- $\gamma$ , IL-4, and IL-2 (Figure 8). CD44, a transmembrane protein that facilitates T-cell migration and recruitment, expression was used as an indicator of activated and antigen-experienced CD4 T cells (Baaten et al., 2012).

No significant differences were observed between vaccination groups in IFN- $\gamma$  and IL-4 production by CD44<sup>+</sup>/CD4<sup>+</sup> T cells in the DLN 2 days after homologous challenge (Figure 8a,b). However, mice vaccinated with formulations that contained HMW CS-induced



**FIGURE 8** HMW CS as an adjuvant increases IL-2 production by DLN CD4 T cells 2 days after challenge. Mice ( $n = 5$ ) were vaccinated i.m. with 1  $\mu$ g H1N1 PR8 HA  $\pm$  40  $\mu$ g CS as an adjuvant. As positive control mice were infected with low-dose PR8 (500 EID<sub>50</sub>). Four weeks after vaccination mice were challenged with 5000 EID<sub>50</sub> H1N1 PR8 virus. Two days after the challenge, mice were killed, and DLN T-cell cytokine production was examined using flow cytometry. IFN- $\gamma$  (a), IL-4 (b), and IL-2 (c) production by CD44<sup>+</sup>/CD4<sup>+</sup> T cells was examined. \* $p \leq .05$ , \*\* $p \leq .01$ , \*\*\* $p \leq .001$ , \*\*\*\* $p \leq .0001$  by one-way ANOVA with Tukey's multiple comparisons test. ANOVA, analysis of variance; CS, chitosan; DLN, draining lymph node; EID, egg infective dose; HA, hemagglutinin; HMW, high molecular weight; IFN- $\gamma$ , interferon- $\gamma$ ; IL-4, interleukin 4; i.m., intramuscularly; LMW, low molecular weight [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



significantly higher frequencies of IL-2<sup>+</sup> CD44<sup>+</sup>/CD4<sup>+</sup> T cells compared to mice receiving LMW CS (Figure 8c). No significant differences were observed between mice receiving HA alone and those receiving either LMW or HMW CS (Figure 8c). Examination of DLN CD4 T-cell cytokine production indicated that HMW CS results in elevated IL-2 production by CD44<sup>+</sup>/CD4<sup>+</sup> T cells compared to LMW CS adjuvantation, suggesting that HMW and LMW CS adjuvantation during vaccination induce distinct T-cell responses the following challenge. Therefore, the observed LMW and HMW CS mediated protection against weight loss after challenge may be mediated through different mechanisms.

## 4 | DISCUSSION

In this study, CS was investigated as a potential vaccine adjuvant, examining the effect of CS MW on innate APC activation in vitro and adaptive immune responses in vivo. Innate immune APC activation was observed after CS treatment, evidenced by elevated cytokine/chemokine mRNA levels and activation markers. However, immune effects were dependent on the MW of the CS. LMW CS treatment induced early cytokine mRNA responses in BMDCs, while HMW CS treatment resulted in later cytokine mRNA responses and elevated expression of BMDC activation markers. The innate immune activation resulting from LMW and HMW CS treatment appeared to be mediated by the IRF pathway, but not the NF- $\kappa$ B pathway, and was not associated with high levels of cell death after 24 h treatment.

These distinct innate immune responses in vitro were accompanied by different effects in vivo based on CS MW. Although mice receiving either LMW or HMW CS as an adjuvant during vaccination displayed decreased morbidity following homologous viral challenge as evidenced by lower weight losses, LMW and HMW CS adjuvantation resulted in distinct adaptive immune responses. LMW CS resulted in significantly elevated antigen-specific IgG antibody titers over unadjuvanted vaccines, while HMW CS did not. However, HMW CS adjuvantation resulted in significantly increased CD69<sup>+</sup>/CD103<sup>+</sup> CD4 and CD8 T cells in the lung and cytokine production by CD4 T cells compared to mice receiving LMW CS. Observed immune responses are summarized in Table 1.

Previously, in vitro, CS has been shown to induce and enhance cytokine production in a variety of immune cells including BMDCs, bone marrow-derived macrophages, and peritoneal macrophages (Bueter et al., 2011; Carroll et al., 2016; Da Silva et al., 2009; Mori et al., 2012). Correspondingly, increased *Il6*, *Ifnb1*, and *Cxcl10* mRNA levels in BMDCs were observed here after 24 and 48 h treatment with LMW (MW: 50–190 kDa) and HMW CS (MW: 310–375 kDa). We did observe a discrepancy in *Il6* mRNA expression at 24 h, when low-dose (1  $\mu$ g/ml) LMW and HMW CS-induced higher levels of *Il6* expression than 10  $\mu$ g/ml CS, yet at 48 h post LMW and HMW CS treatment, *Il6* expression in the 10  $\mu$ g/ml dose was quite high (6- and 12-fold, respectively). Without an extensive time course study, it is difficult to conclude whether the lack of *Il6* transcripts at 24 h in the 10  $\mu$ g/ml dose represents a decrease in transcripts, or whether at that time point, mRNA transcription has yet to begin. Alternatively,

**TABLE 1** Summary of immune responses observed after LMW and HMW CS treatment

Response (compared to negative control)		LMW CS	HMW CS	Positive control
BMDC cytokine mRNA	24 h:	++++	+++	++ (MPLA)
	48 h:	No significant effect	+++	+++ (MPLA)
BMDC surface activation markers		No significant increase	++	+++ (MPLA)
IRF pathway		++	++	++++ (MPLA)
IgG production		+	No significant increase	++++ (MPLA)
Protection		+	+	+++ (PR8)
Lung CD69 <sup>+</sup> /CD103 <sup>+</sup> CD4 and CD8 T cells		–	No significant effect	
IL-4 (lung CD4 T cells)		No significant effect	++	
IL-4 (lung CD8 T cells)		No significant effect	No significant effect	
IFN- $\gamma$ (lung CD4 T cells)		No significant effect	No significant effect	
IFN- $\gamma$ (lung CD8 T cells)		No significant effect	No significant effect	
IL-4 (DLN CD44 <sup>+</sup> /CD4 <sup>+</sup> T cells)		No significant effect	No significant effect	
IFN- $\gamma$ (DLN CD44 <sup>+</sup> /CD4 <sup>+</sup> T cells)		No significant effect	No significant effect	
IL-2 (DLN CD44 <sup>+</sup> /CD4 <sup>+</sup> T cells)		–	No significant effect	

Note: + indicates increased responses compared to the negative control (no treatment/unadjuvanted vaccination). The number of +'s indicates the magnitude of the response compared to the negative control.

Abbreviations: BMDC, bone marrow-derived dendritic cell; CS, chitosan; DLN, draining lymph node; EID, egg infective dose; HA, hemagglutinin; HMW, high molecular weight; IFN- $\gamma$ , interferon- $\gamma$ ; IgG, immunoglobulin G; IL-4, interleukin 4; IRF, interferon regulatory factor; LMW, low molecular weight; mRNA, messenger RNA.

low levels of *Il6* mRNA expression in the 10 µg/ml condition may be the result of negative feedback signals induced by dose treatment, although further investigation would be required to examine this effect. Carroll et al. (2016) also reported increased *Il6* and *Ifnb1* mRNA and protein expression after treating BMDCs with CS (MW: 150–400 kDa). This effect was dependent on a cyclic-di-GMP-AMP synthase (cGAS) and stimulator of IFN genes (STING). While elevated cytokine and chemokine mRNA after both LMW and HMW CS treatment was observed here, LMW and HMW CS treatment-induced differential responses at 24 and 48 h. The difference in cytokine mRNA level after LMW versus HMW CS treatment suggests a disparity in the kinetics of the response to LMW CS and HMW CS, indicating APC may have differing abilities to activate adaptive immune responses based on the MW of CS used. It has been hypothesized that CS adjuvant effects may be mediated through interactions with the cellular membrane or currently undefined host receptors (Moran et al., 2018). Because CS MW has been shown to impact both CS protein interactions and phospholipid bilayer disruption, these functions could be mediating the differences observed in the immune responses induced by LMW and HMW CS (Bekale et al., 2015; Fang et al., 2001). Further studies should be conducted to elucidate the mechanism through which CS activates immune responses and how MW impacts this mechanism.

In addition to cytokine and chemokine mRNA expression, BMDC surface activation marker expression was also assessed. Significantly increased expression of CD80, CD86, and MHC class II on BMDCs treated with HMW CS were observed, consistent with BMDC activation. In contrast, this increase in BMDC surface activation markers was not observed when cells were treated with LMW CS, again supporting that MW of the CS impacts BMDC activation in vitro. Previous reports have also demonstrated that CS and CS-coated culture surfaces are able to induce expression of activation markers in BMDCs (Carroll et al., 2016; Jia et al., 2014; Lin et al., 2014; Oliveira et al., 2012; Villiers et al., 2009). The mechanism through which CS induces the upregulation of CD86 and MHC class II is unclear; however, previous reports have implicated multiple host cellular components including the IFN- $\alpha$ :IFN- $\beta$  receptor, STING, and TLR4 (MW: 150–400 kDa (Carroll et al., 2016) or MW: unreported (Dang et al., 2011; Villiers et al., 2009)). In contrast to the results reported here, Carroll et al. (2016) (24 h CS treatment, dose: 8 µg/ml, MW: 150–400 kDa with 75%–90% deacetylation) and Jia et al. (2014) (48 h CS treatment, dose: 1 µg/ml, MW: unknown) report modest, but significant increases in CD40 expression in BMDCs after CS treatment. Differences between those reports and the CD40 expression reported here may be due to longer treatment durations compared to our study (48 vs. 24 h) and CS properties, with our study examining LMW CS (50–190 kDa with 75%–85% deacetylation) and HMW CS (310–375 kDa with greater than 75% deacetylation) (Carroll et al., 2016; Jia et al., 2014). The lack of CD40 upregulation after LMW and HMW CS treatment may be responsible for the lack of enhanced IFN- $\gamma$  responses seen after vaccination with either variant of CS over unadjuvanted vaccines. Although CD40, unlike CD80/86, is not required for activation of T cells, CD40

interactions with CD40-ligand on T cells are involved in optimal induction of T helper 1 (Th1) polarizing cytokine production and costimulatory molecule expression by DCs and subsequently IFN- $\gamma$  production by both CD8 and CD4 T cells (Caux et al., 1994; Cella et al., 1996; Fujii et al., 2004; Ma & Clark, 2009; Mackey et al., 1998; McLellan et al., 1996; Van Kooten & Banchereau, 1997). CD40:CD40-Ligand interaction between DCs and CD8 T cells, respectively, are also important for cross-priming of CD8 T cells (Bennett et al., 1998; Schoenberger et al., 1998). The work reported here supports previous work that suggests CS treatment can increase BMDC surface activation marker expression in response to CS treatment, although there are differences observed between this report and previous reports by other groups, presumably due to differences in the properties of the CS used and the treatment duration. Additionally, CS MW appears to impact the resulting BMDC marker expression, with HMW CS inducing significantly higher CD80, CD86, and MHC class II expression in BMDCs over cells treated with LMW CS; this again supports that MW has the potential to impact how APC function, and, therefore, how the APC may activate the adaptive immune response during vaccination.

Despite observing differences in cytokine/chemokine mRNA production kinetics and induction of BMDC activation markers between LMW and HMW CS treatment, both types of CS-induced similar patterns and levels of IRF activation. In contrast, neither LMW nor HMW CS activated the NF- $\kappa$ B pathway, despite increases in *Il6* mRNA. Activation of the IRF pathway by CS treatment observed here supports work done by other groups that have found that 2–8 µg/ml CS (MW: 150–400 kDa) treatment of BMDCs induced cGAS and STING activation, upstream of the IRF pathway, indirectly as a result of mitochondrial DNA release (Cai et al., 2014; Carroll et al., 2016; Chen et al., 2016). In further support of indirect activation of the IRF pathway, delayed IRF signaling in response to CS treatment was observed here and by Carroll et al. (2016) (CS: 2 µg, MW: 150–400 kDa) compared to the control MPLA, a known agonist of TLR4. Importantly, our results do not suggest that CS treatment is accompanied by high levels of cell death, which could result in the release of DNA from the mitochondria. Taken together, the work reported here examining IRF activation after CS treatment supports the work done by others suggesting LMW and HMW CS may act by activating cGAS/STING.

The results reported here also support previous work suggesting that CS can successfully act as an adjuvant in an IAV vaccine model (Chang et al., 2010; Wang et al., 2012). Mice vaccinated with formulations containing either LMW or HMW CS experienced significantly less weight loss after the homologous IAV challenge. Despite varied innate effects, LMW- and HMW-induced protection from weight loss did not significantly differ. The observed decrease in weight loss after the homologous challenge is consistent with other reports of enhanced protection from weight loss in vivo after IAV infection in mice receiving CS adjuvanted vaccination (Chang et al., 2010; Wang et al., 2012). However, those studies used live attenuated (Wang et al., 2012) or whole inactivated (Chang et al., 2010) vaccines and CS with

unreported MWs. Here, a low 1 µg dose of recombinant HA protein was used, limiting the antigen quantity and available epitopes. The use of a low antigen dose may allow for the differential effects of LMW and HMW CS treatment to be observed when high levels of antigen are not available to mask modest MW effects. Our studies highlight that both LMW and HMW CS provide increased protection with low antigen dose; however, this protection may be mediated by different mechanisms as distinct antibody response magnitudes and T-cell responses were observed here between LMW and HMW CS.

Accompanying the enhanced protection observed after i.m. vaccination containing LMW and HMW CS, mice receiving LMW CS also displayed increased antigen-specific IgG responses 4 weeks after vaccination compared to unadjuvanted vaccines. In addition, a trend toward elevated IgG1 after both LMW and HMW CS vaccination was observed compared to antigen alone. Previously reported work has shown that both IgG1 and IgG2a subtypes contribute distinctly to protection against IAV (Huber et al., 2006). However, expression of both subtypes, as reported here in mice previously infected with low-dose PR8, provides superior protection (Huber et al., 2006). IL-4 is known to regulate and induce IgG1 isotype switching (Moon et al., 1989). Therefore, the trend of elevated IgG1 expression after CS adjuvantation may be a result of the observed increase in IL-4 production by CD4 T cells in CS, particularly HMW CS, vaccinated mice. In this study, the increased IL-4 production by CD4 T cells, which correlates with elevated IgG1 in CS vaccinated mice, may contribute to enhanced protection observed in CS vaccinated mice compared to mice administered unadjuvanted vaccines. However, the majority of IL-4 expressing CD4 cells were observed in the lung, as opposed to the LN, suggesting IL-4 upregulation may also have a direct effect on the lung microenvironment. Typically, cytokines such as IFN-γ secreted by both CD4 and CD8 T cells are important for IAV clearance during infection (Brown et al., 2004), but IL-4 may be important in blocking some proinflammatory responses in the lung as it has been shown to have a dual role in lung injury (Huaux et al., 2003). In addition, Bueter et al. (2011) reported that CS activated the NLRP3 inflammasome, resulting in IL-1β production. With the IL-1R1 playing a role in the induction and sustainment of the CD4 Th2 (IL-4 producing) subtype (Santarlaschi et al., 2013), activation of the inflammasome may be contributing to the IL-4 production reported here. Although early work suggested that the presence of IL-6, which was induced in BMDCs hereafter CS treatment, increased IL-4 production by CD4 T cells (Diehl et al., 2000; Dienz & Rincon, 2009; Garbers et al., 2018; Rincón et al., 1997), more recent work has shown that IL-6 in the presence of other polarizing cytokines, such as transforming growth factor-β/IL-23 or tumor necrosis factor-α, induces production of other CD4 T cell subset defining cytokines, IL-17 and IL-22, respectively (Dienz & Rincon, 2009; Garbers et al., 2018; Snapper et al., 1988). Therefore, the entire cytokine milieu is critical in defining CD4 T-cell response, and further work is required to elucidate the effect of CS adjuvantation on CD4 T-cell activation, and the subsequent impact on the immune response including B-cell responses.

Although we, like other groups (Carroll et al., 2016; Chang et al., 2010; Ghendon et al., 2008, 2009; Heffernan et al., 2011; Sui, Chen, Wu, et al., 2010; Wang et al., 2012), report enhanced antibody responses in mice vaccinated with CS as an adjuvant compared to those receiving antigen alone, weight loss trends observed in our report indicate that mice vaccinated with 1-µg HA protein combined with LMW or HMW CS are not afforded to sterilize immunity against homologous IAV challenge. As discussed above this result may be connected to the low dose of antigen used in our study resulting in antigen being the dose-limiting factor. However, this weight loss could also be indicative of cell-mediated immunity, as opposed to immunity mediated by neutralizing antibodies. Significantly different CD69<sup>+</sup>/CD103<sup>+</sup> CD4 and CD8 T-cell populations were observed in the lungs of LMW and HMW CS vaccinated mice 5 days after homologous challenge. In addition, elevated IL-4 production was observed by CD4 T cells in the lung 5 days after challenge in mice vaccinated with HMW CS compared to mice receiving unadjuvanted vaccines. This elevated frequency of IL-4 CD4 T cells was not observed in mice receiving LMW CS. In the DLN 2 days after the challenge, it was also observed that HMW CS vaccinated mice displayed significantly higher frequencies of IL-2 producing CD44<sup>+</sup>/CD4<sup>+</sup> T cells compared to mice receiving LMW CS. The predominant Th2-like IL-4 and IL-2 producing T-cell response demonstrated in this study is opposite to what Carroll et al. (2016) observed in their report, in which the predominant response was more Th1-like with high levels of IFN-γ expressing CD4 cells and IgG2c responses. This difference may be due to differences in the properties of the CS preparations as discussed previously, but also may represent a difference in the in vivo experimental conditions between the two reports. Carroll et al. (2016) used 2 µg of antigen per mouse together with 100 µg of CS, which is a twofold higher antigen and adjuvant dose than our report. In addition, Carroll et al. (2016) injected antigen and adjuvant intraperitoneally, as well as used a prime/boost vaccination scheme with injections at Days 0 and 14. Our results are after a single, low antigen dose (1 µg per mouse) vaccination scheme, in which we model an IAV vaccine delivered i.m. and test immunity in the T-cell compartment at mucosal sites after challenge. It has been shown that the level of antigenic stimulation can influence the development of Th2 (IL-4 producing) and Th1 (IFN-γ producing) CD4 effectors with moderate levels of antigen contributing to the differentiation of IL-4 secreting CD4 cells and high levels of antigen promoting more IFN-γ secreting CD4 cells (Kaiko et al., 2008). Clearly, many factors contribute to vaccine-induced responses after antigen and CS administration, including the properties of CS, antigen dose, vaccination site, and prime/boost schemes.

Together the analysis of the T-cell populations and cytokine production in LMW and HMW CS vaccinated mice suggests that the two MW variants of CS have distinct effects on T-cell responses when acting as vaccine adjuvants. These distinct effects may be the result of differential APC activation following LMW and HMW CS adjuvantation, as was observed in vitro. Another possibility to explain these differences is the antigen depot effect that has been attributed to some CS preparations (Markushin et al., 2018; Zaharoff

et al., 2007). This depot effect is thought to enhance and sustain vaccine-induced immunity by providing a slow release of antigen at the injection site, and was originally reported as a potential mechanism of action of alum, a well-known adjuvant used in human vaccines (Marrack et al., 2009). However, there is still some controversy over whether alum does provide a depot effect, with opposing effects reported in the literature (Didierlaurent et al., 2009; Hutchison et al., 2012; Marrack et al., 2009). Nevertheless, more investigation into the potential for an antigen depot effect in HMW CS may be warranted, especially for limiting antigen concentrations, such as the 1 µg per mouse dose of IAV HA we use in this report. Taken together, the adaptive in vivo immune responses following LMW and HMW CS adjuvantation suggest that LMW and HMW CS may impart protection from infection using different mechanisms, with LMW CS inducing higher IgG antibody responses and HMW CS increasing T-cell cytokine production.

## 5 | CONCLUSION

In this study, the differential ability of LMW and HMW CS to act as vaccine adjuvants was investigated. While both LMW and HMW CS were able to induce elevated cytokine and chemokine mRNA levels in BMDCs, the kinetics of this response was impacted by the MW of the CS used for treatment. LMW CS resulted in higher cytokine and chemokine mRNA levels at 24 h after treatment compared to HMW CS, by 48 h after treatment HMW CS-induced elevated cytokine and chemokine mRNA compared to LMW CS. LMW and HMW CS treatment also resulted in differing activation marker expression in BMDCs, with HMW CS resulting in increased CD80, CD86, and MHC class II compared to LMW CS. Despite these differences in cytokine production and activation of BMDCs, both LMW and HMW CS-induced similar levels of IRF pathway activation in a macrophage-like cell line. In vivo, it was observed that both LMW and HMW used as adjuvants in an IAV vaccine-induced protection from a homologous challenge and increased IgG antibody responses. However, the observed increase in IgG titer with CS adjuvantation only reached significance compared to unadjuvanted vaccines with LMW adjuvantation. Finally, it was observed that HMW CS vaccinated mice displayed elevated T-cell activation and cytokine production in the lung and DLN following homologous challenge compared to LMW CS vaccinated mice, suggesting that LMW and HMW CS may induce protection during IAV challenge through different mechanisms. Together these results suggest that while both LMW and HMW CS are able to act as adjuvants and increase protection against homologous IAV challenge, LMW and HMW CS induce distinct innate and adaptive immune responses. However, this report investigated only two MW ranges, and, therefore further investigation of more CS MWs and more finetune MW ranges must be conducted to continue examining the effects of MW on CS adjuvanticity. In addition, CS properties are heavily influenced by other parameters including the degree of deacetylation, charge density, and branching (Aggarwal & Matthew, 2009; Huang et al., 2004; Kiang et al., 2004;

Maurstad et al., 2007; Ravi Kumar, 2000; Shukla et al., 2013), which should also be carefully studied in future studies to fully understand how CS properties can affect its use as a vaccine adjuvant.

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## CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

## AUTHOR CONTRIBUTIONS

*Conceived and designed the experiments:* Anna T. Lampe, Eric J. Farris, Deborah M. Brown, and Angela K. Pannier. *Performed the experiments:* Anna T. Lampe. *Data analysis and acquisition:* Anna T. Lampe. *Drafted the work:* Anna T. Lampe. *Critical revisions and final approval of the version to be published:* Eric J. Farris, Deborah M. Brown, and Angela K. Pannier.

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## SUPPORTING INFORMATION

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