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## Novel recombinant vaccinia virus-vectored vaccine affords complete protection against homologous *Borrelia burgdorferi* infection in mice

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### ABSTRACT

The rising prevalence of Lyme disease (LD) in North America and Europe has emerged as a pressing public health concern. Despite the availability of veterinary LD vaccines, no vaccine is currently available for human use. Outer surface protein C (OspC) found on the outer membrane of the causative agent, *Borrelia burgdorferi*, has been identified as a promising target for LD vaccine development due to its sustained expression during mammalian infection. However, the efficacy and immunological mechanisms of LD vaccines solely targeting OspC are not well characterized. In this study, we developed an attenuated Vaccinia virus (VV) vectored vaccine encoding type A OspC (VV-OspC-A). Two doses of the VV-OspC-A vaccine conferred complete protection against homologous *B. burgdorferi* challenge in mice. Furthermore, the candidate vaccine also prevented the development of carditis and lymph node hyperplasia associated with LD. When investigating the humoral immune response to vaccination, VV-OspC-A was found to induce a robust antibody response predominated by the IgG2a subtype, indicating a Th1-bias. Using a novel quantitative flow cytometry assay, we also determined that elicited antibodies were capable of inducing antibody-dependent cellular phagocytosis *in vitro*. Finally, we demonstrated that VV-OspC-A vaccination generated a strong antigen-specific CD4<sup>+</sup> T-cell response characterized by the secretion of numerous cytokines upon stimulation of splenocytes with OspC peptides. This study suggests a promising avenue for LD vaccine development utilizing viral vectors targeting OspC and provides insights into the immunological mechanisms that confer protection against *B. burgdorferi* infection.

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**KEYWORDS** Vaccinia virus; viral-vector vaccine; *Borrelia burgdorferi*; outer surface protein C; Lyme disease; antibodies; phagocytosis

### Introduction

The increasing prevalence of Lyme disease (LD) in Canada and the United States poses a significant public health concern. Recent estimates suggest that nearly half a million people in North America develop LD each year [1,2]. In Canada, the number of reported LD cases has risen drastically since monitoring began, due, at least in part, to the northern migration of associated tick-vectors [3–6]. The causative agents of LD, bacteria of the *Borrelia burgdorferi sensu lato* complex, are transmitted to humans by ticks of the genus *Ixodes*. Infection with *B. burgdorferi* leads to multisystemic inflammation in the host, presenting as a range of symptoms, including *erythema migrans*, arthritis, carditis, and neurological conditions [7,8]. Despite the efficacy of commercially available canine LD vaccines, there has been no vaccine available for human use since the withdrawal of the LYMERix<sup>TM</sup> vaccine from the market in 2002 [9].

Two prominent outer surface proteins of *Borrelia burgdorferi*, outer surface protein A (OspA) and outer surface protein C (OspC), have garnered significant attention for LD vaccine development. OspA, the target of the LYMERix<sup>TM</sup> vaccine, is primarily expressed by the bacterium while it resides in the tick vector but is downregulated during transmission into the mammalian host [10]. Consequently, OspA-based vaccines may require multiple booster doses to achieve circulating antibody levels capable of preventing tick-to-host transmission [11]. In addition, molecular similarity between OspA and human leukocyte function-associated antigen (hLFA1) has evoked concerns of vaccine-induced autoimmunity [12,13]. While a different OspA-based vaccine currently in Phase 3 clinical trial, VLA15, excludes this potentially cross-reactive epitope, vaccines targeting OspC alone may be a promising alternative strategy to reduce vaccine hesitancy [14,15]. In contrast to

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OspA, OspC is induced during transmission to the mammalian host and plays a crucial role in early mammalian infection and bacterial dissemination [10,16]. Its sustained expression throughout host infection renders OspC an attractive target for vaccine development [11,17,18]. Still, significant knowledge gaps remain regarding the efficacy of vaccines solely targeting OspC, without the inclusion of an OspA antigen.

In this study, we employ an attenuated Vaccinia virus (VV) vector for delivery of the OspC gene. VV is a double-stranded DNA poxvirus with several advantages for use as a vaccine vector, including its capacity for incorporation of heterologous DNA, ability to induce both cellular and humoral immune response, thermostability, cytoplasmic replication, and ease of propagation [19]. It has been widely used for smallpox vaccination campaigns, eventually leading to the development of several attenuated strains that cause fewer adverse reactions [20]. We previously described a highly efficient method to generate a recombinant VV vector containing deletion of the E3L gene and modification of the K3L gene [21]. E3L gene deletion prevents the virus from suppressing the host type 1-interferon response, leading to dramatic attenuation of the virus, while swapping of the K3L gene with a poxvirus ortholog serves as a positive selection marker for recombination during vaccine production [22]. A previous study by our lab demonstrated that this modified vector conferred protection against respiratory syncytial virus after a single intramuscular injection [23]. The attenuated VV vector was also found to be mostly avirulent in mice with a promising safety profile, demonstrating only mild and transient weight loss following intranasal administration [23,24].

Here, the same VV vector with E3L deletion and K3L substitution was used to deliver the type A OspC gene of *B. burgdorferi sensu stricto* B31-A3 (VV-OspC-A). C3H/HeN mice were vaccinated in a prime-boost regimen and then challenged by needle injection of homologous *B. burgdorferi* B31-A3 expressing genetically identical OspC. Following the challenge, the bacterial burden was evaluated across a range of tissues. Clinical presentations of LD, including pathology in the heart and lymph nodes, were also assessed. Finally, the humoral and cellular immune responses were evaluated following boost vaccination.

## Methods

### Bacterial and mammalian cell culture

All *B. burgdorferi* strains used in this study were kindly provided by Dr. Patricia Rosa (National Institute of Allergy and Infectious Disease, Montana, US). Wild-type *B. burgdorferi* strain B31-A3 and strain B31-A3

constitutively expressing OspC (*B. burgdorferi*-OspC) were prepared as previously described [25–27]. In brief, strains were cultured from glycerol stocks in complete Barbour-Stoenner-Kelly (BSK)-H media containing 6% rabbit serum (Millipore Sigma, Burlington, ON) and supplemented with 5 µg/mL Amphotericin B, 100 µg/mL Phosphomycin, and 50 µg/mL Rifampicin. Cultures were incubated at 35°C and 1.5% CO<sub>2</sub> until mid-log phase and enumeration was performed using a Petroff-Hausser counting chamber under dark-field microscopy.

BHK21, HeLa/PKR-knockout, and RK13 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1.5 g/L sodium bicarbonate, 25 mM HEPES, 20 U/mL penicillin, 0.02 mg/mL streptomycin and 10% heat-inactivated fetal-bovine serum.

### Animal care

Six-week-old female C3H/HeN mice were obtained from Charles River, Senneville, Quebec, Canada. All animal procedures were approved by the Animal Care Committee at Health Canada, Ottawa, Ontario (Study #2020-011) and performed in accordance with institutional guidelines. Mice were allowed to acclimatize to experimental locations for one week before procedures began.

### Generation of recombinant vaccinia virus

The type A outer surface protein C (OspC) gene of *Borrelia burgdorferi* B31 (GenBank accession #AAC66329.1) driven by the vaccinia virus mH5 promoter and including the human tyrosinase signal peptide (MLLAVLYCLLWSFQTSAGHFPPRA; GenBank accession #AH003020) at the N-terminus was codon optimized for expression in mice and commercially synthesized in a pUC57 plasmid vector by Bio Basic Inc. (Markham, ON) [28–30]. To generate a recombinant DNA shuttle vector, the OspC gene was fused between the right and left flanking regions containing the poxvirus K3L ortholog taterapox virus 037 (TATV037) using overlapping PCR with Phusion High-Fidelity DNA Polymerase (Life Technologies) and the following primers – 5'-TACGCTACTA-TACCGGCATT-3', 5'-GGATCAGCATCTGGTACAAT-3'.

The recombinant vaccinia virus was generated as previously described [21]. In brief, the shuttle vector was transfected into HeLa/PKR-knockout cells infected with the parental VVΔE3LΔK3L. The virus was harvested 48 h later and then passaged twice in BHK21 cells. The recombined virus was plaque purified by selecting plaques expressing green fluorescent protein, amplified in BHK21 cells, and then purified using a 35% sucrose cushion. The control VV-K8.1

control was generated in the same fashion, as described in detail previously [21].

Viral titres were determined using a standard plaque assay in which serial dilutions of the virus were incubated on RK13 cells for 2 h at 37°C and then overlaid with 2x DMEM media containing 4% FBS and 0.8% agarose. Plaques were counted after a three day incubation at 37°C.

### Western blot analysis

BHK21 cells were infected with VV-OspC-A or VV-K8.1 in a 6-well plate. Plates were incubated for 48 h at 37°C and then cells were collected by scraping into the media. Cells were freeze-thawed three times then centrifuged at 250 × g for 10 min. The cell pellet was lysed in sodium dodecyl sulphate (SDS) lysis buffer (50 mM Tris-HCl pH 6.8, 2% (W/V) SDS, 0.1% (W/V) bromophenol blue, 10% (W/V) glycerol, 5% fresh β-mercaptoethanol) and then passed through a QiaShredder (Qiagen). Lysates were heated to 95°C for 5 min before electrophoresing on a 4–15% TGX stain-free SDS-PAGE gel (Bio-Rad, Saint-Laurent, QC). Proteins were transferred to a polyvinylidene difluoride membrane then blocked with tris-buffered saline (TBS) containing 5% (W/V) non-fat milk powder for 1 h at room temperature. The membrane was then incubated overnight at 4°C in blocking buffer containing polyclonal rabbit anti-OspC antibody (1:1000 dilution) (Rockland Immunochemicals, Pottstown, PA). The membrane was washed five times with TBS prior to incubating for 1 h at room temperature with blocking buffer containing goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:15,000 dilution) (ThermoFisher, Ottawa, ON). The membrane was washed four more times with TBS and then developed using SuperSignal™ West Femto Maximum Sensitivity Substrate (ThermoFisher) and imaged on a ChemiDoc MP imaging system (Bio-Rad).

### Immunizations and bacterial challenge

Eight-week-old mice were randomly assigned to groups and injected subcutaneously with 50 µL of the Vanguard® CrLyme vaccine (Zoetis, NJ) ( $n = 13$ ) or intramuscularly with 50 µL of the VV-OspC-A ( $n = 14$ ) or VV-K8.1 ( $n = 14$ ) vaccines at a dose of  $5 \times 10^6$  PFU. An identical dose of the vaccine was administered four weeks after the initial injection. Four weeks after boost injection, half the mice in each group were injected subcutaneously between the shoulder blades with  $10^6$  wild-type *B. burgdorferi* B31-A3 cells in 100 µL of incomplete BSK-H media without rabbit serum (Millipore Sigma). These mice were sacrificed 14 days post-challenge for collection of blood and tissues. The remaining half of each

group ( $n = 7$ ) was sacrificed four weeks after the boost injection for collection of blood and tissues without bacterial challenge. All vaccine and challenge injections were performed under isoflurane anaesthesia.

### Tissue culture for bacterial identification

Two weeks after bacterial challenge, the right ear and skin surrounding the injection site were collected and cultured as previously described with one exception: cultures in this study were incubated for 28 days at 35°C and 1.5% CO<sub>2</sub> before examination by darkfield microscopy [25]. Cultures were considered positive if at least one mobile spirochaete was identified across five fields of view.

### qPCR for bacterial burden

Two weeks after bacterial challenge, the bladder, left ear, left tibiotarsal joint, inferior half of the heart, and left inguinal and medial iliac lymph nodes were collected and frozen in liquid nitrogen. DNA was extracted from tissues using a DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. qPCR analysis of the extracted DNA was performed as previously described using a *Borrelia burgdorferi* FlaB custom gene assay (Forward primer: 5'-TCTTTTCTCTGGTGAGGGAGCT-3', Reverse primer: 5'-TCCTTCCTGTTGAACACCCTCT-3', ThermoFisher) or mouse β-actin gene assay (ID: Mm02619580\_g1, ThermoFisher) [25]. Standard curves of plasmids encoding mouse β-actin or extracted *B. burgdorferi* genomic DNA were used to determine the number of FlaB copies per  $10^6$  β-actin copies.

### Histopathology

Two weeks after bacterial challenge, the heart and right axillary lymph node were collected for histopathology analysis. The heart was bisected horizontally and the superior half of the heart, including the origin of the great vessels, heart base, and top halves of the ventricles and atria, was collected. The tissue processing and haematoxylin and eosin staining was performed as previously described [25]. Inflammation in the heart and lymph nodes was assessed by a certified veterinary pathologist according to the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice (INHAND) standards [31,32]. Each heart was assigned a numeric grade (0–5) based on the severity of inflammation where 0 was normal, 1 was minimal, 2 was mild, 3 was moderate, 4 was marked, and 5 was severe. Each lymph node was assigned three numeric grades (0–5) indicating the severity of follicular hyperplasia, paracortical hyperplasia, and cord plasma cell hyperplasia where 0 was normal, 1 was minimal, 2

was mild, 3 was moderate, 4 was marked, and 5 was severe. The combined pathology score represents the sum of these three lymph node pathology scores.

### Determination of lymphadenopathy

Two weeks after bacterial challenge, the left axillary lymph node was collected and placed in cold RPMI media supplemented with 0.15% (w/v) sodium bicarbonate, 1 mM sodium pyruvate, 5 mM HEPES, 20 U/mL Penicillin, 0.02 mg/mL Streptomycin, 0.1% (v/v) 2-mercaptoethanol, and 10% (v/v) heat-inactivated fetal-bovine serum (FBS). The lymph nodes were pressed between two microscope slides to create a single cell suspension then passed through a 70 µm cell strainer before centrifugation at 300 g for 5 min. The cells were resuspended in media and then the white blood cells were counted using a Sysmex XT-2000iV haematology analyzer.

### Enzyme linked immunosorbent assay (ELISA)

ELISAs were performed as previously described [25]. In brief, 96-well Nunc Maxisorp™ flat bottom plates (ThermoFisher) were coated with 0.5 µg/mL of recombinant OspC-type A (Native Antigen, Oxfordshire, UK) overnight at 4°C and then blocked with 1% (w/v) Bovine Serum Albumin (IgG-Free, Protease-Free, Jackson Immuno Research, West Grove, PA). Serum obtained from mice three weeks after prime or boost vaccination was then added to the plate in serial dilutions. For total IgG analysis, serum was diluted two-fold ranging from 1:50 to 1:102,400. For IgG2a and IgG1 analysis, serum was diluted three-fold between the range of 1:100 to 1:17,714,700. Following washes, plates were then incubated with HRP-conjugated goat anti-mouse IgG (Cytiva, Marlborough, MA), HRP-conjugated goat anti-mouse IgG1 (Jackson Immuno Research), or HRP-conjugated goat anti-mouse IgG2a (Jackson Immuno Research). Plates were washed, incubated for 5 min with Tetramethylbenzidine (TMB) substrate (Cell Signaling Technology, Danvers, MA), and then terminated with 0.16 M sulphuric acid. Absorbance was read at 450 nm using a spectrophotometer and endpoint titres were defined as the reciprocal of the highest dilution that resulted in an optical density (OD) greater than the average OD of all wells containing serum from mice vaccinated with the VV-K8.1 vaccine plus three times the standard deviation.

### Antibody-dependent cellular phagocytosis assay

RAW 264.7 cells were obtained from ATCC (ATCC TIB-71, Manassas, VA) and cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented

with 1.5 g/L sodium bicarbonate, 20 U/ml Penicillin, 0.02 mg/ml Streptomycin, and 10% FBS.

Cultures of *B. burgdorferi*-OspC were grown to mid-log phase then centrifuged at 5000 g for 15 min. The bacterial pellet was washed with PBS and resuspended in 200 µL of 1:50 diluted anti-*Borrelia* antibody conjugated to FITC (PA1-73005, ThermoFisher) in PBS prior to incubation for 30 min at 37°C. The bacteria was centrifuged and washed twice with PBS before diluting to  $3.75 \times 10^7$  cells/mL in DMEM containing no antibiotics or serum. 20 µL of the diluted and stained *B. burgdorferi*-OspC was then added to each well of 96-well plate. Next, 20 µL of mouse serum collected 28 days post-boost was added to each well. Before addition, serum was heat-inactivated at 56°C for 30 min and then diluted 1:10 in DMEM containing no antibiotics or serum. The plate was then shaken at 300 rpm in a 37°C incubator for 15 min. 10 µL of RAW 264.7 cells at a concentration of  $7.5 \times 10^6$  cells/mL in DMEM containing no antibiotics or serum were then added to each well before shaking again at 300 rpm in a 37°C incubator for 15 min. The plate was then incubated for 1 h at 37°C without shaking. To arrest the phagocytosis, 100 µL of cold PBS was added to each well and plates were analyzed immediately by flow cytometry using a BD FACSymphony™ A1.

### Multiplex cytokine ELISA

Four weeks after boost-vaccination, mice were sacrificed for collection of the spleens. Single cell suspensions were created by gently pressing the spleen between two microscope slides and then passing through a 70 µm cell strainer. The cells were centrifuged at 300 g for 5 min and then resuspended in RPMI media before cell counting using a Sysmex XT-2000iV haematology analyzer.

Two million splenocytes were then stimulated with a custom 15-mer overlapping peptide pool (GenScript, Piscataway, NJ) of OspC type A containing 1 µg of each peptide for 24 h at 37°C in RPMI media. After incubation the plate was centrifuged at 1800rpm for 5 min and the supernatant was collected and frozen at -80°C. Secreted cytokine levels in the supernatant were measured using a ProcartaPlex 17-plex Immunoassay kit (EPX170-26087-901, ThermoFisher) according to the manufacturer's instructions. The plates were read on a Luminex 200 system (Millipore-Sigma) and data analysis was performed using MILLI-PLEX Analyst version 5.1 software to determine the concentration of each cytokine.

### Flow cytometry of antigen-specific T cells

Two million splenocytes were stimulated with a custom 15-mer overlapping peptide pool (GenScript, Piscataway, NJ) of OspC type A containing 1 µg of each

peptide for 5 h at 37°C, 5% CO<sub>2</sub> in RPMI media. Unstimulated samples were included as a negative control and samples stimulated with phorbol 12-myristate-13-acetate (Sigma-Aldrich) at 20 ng/mL and ionomycin (Sigma-Aldrich) at 1 µg/mL were included as a positive control. After 1 h of stimulation, 0.2 µL of GolgiPlug™ (Brefeldin A, BD Biosciences) was added to each well. After stimulation, cells were washed with PBS and then blocked with rat anti-mouse CD16/CD32 (Mouse Fc Block™, BD Biosciences) for 15 min at 4°C before surface straining with anti-CD3 APC-Cy7 (Clone 17A2, BD Biosciences), anti-CD4 BUV395 (Clone GK1.5, BD Biosciences) anti-CD8 BV510 (Clone 53-6.7, BD Biosciences) and LIVE/DEAD™ Fixable Red Dead Cell Stain (ThermoFisher) diluted in Brilliant Stain Buffer (BD Biosciences) for 30 min in the dark at 4°C. Cells were then washed twice in cold PBS, fixed using CytoFix™ fixation buffer (BD Biosciences), and then permeabilized with Perm/Wash buffer (BD Biosciences). Cells were then incubated for 30 min at 4°C in the dark with intracellular stain containing anti-IFN-γ BUV737 (Clone XMG1.2, BD Biosciences) and anti-TNF (Clone MP6-XT22, BD Biosciences) diluted in Brilliant Stain Buffer (BD Biosciences). After staining, cells were washed twice with Perm/Wash buffer, and then resuspended in PBS and immediately analysed by flow cytometry using a BD FACSAria™ Fusion. Data were analyzed using FlowJo v10 software (TreeStar). A representative gating strategy is depicted in Figure S1.

### Statistical analysis

Statistical significance was calculated using a Kruskal–Wallis test with Dunn’s multiple comparison post-hoc test. All statistical analyses were performed using GraphPad Prism 9. \* *p*-value < 0.05, \*\* *p*-value < 0.01, \*\*\* *p*-value < 0.001, \*\*\*\* *p*-value < 0.0001.

## Results

### VV-OspC-A protects against homologous *B. burgdorferi* infection and dissemination

To assess the protection conferred by the candidate vaccine, mice were vaccinated on day 0 and day 28 with one of three vaccine constructs: (1) the modified VV-vector encoding OspC (VV-OspC-A), (2) the modified VV-vector encoding an unrelated herpes protein (VV-K8.1) serving as a negative control, or (3) a commercially available canine LD vaccine, Vanguard® CrLyme (CrLyme), serving as a positive control (Figure 1a). CrLyme is a subunit vaccine containing recombinant OspA in addition to a recombinant chimeric OspC composed of the L5 and H5 epitopes of seven different OspC types, including type A [33].

Prior to vaccination, OspC expression was confirmed *in vitro* by western blotting of VV-OspC-A infected BHK21 cells (Figure S2). Four weeks after the boost vaccination (day 56), mice were challenged by subcutaneous injection of 10<sup>6</sup> *B. burgdorferi sensu stricto* strain B31-A3. Finally, mice were sacrificed 14 days post-challenge (day 70) for tissue collection (Figure 1b).

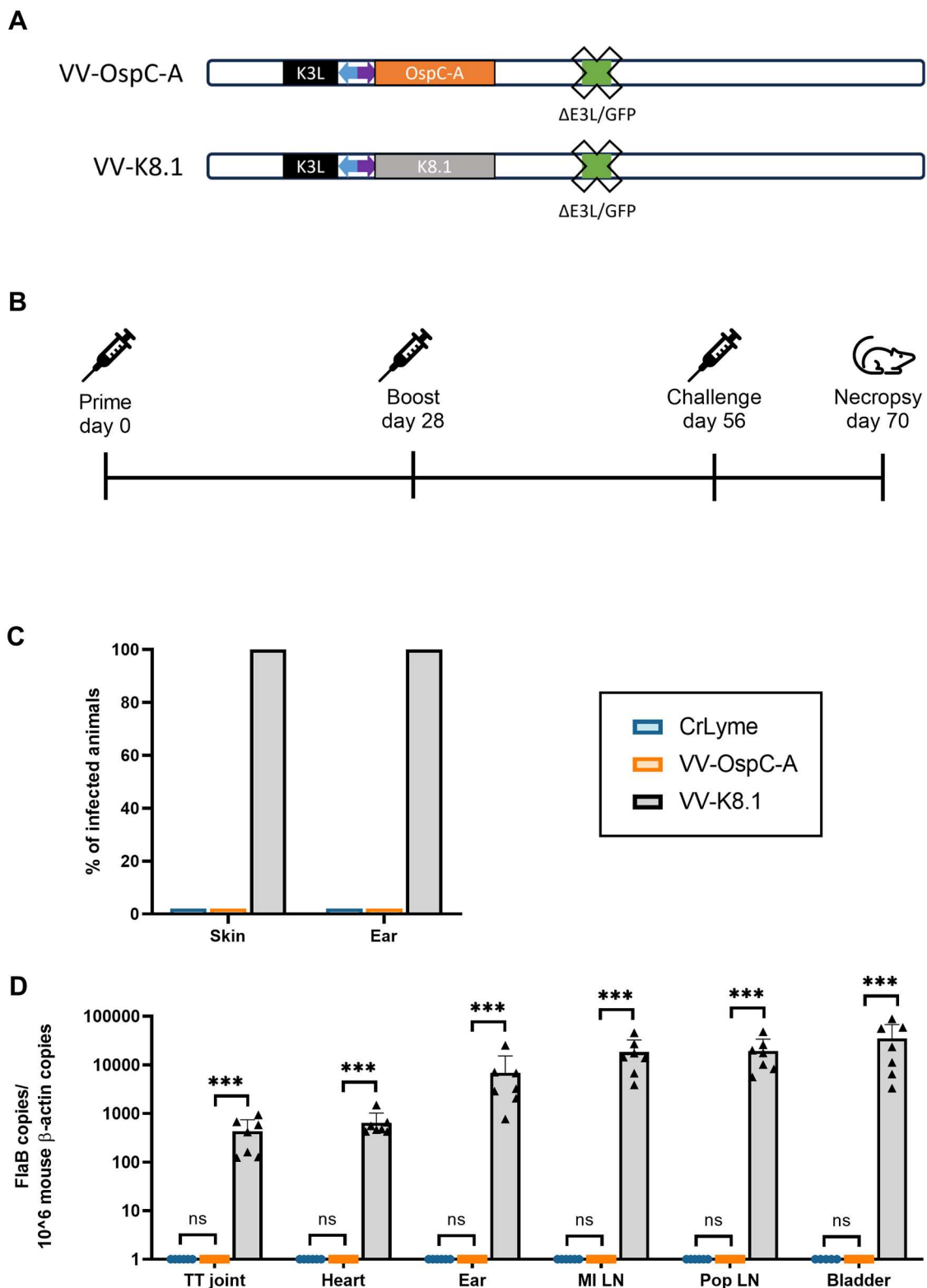
At necropsy, the skin and ears were placed in bacterial culture media and incubated for 28 days before examining for the presence of *B. burgdorferi* by darkfield microscopy. As expected, all skin and ear cultures from mice vaccinated with the control VV-K8.1 vaccine were positive for *B. burgdorferi* infection (Figure 1c). In comparison, no bacterial growth was detected in any mouse vaccinated with either CrLyme or VV-OspC-A. Furthermore, a range of organs were collected at necropsy for quantification of bacterial burden by qPCR. In accordance with the culture results, all mice vaccinated with VV-K8.1 had significantly increased quantities of *B. burgdorferi* DNA across all organs tested in comparison to the CrLyme and VV-OspC-A groups (Figure 1d). Importantly, spirochaete DNA was not detected in any tissues from mice vaccinated with either CrLyme or the candidate VV-OspC-A. Taken together, these results demonstrate that two doses of the candidate VV-OspC-A vaccine afforded complete protection against *B. burgdorferi* infection at a level similar to that afforded by the CrLyme vaccine.

### VV-OspC-A prevents the development of Lyme carditis

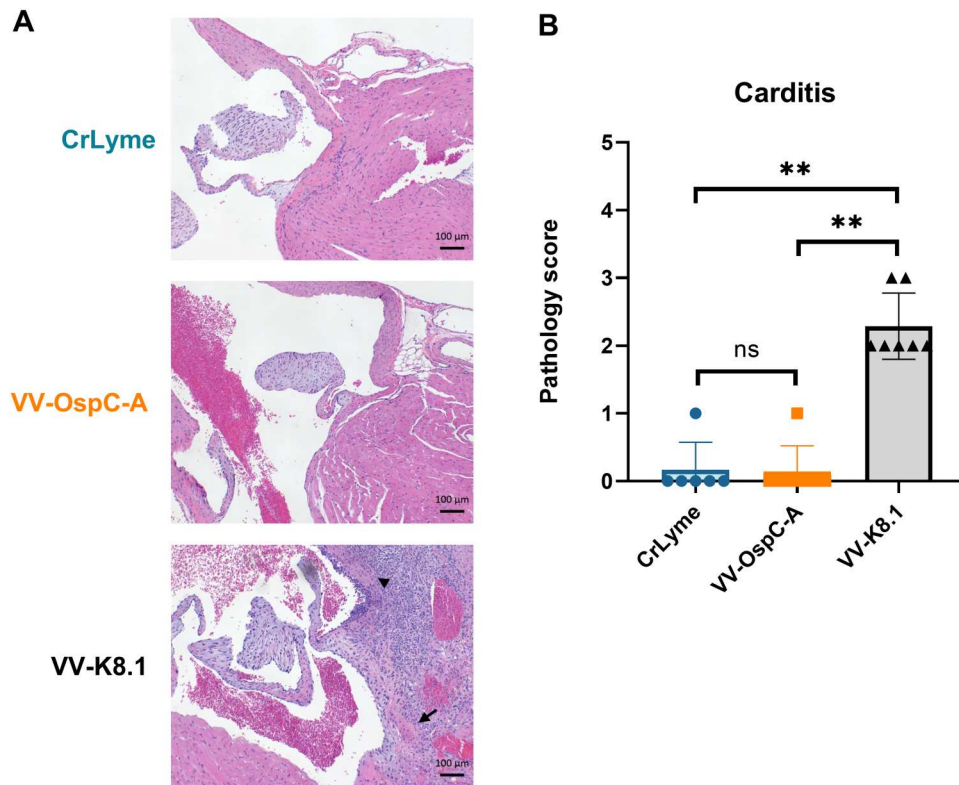
Given that we observed significant infection in the heart tissue and that disseminated infection with *B. burgdorferi* is known to cause carditis in both humans and C3H/HeN mice, we performed histopathological assessment of the heart tissue two weeks post-challenge. Mice inoculated with the control VV-K8.1 vaccine consistently exhibited inflammation of the heart tissue, characterized by mild-to-moderate mixed mononuclear-neutrophilic infiltration of the heart base with variable involvement of adjoining structures, including the aorta (Figure 2a, b). Conversely, mice immunized with VV-OspC-A or CrLyme were protected against the development of Lyme carditis, with the candidate vaccine performing as well as the veterinary standard. These findings are consistent with the ability of the VV-OspC-A vaccine to effectively prevent infection of the heart.

### VV-OspC-A prevents the development of lymphadenopathy

*B. burgdorferi* infection induces severe swelling of the lymph nodes with the most severe lymphadenopathy observed closest to the site of infection [34–36]. At



**Figure 1.** Vaccination with VV-OspC-A affords protection against *B. burgdorferi* challenge in mice. (a) Schematics depicting the VV-vector vaccines encoding outer surface protein C type A (OspC-A) or an unrelated protein from herpes virus (VV-K8.1) showing the taterapox virus K3L ortholog (black box), K3L ortholog promoter (blue reverse arrow), synthetic early/late promoter (purple forward arrow), and E3L gene deletion and substitution for GFP. (b) Schematic depicting the immunization and challenge schedule used in the protection study. On day 0 and day 28, groups of C3H/HeN mice were vaccinated subcutaneously with CrLyme ( $n = 6$ ) or intramuscularly with VV-OspC-A ( $n = 7$ ) or VV-K8.1 ( $n = 7$ ). Mice were challenged subcutaneously with  $1 \times 10^6$  *B. burgdorferi* cells on day 56 and sacrificed on day 70. (c) The right ear and skin surrounding the bacterial injection site were collected at necropsy and incubated for 28 days in BSK-H growth media. Cultures were considered positive if at least one spirochaete was identified by dark-field microscopy across five fields of view. (d) qPCR was used to determine bacterial burden in a variety of mouse tissues 14 days after *B. burgdorferi* challenge. Bacterial burden is represented by copies of the *B. burgdorferi* FlaB gene per  $1 \times 10^6$  mouse  $\beta$ -actin gene copies. Samples below the threshold of amplification were assigned a value of one. TT joint, tibiotarsal joint; MI LN, medial iliac lymph node; Pop LN, popliteal lymph node. Error bars represent standard deviation. ns, not significant, \*\*\*  $p$ -value < 0.001, \*\*\*\*  $p$ -value < 0.0001.



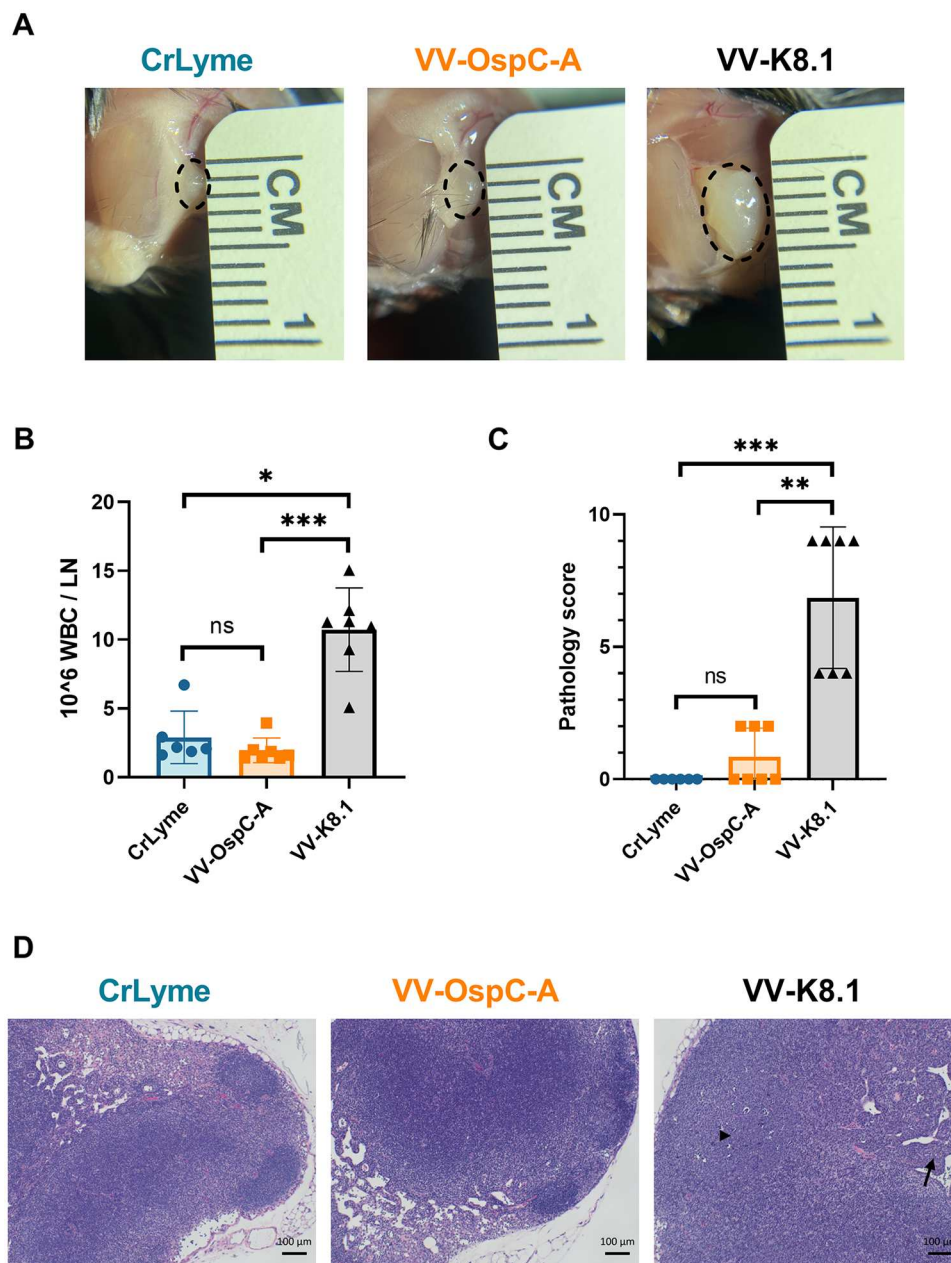
**Figure 2.** Vaccination with VV-OspC-A prevents the development of Lyme carditis. (a) Representative images of H&E stained heart tissue collected from mice vaccinated with CrLyme, VV-OspC-A, or VV-K8.1 14 days post-challenge. Arrowhead indicates inflammation of the aorta wall. Arrow indicates subjacent inflammation in the connective tissue. Scale bar represents 100 µm. (b) Summary of carditis pathology scores as determined by trained histopathologist. Scoring criteria is described in full in the methods section of this article. Error bars represent standard deviation. ns, not significant, \*\*  $p$ -value < 0.01.

necropsy, we observed dramatic enlargement of the axillary lymph nodes in mice vaccinated with the VV-K8.1 control, but not in mice vaccinated with VV-OspC-A or CrLyme (Figure 3a). We then collected the lymph nodes for enumeration of the white blood cells. In line with the visual observations, mice vaccinated with VV-K8.1 had significantly increased white blood cell counts per lymph node that were not observed in the VV-OspC-A and CrLyme vaccinated groups, indicating that these vaccines prevented the development of lymph node hyperplasia (Figure 3b). We also performed histopathology analysis on the axillary lymph nodes. Mice vaccinated with the VV-K8.1 control were found to have moderate-to-severe lymph node pathology characterized by marked follicular hyperplasia with or without severe medullary cord hyperplasia (Figure 3c,d). In contrast, mice vaccinated with VV-OspC-A or CrLyme were equally effectively protected against the development of lymph node hyperplasia. Taken together, these results suggest that the candidate vaccine protects against the development of lymphadenopathy associated with *B. burgdorferi* infection.

#### **VV-OspC-A induces a robust OspC-specific antibody response with Th1-bias**

To shed light on the immunological mechanisms underlying vaccine protection, a second set of mice

were vaccinated on day 0 and day 28 with the same vaccine constructs and were sacrificed on day 56 without challenge, for collection of blood and spleens. Mice were also bled three weeks after each immunization for evaluation of the serum immune response (Figure 4a). The OspC-specific antibody titre in the serum was quantified by enzyme linked immunosorbent assay (ELISA). After prime vaccination, mice vaccinated with VV-OspC-A had significant anti-OspC antibody titres (mean endpoint titre (mEPT) = 293), in comparison to mice vaccinated with VV-K8.1 (mEPT = 32) (Figure 4b). In contrast, mice vaccinated with one dose of CrLyme did not generate significantly higher antibodies than the control (mEPT = 36). Following boost vaccination, the antibody titres for mice vaccinated with CrLyme increased (mEPT = 8663), however, the titres were highly variable with some mice producing very low anti-OspC titres (Figure 4c). Yet, it should be clarified that CrLyme is a subunit vaccine comprised of both OspC epitopes and OspA, while our viral-vectored vaccine carries only full-length OspC. As such, no direct comparison of antibody titres is intended. Nonetheless, the highest antibody titres were seen in the VV-OspC-A group following boost vaccination (mEPT = 34,743), suggesting that the candidate vaccine is highly immunogenic in mice.

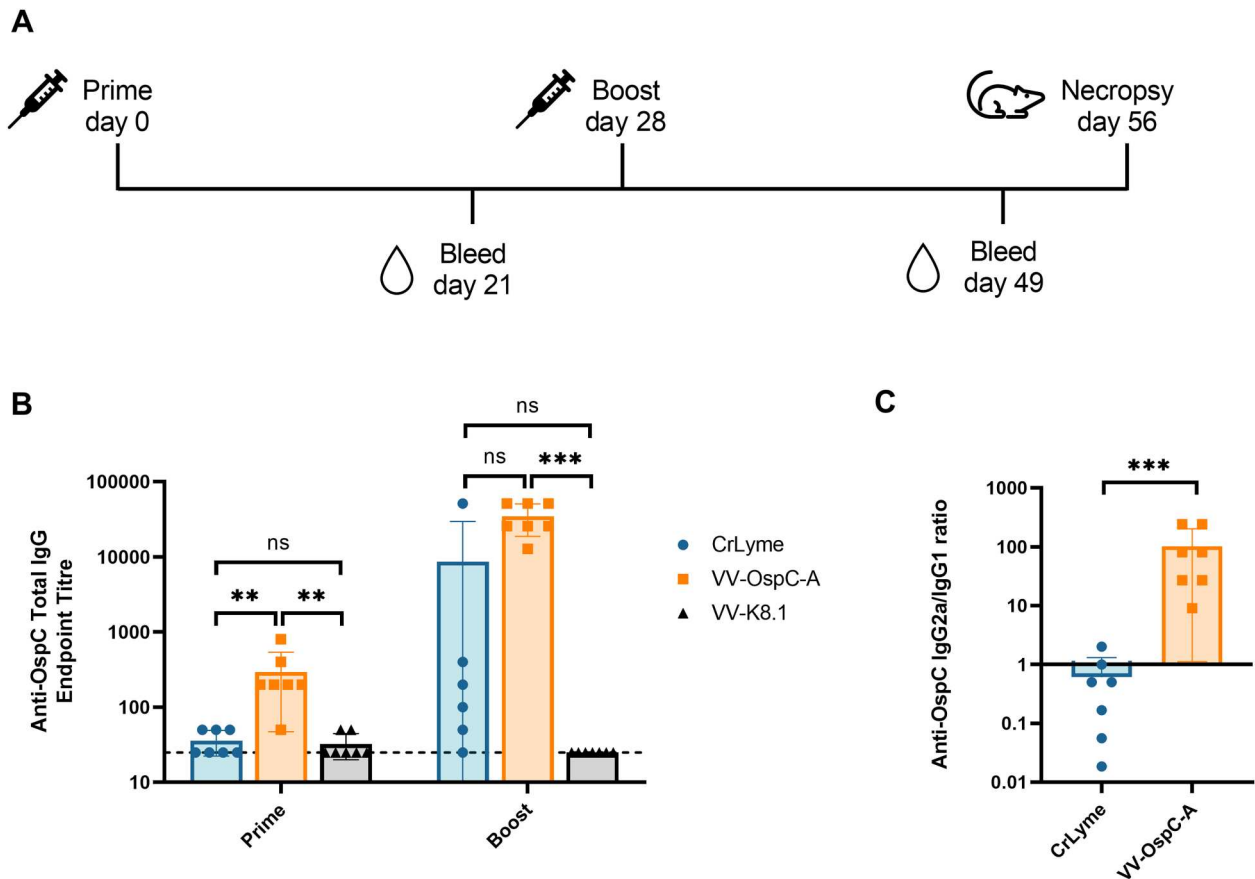


**Figure 3.** Vaccination with VV-OspC-A prevents the development of lymphadenopathy. (a) In situ images of the left axillary lymph node from mice vaccinated with CrLyme, VV-OspC-A, or VV-K8.1 at the time of necropsy (day 70). The node is encircled by a dashed black line. (b) White blood cell (WBC) counts from left axillary lymph node (LN) extracted at necropsy. (c) Summary of combined lymph node hyperplasia scores as determined by a trained histopathologist. Scoring criteria is described in full in the methods section of this article. (d) Representative images of H&E stained lymph node tissue collected from mice vaccinated with CrLyme, VV-OspC-A, or VV-K8.1 14 days post-challenge. Arrowhead indicates marked follicular hyperplasia and arrow indicates severe medullary cord hyperplasia. Scale bar represents 100  $\mu$ m. Error bars represent standard deviation. ns, not significant, \*\*  $p$ -value < 0.01, \*\*\*  $p$ -value < 0.001.

To further characterize the antibody response, the serum levels of OspC-specific IgG subtypes were determined. Interestingly, the CrLyme vaccine elicited significantly more IgG1 than IgG2a antibodies, while the VV-OspC vaccine predominantly elicited IgG2a antibodies (Figure 4d). These results reveal that the two vaccine forms elicit different types of immune responses to OspC; the CrLyme vaccine induces a Th2-biased humoral immune response, whereas the candidate vaccine induces a Th1-biased response.

#### **Antibodies elicited by VV-OspC-A are capable of inducing phagocytosis in vitro**

A major mechanism of *B. burgdorferi* clearance by the host is phagocytosis. Therefore, we developed a novel assay to quantify vaccine-induced antibody-dependent cellular phagocytosis (ADCP) of *B. burgdorferi* *in vitro* using a fluorescently stained bacterial strain that constitutively expresses OspC-A (Figure 5a). Flow cytometry was used to measure the percentage of fluorescent macrophages as an indicator of



**Figure 4.** Vaccination with VV-OspC-A induces a robust antibody response. (a) Schematic depicting the procedure schedule used in the immunogenicity study. Groups of seven C3H/HeN mice were vaccinated subcutaneously with CrLyme or intramuscularly with VV-OspC-A or VV-K8.1 on day 0 and day 28, bled on day 21 and day 49, and sacrificed on day 56. (b) ELISA determination of OspC-specific IgG antibody titres in serum of mice three weeks after prime (day 21) or boost (day 49) injections. Dashed line indicates the limit of detection. Samples pertaining to data points on the dashed line have antibody titres below the limit of detection. (c) Ratio of IgG2a to IgG1 OspC-specific serum antibodies three weeks after boost immunization (day 49). Error bars represent standard deviation. ns, not significant, \*\*  $p$ -value < 0.01, \*\*\*  $p$ -value < 0.001.

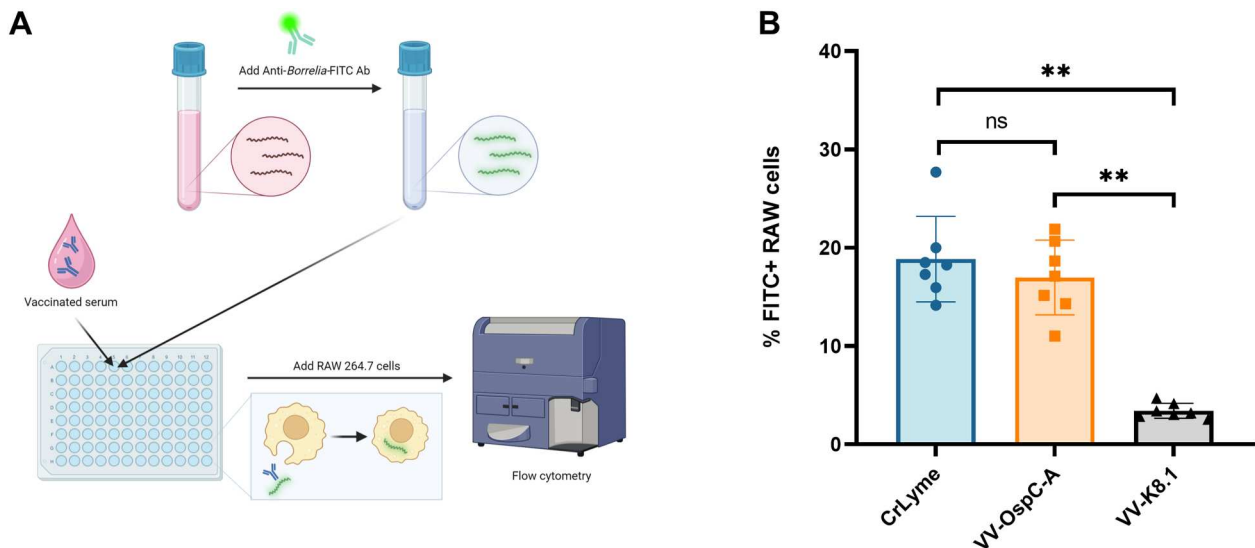
phagocytosis. Serum from mice vaccinated with two doses of VV-K8.1 induced very low levels of phagocytosis activity, while both the CrLyme vaccine and VV-OspC-A vaccine resulted in a remarkable elevation of phagocytic activity (5.5-fold and 5-fold increase compared to VV-K8.1, respectively) (Figure 5b). Taken all together, these results indicate that the VV-OspC-A vaccine is highly immunogenic and elicits functional antibodies capable of inducing antibody-dependent cellular phagocytosis.

#### VV-OspC-A induces a potent Th1-skewed cellular immune responses

Finally, we sought to investigate the cellular immune response to VV-OspC-A vaccination, which has not been well-characterized for vaccines against LD. To this end, we collected splenocytes from vaccinated mice 28 days after boost and stimulated them with an overlapping peptide library of type A OspC. The supernatant of the stimulated splenocytes was then collected for quantification of the secreted cytokines using a multiplex ELISA-like immunoassay.

Vaccination with two doses of VV-OspC-A induced an extensive cellular immune response, significantly increasing production of Th1 cytokines, including IL-2, IFN- $\gamma$ , and TNF- $\alpha$ , and IL-18 (Figure 6). Expression of one Th2 cytokine, IL-13, was also significantly increased compared to the VV-K8.1 control. Vaccination with VV-OspC-A also induced expression of other cytokines that have been implicated in the immune response to extracellular bacterial including, IL-6, IL-10, IL-22, and GM-CSF [37–40]. In comparison, vaccination with the CrLyme vaccine did not lead to a significant increase in the production of any measured cytokine when stimulated with the OspC peptide pool.

We further characterized the antigen-specific T cells responses using flow cytometry. In accordance with the multiplex ELISA results, CD4<sup>+</sup> T cells isolated from mice vaccinated with VV-OspC-A were found to express elevated levels of the Th1-associated cytokines, IFN- $\gamma$  and TNF- $\alpha$ , following stimulation with an OspC peptide library when compared to the VV-K8.1 control (Figure 7a). Significant IFN- $\gamma$  and TNF- $\alpha$  expression was not observed in CD8<sup>+</sup> T cells



**Figure 5.** Vaccination with VV-OspC-A induces antibody-dependent phagocytosis (ADCP) *in vitro*. (a) Schematic depicting the workflow for the ADCP assay created with BioRender.com. See methods section for details. (b) Percentage of RAW 264.7 cells emitting FITC signal following incubation with FITC-stained *B. burgdorferi*-OspC and serum from mice vaccinated with CrLyme, VV-OspC-A, or VV-K8.1. Error bars represent standard deviation. ns, not significant, \*\*\*  $p$ -value < 0.001.

isolated from the VV-OspC-A vaccinated mice (Figure 7b). Additionally, the CrLyme vaccine did not induce significant IFN- $\gamma$  or TNF- $\alpha$  production by both CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells, possibly due to limited OspC epitopes present in the subunit vaccine. Taken together, these results suggest that the candidate VV-OspC-A vaccine induces antigen-specific cellular immune responses in addition to humoral immunity.

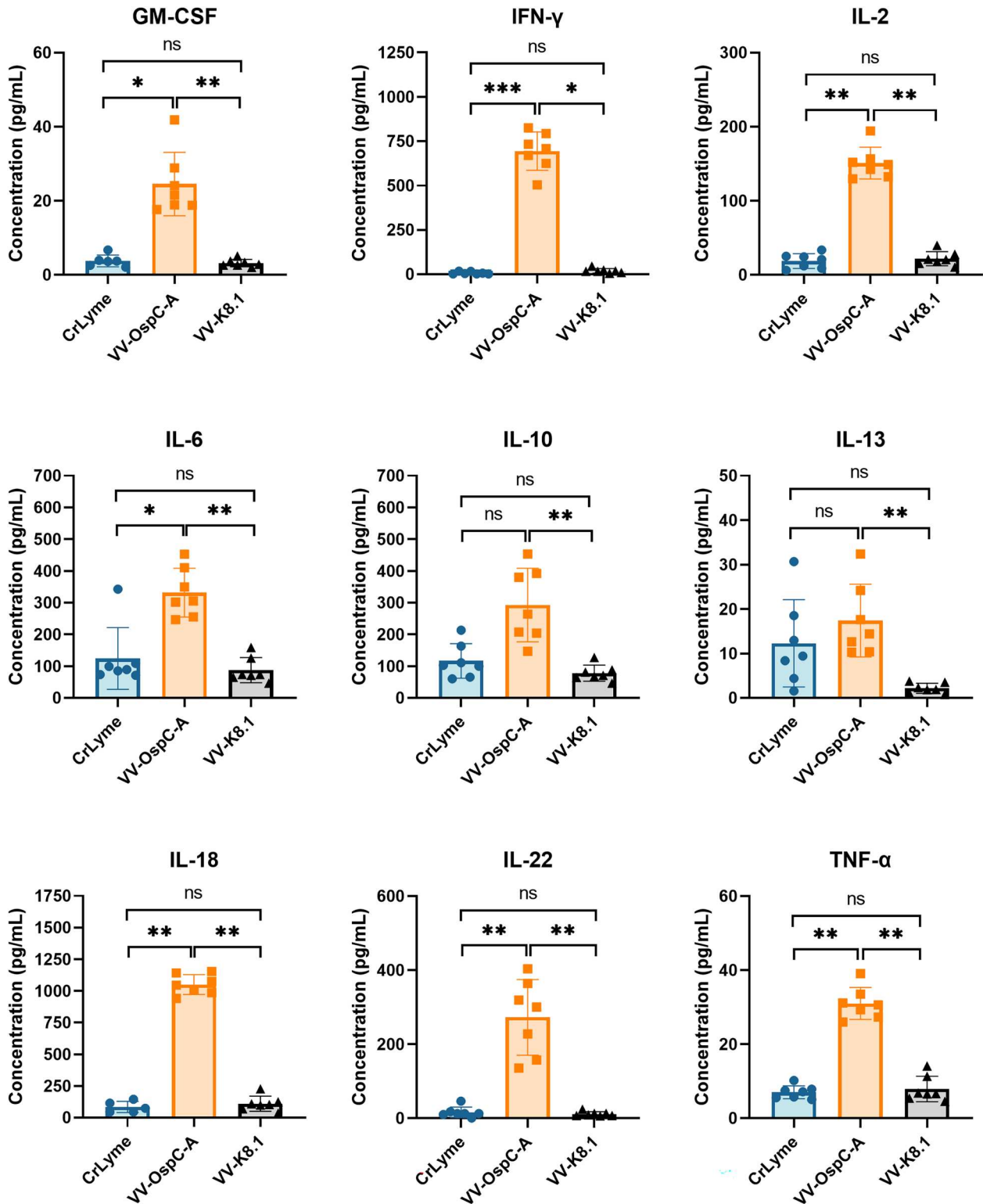
## Discussion

Despite rising rates of LD across North America, there continues to be no vaccine available for use in humans. In addition, the success of antibiotic treatments for LD is hindered by diagnostic challenges, compliance, and post-treatment symptoms [41]. It is therefore imperative to investigate novel LD vaccine strategies. Furthermore, the protective immune responses conferred by LD vaccination have yet to be thoroughly characterized. In this study, we employed a highly immunogenic modified Vaccinia-virus vector for the delivery of a type A OspC gene. Following administration in mice, we evaluated the protection conferred against Lyme disease and characterized the underlying immune responses.

To assess the protection against infection, we employed an attenuated VV-vector developed by our group and compared it to a negative control VV-vector encoding an irrelevant viral antigen (VV-K8.1) and a positive control of a canine vaccine known to afford robust protection in animals (CrLyme). Strikingly, we found that two doses of the candidate VV-OspC-A vaccine afforded complete protection against homologous *B. burgdorferi* needle challenge in mice.

All tissue samples extracted 14 days post-challenge from mice vaccinated with the candidate vaccine were negative for both live *B. burgdorferi* spirochaetes and *B. burgdorferi* DNA (Figure 1). In comparison, the negative control group vaccine displayed high bacterial burden in all evaluated tissues, including the heart and lymph nodes. Accordingly, mice in this group developed severe lymphadenopathy and carditis, which have been associated with *B. burgdorferi* infection in both mice and humans [8,34,36,42,43]. Carditis and lymph node hyperplasia were both significantly reduced in mice vaccinated with VV-OspC-A (Figures 2 and 3). Furthermore, the VV-OspC-A vaccine performed as well as the clinically available CrLyme vaccine at reducing bacterial burden and pathology resulting from challenge with the *B. burgdorferi* strain B31-A3.

In this work, we investigated whether a viral-vectored vaccine is capable of protecting mice against homologous *B. burgdorferi* infection. Although traditional vaccine platforms targeting OspC have demonstrated pre-clinical efficacy, a previous attempt at an OspC-based vaccine employing Newcastle disease virus (NDV)-vector by Xiao *et al.* failed to confer protection in hamsters against a challenge of the same *B. burgdorferi* strain used in our study [44–46]. Intranasal prime and boost inoculations with NDV expressing OspC, with or without a tPA secretion signal, failed to induce an immune response in C3H mice or reduce bacterial burden following challenge in hamsters. In contrast to this study, our candidate vaccine uses an alternative secretion signal, is administered intramuscularly, and provides the boost vaccination at day 28 rather than day 14, which could explain the observed discrepancies between

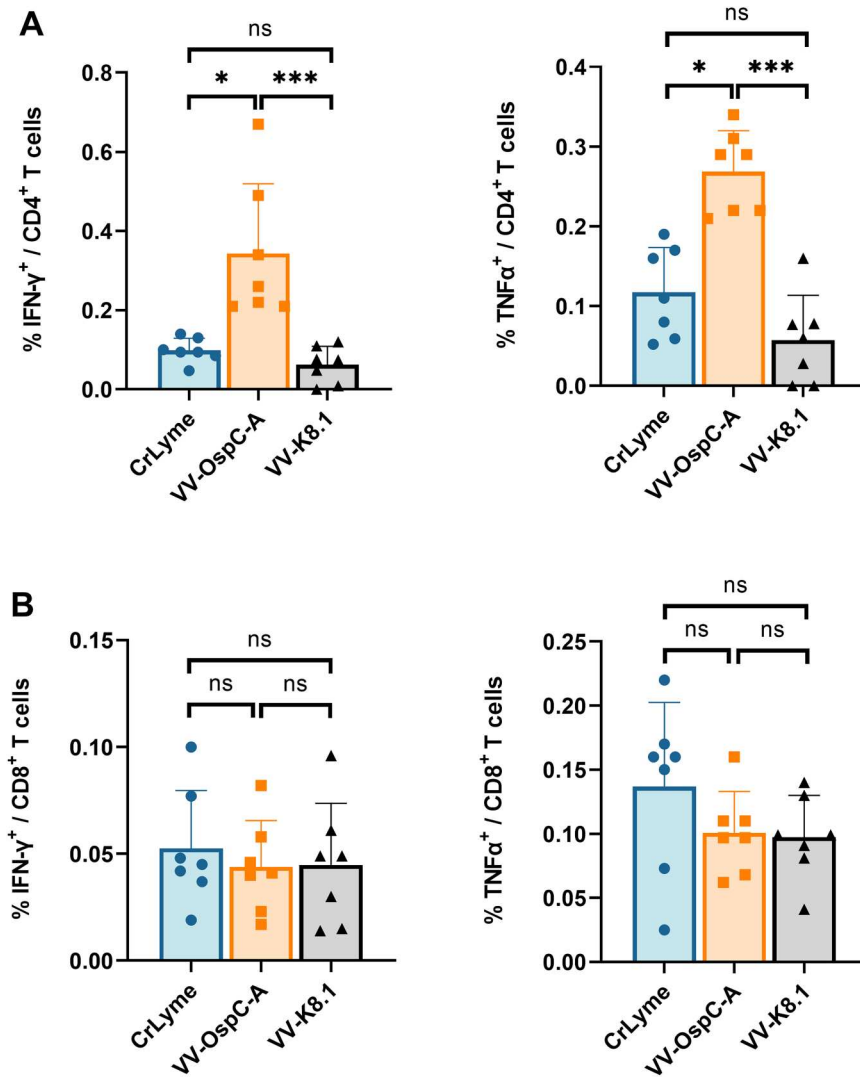


**Figure 6.** Vaccination with VV-OspC-A induces cytokine expression in splenocytes following stimulation with OspC. Splenocytes harvested from mice 28 days after vaccination with CrLyme, VV-OspC-A, or VV-K8.1 were stimulated with an overlapping peptide library of OspC for 24 h. The concentration of each cytokine in the supernatant was determined by ProcartaPlex 17-plex Immunoassay kit. Error bars represent standard deviation. ns, not significant, \*  $p$ -value < 0.05, \*\*  $p$ -value < 0.01, \*\*\*  $p$ -value < 0.001.

the two studies with regards to protection and immunogenicity.

Having observed complete protection conferred by the VV-OspC-A vaccine, we then sought to investigate the underlying immunological mechanisms of protection. Although the mechanistic investigation in this study is not exhaustive, we employed new tools to

analyze antibody effector functions and cellular immune responses along with the well-documented antibody binding assays. Furthermore, while CrLyme and our candidate vaccine equally afforded complete protection, we revealed distinct immunological profiles induced by the two vaccines. Specifically, two doses of VV-OspC-A elicited high levels of anti-



**Figure 7.** VV-OspC-A induces antigen specific CD4 $^+$  T cell responses in mice. Splenocytes harvested from mice 28 days after vaccination with CrLyme, VV-OspC-A, or VV-K8.1 were stimulated with an overlapping peptide library of OspC. The percentage of (a) CD4 $^+$  and (b) CD8 $^+$  T cells producing IFN- $\gamma$  and TNF- $\alpha$  were analyzed by flow cytometry. Error bars represent standard deviation. ns, not significant, \*  $p$ -value < 0.05, \*\*\*  $p$ -value < 0.001.

OspC antibody titres predominated by the IgG2a subtype, indicating a Th1-skewed immune response (Figure 4). In contrast, the OspC-specific antibodies following two doses of CrLyme were predominated by IgG1, indicative of a Th2-skewed response. It is not unexpected that the CrLyme vaccine induced a Th2-biased response since this subunit vaccine contains an alum adjuvant known to stimulate Th2 responses [47–51]. In comparison, viral-vector vaccines, including VV, are known to induce Th1 responses [52–54]. Although it is not well understood whether Th1-biased or Th2-biased responses to are preferred in the context of *B. burgdorferi* clearance, IgG2a antibodies have been implicated in the clearance of extracellular bacteria via Fc receptor binding [55–57].

To assess the cellular immune response to each vaccine, we stimulated splenocytes from vaccinated mice with an OspC overlapping peptide pool and then measured cytokine production by multiplex ELISA

and flow cytometry. Vaccination with CrLyme did not result in elevated levels of any of the 17 cytokines measured by ELISA or significant IFN- $\gamma$ - or TNF- $\alpha$ -producing T cells (Figures 6 and 7). This lack of response may indicate that the L5 and H5 domains included in the OspC portion of the CrLyme vaccine do not include T cell epitopes and therefore protection conferred by CrLyme may be predominated by OspA-targeted immune responses that were not evaluated in this study. In comparison, the VV-OspC-A group had significant cytokine production following OspC stimulation, with especially high levels of the Th1 cytokines, IFN- $\gamma$  and IL-18 (Figure 6). Notably, while VV-OspC-A did result in elevated levels of the Th2 cytokine, IL-13, two major Th2 cytokines, IL-4 and IL-5, were not detected following stimulation (data not shown) [58]. CD4 $^+$  T cells expressing IFN- $\gamma$  or TNF- $\alpha$  were also increased in VV-OspC-A-vaccinated mice following stimulation (Figure 7). These results are in agreement with the Th1-bias observed in the humoral response

and suggest that VV-OspC-A also induces potent Th1-biased cellular immune responses.

Phagocytosis is a key mechanism of *B. burgdorferi* clearance by the mammalian host [59–62]. To our knowledge, the ability of vaccine-induced antibodies to facilitate antibody-dependent cellular phagocytosis (ADCP) of *B. burgdorferi* has never been investigated as a correlate of vaccine-induced protection. In this study, we employed a novel *in vitro* ADCP assay for *B. burgdorferi* using a strain that constitutively expresses OspC *in vitro* and a murine macrophage cell line. Constitutive OspC expression was required to simulate *in vivo* conditions since most *B. burgdorferi* do not reliably express OspC *in vitro*. We found that serum from mice vaccinated with the candidate VV-OspC-A vaccine induced significant phagocytosis at similar levels to the CrLyme vaccine, indicating, for the first time, that the OspC-binding antibodies elicited by vaccination are also capable of inducing ADCP effector functions (Figure 5). It should also be noted that the *B. burgdorferi* constitutive OspC strain also expresses OspA *in vitro* and therefore the ADCP activity observed in the CrLyme group could be mediated by anti-OspA antibodies elicited against the OspA portion of this vaccine, which were not measured in this study [26,27].

Finally, it should be acknowledged that the OspC antigen is genetically diverse. Over 20 different OspC types have been identified with limited-to-no cross-protection between types [63]. Consequently, our intention was not to evaluate the breadth of the vaccine-induced protection. In this study, we vaccinated using only an OspC type A antigen with a genetically homologous type A challenge. While chimeric antigens developed by other groups could likely be adapted to our vaccine platform, it would require further investigation to assess the breadth and duration of protection [33]. Furthermore, other animal species, such as non-human primates, could be employed in future studies to ensure the safety and efficacy of the candidate vaccine. Overall, this report represents a proof-of-concept study using a modified recombinant VV-vector vaccine that affords complete protection against homologous challenge to elucidate the protective immunological mechanisms for LD vaccination.

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## Disclosure statement

No potential conflict of interest was reported by the author(s).

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## Ethics statement

The animal study was reviewed and approved by the Health Canada Institutional Animal Care Committee (Study #2020-011).

## Data availability statement

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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