

# **Evaluation of OspC as a Vaccine Antigen for Lyme Disease**

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# Abstract

Lyme disease (LD), caused by bacteria of the *Borrelia burgdorferi* sensu lato complex, is the most common vector-borne illness in North America. While effective antibiotic treatments are available for LD, 10-20% of patients will develop post-treatment Lyme disease syndrome, significantly reducing their quality of life and emphasizing the need for novel prophylactics. Notably, there is currently no commercially available vaccine for the prevention of LD in humans. Outer surface protein C (OspC), an immunogenic lipoprotein found on the surface of *B. burgdorferi*, is a promising antigen for LD vaccine development as it is highly expressed by the bacteria during early mammalian infection. However, the development of a broadly protective OspC-targeting vaccine is challenged by the vast antigenic variation of OspC, with over 20 different *ospC* major allele groups.

In this thesis, I evaluate the protective efficacy of LD vaccines targeting OspC using a variety of next-generation vaccine platforms in a C3H/HeN mouse model. I demonstrate that monovalent DNA and mRNA vaccines encoding OspC type A delivered by lipid nanoparticles (LNPs) elicit a functional OspC-specific antibody response that confers complete protection against homologous *B. burgdorferi* infection, dissemination, and pathologies. This work highlights the applicability of clinically approved LNP formulations for the delivery of pDNA vaccines in addition to conventional RNA payloads. Furthermore, I show that a modified vaccinia virus-vectored vaccine encoding OspC type A can confer complete protection against homologous *B. burgdorferi* infection comparable to a veterinary LD vaccine through the induction of potent OspC-specific antibody and CD4<sup>+</sup> T cell responses.

Finally, this thesis demonstrates the protective efficacy of a polyvalent mRNA vaccine encoding five common OspC types. The optimal LNP formulation was first evaluated by

comparing the potency, expression kinetics, biodistribution, and immunogenicity of different LNP formulations delivering either DNA or mRNA encoding firefly luciferase. Additionally, the infection profiles of the five corresponding *B. burgdorferi* strains were characterized to identify strain-specific differences before systematic challenge of the pentavalent OspC mRNA vaccine. The candidate mRNA vaccine afforded protection against multiple strains expressing different OspC types and reduced associated pathologies. This study is the first demonstration of multi-strain protection of an OspC vaccine in the absence of outer surface protein A. Overall, this thesis illustrates the efficacy and immune mechanisms of next-generation vaccines targeting OspC and provides a foundation for the rational design of future LD vaccines.

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## Abbreviations

|          |                                                                      |
|----------|----------------------------------------------------------------------|
| ADCP     | antibody-dependent cellular phagocytosis                             |
| AIM      | activation-induced marker                                            |
| ALC-0315 | [(4-Hydroxybutyl)azanediyl]di(hexane-6,1-diyl) bis(2-hexyldecanoate) |
| ALC-0519 | 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide             |
| ANOVA    | analysis of variance                                                 |
| AUC      | area under the curve                                                 |
| Bbsl     | <i>Borrelia burgdorferi</i> sensu lato                               |
| Bbss     | <i>Borrelia burgdorferi</i> sensu stricto                            |
| BCA      | bicinchoninic acid                                                   |
| BCR      | B cell receptor                                                      |
| BGH pA   | bovine growth hormone polyadenylation                                |
| BmpA     | Basic membrane protein A                                             |
| BSK      | Barbour-Stonner-Kelly                                                |
| CDC      | Centers for Disease Control and Prevention (CDC)                     |
| CIHR     | Canadian Institutes of Health Research                               |
| CMV      | cytomegalovirus                                                      |
| COVID-19 | coronavirus disease of 2019                                          |
| cp       | circular plasmid                                                     |
| crOspC   | chimeric recombinant Outer surface protein C                         |
| CSF      | cerebrospinal fluid                                                  |
| DbpA     | Decorin-binding protein A                                            |

|             |                                                                |
|-------------|----------------------------------------------------------------|
| DC          | dendritic cell                                                 |
| DEET        | N,N-diethyl-m-toluamide                                        |
| dLN         | draining lymph node                                            |
| DLS         | dynamic light scattering                                       |
| DMEM        | Dulbecco's modified eagle medium                               |
| DMG-PEG2000 | 1,2-dimyristoyl-rac-glycerol-3-methoxypolyethylene glycol-2000 |
| DOPC        | 1,2-dioleoyl-sn-glycerol-3-phosphocholine                      |
| DOTAP       | 1,2-Dioleoyl-3-trimethylammonium                               |
| DOTMA       | N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride |
| DSPC        | 1,2-Distearoyl-sn-glycerol-3-phosphorylcholine                 |
| EGFP        | enhanced green fluorescent protein                             |
| EIA         | enzyme immunoassay                                             |
| ELISA       | enzyme-linked immunosorbent assay                              |
| FACS        | fluorescence-activated cell sorting                            |
| FBS         | fetal bovine serum                                             |
| FDA         | Food and Drug Administration                                   |
| FITC        | fluorescein isothiocyanate                                     |
| GC          | germinal center                                                |
| GM-CSF      | granulocyte-macrophage colony-stimulating factor               |
| HLA         | human leukocyte antigen                                        |
| hLFA1       | human leukocyte function-associated antigen 1                  |
| HRP         | horseradish peroxidase                                         |

|        |                                                                                                     |
|--------|-----------------------------------------------------------------------------------------------------|
| hTPA   | human tissue plasminogen activator                                                                  |
| hTSS   | human tyrosinase signal sequence                                                                    |
| IACI   | infection-associated chronic illnesses                                                              |
| IFN    | interferon                                                                                          |
| Ig     | immunoglobulin                                                                                      |
| IL     | interleukin                                                                                         |
| IM     | intramuscular                                                                                       |
| INHAND | International Harmonization of Nomenclature and Diagnostic Criteria for<br>Lesions in Rats and Mice |
| IVIS   | in vivo imaging system                                                                              |
| KC2    | DLin-KC2-DMA                                                                                        |
| LC     | Langerhans cell                                                                                     |
| LD     | Lyme disease                                                                                        |
| LDL    | low density lipoprotein                                                                             |
| LN     | lymph node                                                                                          |
| LNP    | lipid nanoparticle                                                                                  |
| LoD    | limit of detection                                                                                  |
| lp     | linear plasmid                                                                                      |
| MAC    | membrane attack complex                                                                             |
| MBP    | maltose-binding protein                                                                             |
| MC3    | DLin-MC3-DMA                                                                                        |
| MCS    | multiple cloning site                                                                               |

|              |                                                       |
|--------------|-------------------------------------------------------|
| mDC          | myeloid dendritic cell                                |
| mEPT         | mean endpoint titre                                   |
| MHC          | major histocompatibility complex                      |
| MLST         | multi-locus sequence typing                           |
| NDV          | Newcastle disease virus                               |
| NIAID        | National Institute of Allergy and Infectious Diseases |
| NTA          | nanoparticle tracking analysis                        |
| OD           | optical density                                       |
| OspA         | Outer surface protein A                               |
| OspB         | Outer surface protein B                               |
| OspC         | Outer surface protein C                               |
| PAMP         | pathogen-associated molecular pattern                 |
| PBS          | phosphate-buffered saline                             |
| PC           | phosphatidylcholine                                   |
| PCR          | polymerase chain reaction                             |
| pDNA         | plasmid DNA                                           |
| PE           | phycoerythrin                                         |
| PEG          | polyethylene glycol                                   |
| PIV5         | parainfluenza virus 5                                 |
| PMA          | phorbol 12-myristate 13-acetate                       |
| PRR          | pattern recognition receptor                          |
| <i>PTLDS</i> | post-treatment Lyme disease syndrome                  |

|          |                                                                               |
|----------|-------------------------------------------------------------------------------|
| qPCR     | quantitative polymerase chain reaction                                        |
| RABV     | rabies virus                                                                  |
| RLU      | relative luminescence units                                                   |
| rOspA    | recombinant Outer surface protein A                                           |
| rOspC    | recombinant Outer surface protein C                                           |
| RST      | ribosomal RNA intergenic spacer type                                          |
| RSV      | respiratory syncytial virus                                                   |
| Salp15   | salivary protein 15                                                           |
| SCID     | severe combined immunodeficiency                                              |
| SD       | standard deviation                                                            |
| SDS      | sodium dodecyl sulfate                                                        |
| SDS-PAGE | sodium dodecyl sulfate–polyacrylamide gel electrophoresis                     |
| SFU      | spot forming unit                                                             |
| SM-102   | heptadecan-9-yl 8-((2-hydroxyethyl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate |
| ST       | serotype                                                                      |
| TBS      | tris-buffered saline                                                          |
| TCR      | T cell receptor                                                               |
| Th       | T helper                                                                      |
| TLR      | Toll-like receptor                                                            |
| TMB      | tetramethylbenzidine                                                          |
| TNF      | tumour necrosis                                                               |

|          |                                        |
|----------|----------------------------------------|
| tPA      | tissue plasminogen activator           |
| TROSPA   | tick receptor for OspA                 |
| TT joint | tibiotarsal joint                      |
| VAERS    | Vaccine Adverse Event Reporting System |
| VEEV     | Venezuelan equine encephalitis virus   |
| VlsE     | variable major protein-like sequence E |
| VRP      | virus replicon particle                |
| VV       | vaccinia virus                         |
| WBC      | white blood cell                       |

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# Chapter 1: Introduction

## 1.1. Lyme disease

### 1.1.1. Epidemiology

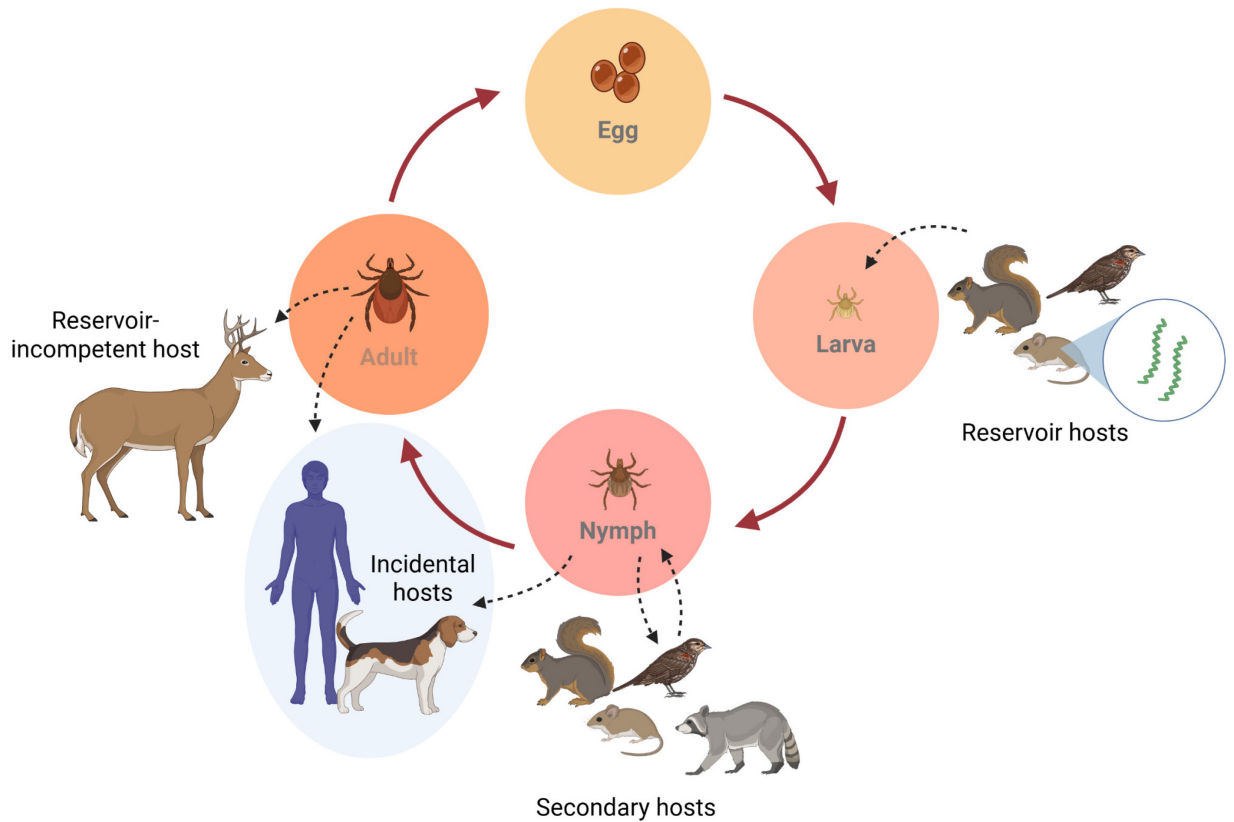
Lyme disease (LD), caused by *Borrelia burgdorferi sensu lato* (Bbsl), is the most common vector-borne illness in North America and Europe. Across Europe, over 120,000 cases are reported each year [1]. In 2023, there were 89,000 reported cases in the United States, however, the true incidence is thought to be much higher, with an estimated 476,000 cases per year [2]. The current reported case numbers represent nearly a 10-fold increase in incidence since 1991, when LD became a national notifiable disease in the United States. In Canada, the number of cases has also increased from 144 cases when monitoring began in 2009 to 5,329 reported cases in 2024 [3]. The dramatic rise in cases is thought to be due, at least in part, to the expansion of associated tick habitats driven by climate change [4–9]. In Canada, current climate change models predict that LD will cost the health care system between 0.5 and 2.0 billion Canadian dollars per year by 2050 [10]. Similarly, in the United States, Lyme disease represents a substantial economic burden, costing the health care system between 700 million and 1.3 billion dollars annually [11,12].

The risk of contracting LD is highly dependent on geographic location and influenced by both the circulating strains of the causative bacteria and the feeding habits of associated tick vectors [8]. In Canada, the majority of infections are contracted in Ontario, Quebec, and Nova Scotia [13]. In the United States, cases are concentrated in the Northeast, Mid-Atlantic, and upper Midwest regions [6]. The age distribution of LD cases is bimodal with peaks in the 5-14 and 55-79 year old groups [13–15]. Individuals who spend time outdoors in wooded and grassy areas are more at risk for contracting LD, especially during periods of high tick activity, such as mid-May

to mid-August for nymphal ticks and March to mid-May or mid-August to November for adult ticks [16–18]. Individual risk can be reduced by wearing protective clothing, using N,N-diethyl-m-toluamide (DEET)-based repellents, and performing “tick checks” to identify and remove ticks from the body within 24-72 hours of attachment [19].

### 1.1.2. Tick transmission

The causative agent of LD, *B. burgdorferi*, is maintained in nature via enzootic cycles involving wildlife vertebrate hosts and ticks in the genus *Ixodes* (Figure 1.1) [20,21]. *Ixodes* ticks consume three necessary blood meals across their 2-4 year life cycle [8]. Ticks hatch from eggs as uninfected larvae in the summer months. Shortly after hatching, the larvae consume their first blood meal on a small mammal or bird. The larvae then molt into nymphs and consume a second blood meal, before molting into their final stage as an adult tick [22,23]. Adult ticks preferentially feed on deer, which are not susceptible to *B. burgdorferi* infection, but are critical for sustaining the tick population [8,24]. Ticks can acquire *B. burgdorferi* during a blood meal from an infected host and transmit it to other wildlife or humans during subsequent feedings. *B. burgdorferi* resides in the tick midgut until the next blood meal, which triggers amplification of *B. burgdorferi*, migration from the tick midgut to the salivary glands, and then transmission into the vertebrate host [8,24–26].



**Figure 1.1** Enzootic cycle of *B. burgdorferi* and associated *I. scapularis* tick vector. Dashed lines indicate transmission of *B. burgdorferi* between tick and vertebrate hosts. Image created using BioRender and adapted from Kurokawa et al. 2020 [24] and Radolf et al. 2012 [27] in accordance with the Creative Commons Attribution 4.0 International License (<https://creativecommons.org/licenses/by/4.0/>).

While many *Ixodes* species can transmit *B. burgdorferi* among wildlife hosts, only four are known to feed on humans: *I. ricinus* in Europe, *I. persulcatus* in Asia, *I. scapularis* in Eastern North America, and *I. pacificus* in Western North America [8]. The nymphal stage is thought to be the most common stage for transmission to human hosts [8,23]. Humans are considered incidental or dead-end hosts as they are not involved in the transmission cycle and do not maintain high levels of bacteria in their tissues [27,28].

During human infection, *B. burgdorferi* is first deposited into the dermis or deep epidermis along with a flood of tick saliva. The saliva contains pharmacologically active compounds that enhance infectivity by inhibiting macrophage function, mast cell activation, and wound healing [29]. Initial infection and the responding inflammation causes the characteristic *erythema migrans*, or “bull’s-eye” rash, and flu-like symptoms [30]. Days to weeks later, *B. burgdorferi* spread throughout the host via the skin, lymphatic ducts, and circulatory system to reach diverse tissues, including the heart, joints, and nervous system [31].

### 1.1.3. *Borrelia burgdorferi*

#### 1.1.3.1. Classification

The Bbsl complex consists of 22 genospecies, 10 of which have been detected in humans [8]. The majority of cases are caused by three of these genospecies: *B. burgdorferi* sensu stricto (Bbss) in North America or *Borrelia garinii* and *Borrelia afzelii* in Europe and Asia [32–34]. All Bbsl genospecies are transmitted by ticks of the genus *Ixodes* and maintained in vertebrate wildlife reservoirs.

Bbsl are spirochete bacteria defined by their helical shape and unique endoflagellum-driven motility [20,35]. They are atypical gram negative bacteria, as their outer membrane does not contain lipopolysaccharide, rather it is composed of phosphatidylcholine, phosphatidylglycerol, cholesteryl-6-O-acyl- $\beta$ -D-galactopyranoside, and cholesteryl- $\beta$ -D-galactopyranoside [27,36]. Bbsl also differs from prototypical bacteria due to their large number and variety of lipoproteins present on the outer surface [27,37]. Different strain typing systems are used to classify Bbsl, including multi-locus sequence typing (MLST), the outer surface protein C gene (*ospC*), and ribosomal RNA intergenic spacer type (RST) [38–43].

### 1.1.3.2. Genetic organization

Bbsl has one of the most complex bacterial genomes characterized to date. It contains a 910 kb linear chromosome complemented by a variable set of approximately 9 linear plasmids (lp) and 12 circular plasmids (cp) [40,44]. Roughly 853 genes are encoded by the linear chromosome, including the majority of housekeeping genes [45]. Bbsl does not possess the genes required for amino acid, nucleotide, or fatty acid synthesis, for which it relies entirely on its host [40,45]. Notably, Bbsl also does not encode any toxins or specialized secretion systems involved in causing disease [40,45,46]. In comparison, 6% of the genes encoded by the chromosome are involved in chemotaxis and motility, as these are critical functions required for the transmission of the spirochete between hosts, as well as dissemination within a host [45,47].

The plasmid complement can vary by strain but typically contributes an additional 600 kb and 485 genes, with plasmids ranging in size from 9 to 62 kb [44]. Genes encoding the surface lipoproteins are present mostly on the plasmids and comprise nearly 8% of the open reading frames [40]. These lipoproteins play a critical role in the enzootic cycle and are differentially expressed throughout the cycle to adapt to the variety of hosts [27,48–50]. A set of “core” plasmids are present in nearly all strains. These plasmids include lp54 and cp26, which encode the virulence factors outer surface protein A (OspA) and outer surface protein C (OspC), respectively, as well as lp36, lp28-4, lp28-3, lp25, and lp17 [51–54].

### 1.1.3.3. Surface proteins

#### 1.1.3.3.1. Outer surface protein A (OspA)

##### *1.1.3.3.1.1. Structure and function*

Outer surface protein A (OspA) is an immunogenic lipoprotein found on the surface of Bbsl. It is encoded by lp54 and is composed of 21 anti-parallel beta-strands and one short alpha-

helix [55]. OspA undergoes post-translational modifications, including cleavage of the N-terminal signal peptide and covalent modification of the N-terminal cysteine residue with lipid moieties in order to anchor OspA to the outer membrane. The central region of OspA forms a flat and extended beta-sheet that separates the two terminal globular domains [55].

OspA binds the tick receptor for OspA (TROSPA) in the midgut to facilitate colonization of the tick vector [56,57]. Accordingly, OspA is highly expressed by spirochetes during residence in the tick midgut. Conversely, OspA expression is downregulated following the tick bloodmeal to facilitate migration of the spirochetes to the tick salivary glands and into the vertebrate host [58,59]. Yang et al. demonstrated that deletion of OspA/B in a *B. burgdorferi* 297 strain did not impact infectivity in mice or resulting pathologies, but abolished tick colonization and survival in the tick midgut [60].

#### *1.1.3.3.1.2. Antigenic variability and immunogenicity*

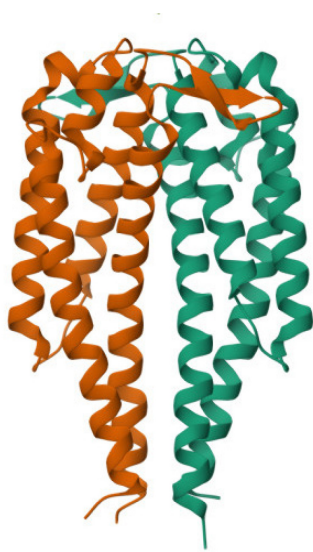
Seven different serotypes of OspA have been described and are referred to as ST1 through ST7. Bbsl genospecies can be categorized by OspA serotype with Bbss categorized as ST1, *B. afzelii* as ST2, *B. garinii* as ST3, ST5, ST6, and ST7, and *B. bavariensis* as ST4 [61]. Within serotypes, the genetic sequence of *ospA* is 98.5-100% similar, while similarity across serotypes can be as low as 75% [62]. The exposed C-terminal domain of OspA, especially amino acid positions 206-230, represents a major source of antigenic variation [63]. Protective conformational epitopes have been identified in the C-terminal domain of OspA and a linear epitope spanning amino acids 221-240 has been identified as surface-exposed, immunogenic, and capable of eliciting bactericidal antibodies [64–68]. Moreover, vaccination with rOspA has been shown to prevent *B. burgdorferi* needle or tick-mediated infection in mice, guinea pigs, canines, and humans [19,69–73]. Protection conferred by rOspA vaccination is most effective against homologous

challenge, with limited cross-serotype protection [72]. Additionally, passive transfer of anti-OspA immune sera also confers protection against infection and pathologies associated with LD [74–76].

### 1.1.3.3.2. Outer surface protein C (OspC)

#### 1.1.3.3.2.1. Structure and function

OspC is another pertinent lipoprotein expressed on the surface of *B. burgdorferi*. The gene for OspC is encoded by cp26 and produces a 22-23 kDa lipoprotein consisting of four long  $\alpha$ -helices (1, 2, 3, and 6), two short  $\alpha$ -helices (4 and 5), and two small  $\beta$ -strands (Figure 1.2) [77–80]. OspC functions as a homodimer, stabilized by interactions between the longest  $\alpha$ -helix of each unit [78]. The N-terminus is triacylated at the amino-proximal cysteine residue, forming a lipid anchor in the outer membrane. Additionally, an 18 amino acid signal sequence is removed during processing before lipidation occurs [78].



**Figure 1.2** Crystal structure of the OspC dimer. Monomers are depicted in orange and green. Figure obtained from Protein Data Bank 1GGQ ([doi.org/10.2210/pdb1GGQ/pdb](https://doi.org/10.2210/pdb1GGQ/pdb)) and deposited by Dunn, J.J., Lawson, C.L. in accordance with the Creative Commons Attribution 4.0 International License (<https://creativecommons.org/licenses/by/4.0/>).

Expression of OspC is regulated by temperature, biochemical changes, and bacterial density [58,59,81–83]. Transcription and translation of OspC are induced between 32°C and 37°C but not at lower temperatures, such as 24°C [84,85]. Expression of OspC is upregulated during tick feeding to promote migration into the tick salivary glands and transmission into the host [86]. OspC is required for early mammalian infection and serves several functions [87,88]. It binds host plasminogen, a precursor to the serine protease plasmin, which conducts fibrinolysis and extracellular matrix degradation. It is thought that OspC immobilizes plasminogen on the spirochete surface to facilitate tissue breakdown and invasion [89]. Additionally, OspC binds to tick salivary protein 15 (Salp15), masking the bacteria from complement- and antibody-mediated killing [90]. OspC has also been shown to block phagocytosis by macrophages in the skin and reduce uptake of spirochetes by murine and human macrophages *in vitro* [91]. Lastly, OspC expression is downregulated approximately two weeks after infection to prevent detection by the host adaptive immune response [81,87,92,93].

#### *1.1.3.3.2.2. Antigenic variability*

OspC is highly polymorphic, with over 20 different *ospC* major allele groups, referred to as OspC types [94–96]. Major groups possess less than 1% sequence variation, while variation across groups is approximately 20% [95]. Even within a single geographic region, OspC types can exhibit as much diversity as that observed across the entire species [95,97]. Much of this variation is located within surface-exposed loop domains that are highly susceptible to immune pressure [78,80]. Cross-protection between OspC types is limited; infected individuals may be protected against infection with strains expressing the same OspC type, but remain susceptible to heterologous OspC types [98–100]. Similarly, recombinant OspC vaccination only affords protection against homologous strains expressing the same OspC type [70,99–101]. Additionally,



a high percentage of infected ticks contain more than one OspC type [97,102–104]. The most prevalent OspC types vary significantly by region. In the Northeastern United States and Canada, the most common OspC types detected in *I. scapularis* ticks are A, K, B, C and N, whereas, in the Midwest United States and West Coast, type H is highly prevalent [97,105,106]. At least 12 different OspC types have been detected in disseminated human infections, including A, B, C, D, E, F, G, H, I, K, M, and N [107]. The most prevalent OspC types detected in human LD infections are A, I, K, and N. In fact, approximately 60% of infections in the Northeastern United States are caused by OspC type A or K, and 90% of cases are caused by just seven types: A, B, F, H, I, K, and N [108].

#### *1.1.3.3.2.3. Immunogenicity*

Since OspA is not highly expressed on the bacterial surface during vertebrate infection, individuals and canines with LD and experimentally infected mice typically mount an antibody response predominantly targeting OspC, rather than OspA [109–113]. Passive transfer of OspC antibodies has been shown to protect against *B. burgdorferi* needle and tick infection and reduce associated pathologies, such as arthritis and carditis [76,100,114,115]. Some mechanisms of OspC antibody-mediated protection have been elucidated, including *in vitro* complement-dependent and complement-independent borreliacidal activity [116–121]. Furthermore, while it was previously thought that OspC antibodies only act on *B. burgdorferi* during vertebrate host infection, recent research suggests that anti-OspC antibodies can also neutralize homologous *B. burgdorferi* in the tick midgut and prevent transmission without direct borreliacidal activity [98]. Further research is required to better characterize the full range of OspC-specific antibody effector functions.

Several immunodominant epitopes of OspC have been proposed. Screening of recombinant OspC type A fragments using sera from *B. burgdorferi* B31MI infected individuals identified two

highly immunogenic linear epitopes, H5, located in the fifth  $\alpha$ -helix between residues 168 and 203, and L5, located in the third  $\alpha$ -helix and fifth loop between residues 136 and 150 [94]. The L5 epitope is thought to be surface-exposed and located in the putative ligand binding domain of the OspC dimer. Both the L5 and H5 genetic sequences are highly variable across OspC types but well-conserved within each major group. Other studies have identified L5 as the binding site for the borreliacidal monoclonal antibodies, 8C1 and 16.22, and as a highly recognized region by human immune sera [122–124]. An approved veterinary LD vaccine, Vanguard® crLyme, targets the L5 and H5 epitopes of multiple OspC types, in conjunction with OspA, to afford protection against *B. burgdorferi* infection [125].

Phage display screening of OspC fragments using sera from infected patients has identified an additional six B cell epitopes, three of which are conserved across *B. burgdorferi* genospecies. One epitope, spanning residues 19-27, includes the immutable cysteine residue required for dimerization [126]. These epitopes were developed into chimeric proteins that demonstrated moderate sensitivity in the diagnosis of LD, but have not yet been evaluated for vaccination [127].

Finally, the importance of immunodominant epitopes found in the N- and C-termini of OspC remains unclear. One protective monoclonal antibody, B5, was thought to bind a conformational epitope involving both N- and C-terminal residues [128,129]. This binding site was recently elucidated as  $\alpha$ -helix 1 and  $\alpha$ -helix 6 with additional interactions at the loop between  $\alpha$ -helices 5 and 6 [79]. Borreliacidal immunoglobulin M (IgM) antibodies in sera of infected and vaccinated individuals have also been shown to bind N- and C-terminal epitopes of OspC [130–132]. In contrast, Earnhart et al. demonstrated that deletion of the 10 most C-terminal (C10) amino acids or extended regions of the N-terminus did not reduce binding of immune sera to OspC [94]. They later confirmed that the C10 region does not contribute to protective immune responses or

borreliacidal activity [133], suggesting that future research efforts should focus on alternative immunodominant epitopes.

#### 1.1.3.3.3. Other surface proteins

Several other *B. burgdorferi* surface proteins have been explored as potential targets for LD vaccine development and therapeutics, including BB0405, BB0172, BBI39, BBI36, CspZ, Outer surface protein B (OspB), FtlA/B, Decorin-binding protein A (DbpA), BBK32, and Basic membrane protein A (BmpA) [134–141]. Of particular note is VlsE, which is highly expressed on the spirochete surface for the duration of infection [40,92]. VlsE is encoded by lp28-1 and contains approximately 15 silent cassettes upstream of the gene locus. The DNA sequence of VlsE undergoes homologous recombination with the upstream cassettes to continually exchange the exposed amino acids and prevent host immune detection [142,143]. This ongoing recombination presents significant challenges to interventions targeting VlsE and has significantly limited any further investigation of VlsE as a vaccine target.

#### 1.1.4. Diagnosis, progression, and treatment

Diagnosis of early localized LD, occurring 3-30 days post-infection, relies on a combination of factors, including tick exposure, clinical symptoms, and laboratory testing [144]. Tick exposure can include a recent tick bite or time spent in a high risk area. Early clinical signs of LD used for diagnosis include flu-like symptoms, lymphadenopathy, and the pathognomonic *erythema migrans* rash [145]. When a tick bite has occurred without accompanying *erythema migrans* rash, laboratory testing is recommended. Diagnostic testing consists of a human combined enzyme immunoassay assay (EIA) to screen for immunoglobulin G (IgG) and IgM antibodies, followed by confirmatory testing, such as polymerase chain reaction (PCR) detection of *B. burgdorferi* DNA, a second IgG and IgM combined EIA, or an IgG and/or IgM immunoblot

[144,146]. Following diagnosis, the recommended treatment for early localized LD is 10-21 days of doxycycline antibiotics. This course of treatment is reported to be 90% effective in patients with *erythema migrans* [146–149]. Additionally, if administered within 72 h of a tick bite, a single dose of doxycycline can reduce the development of LD by 87% [150].

Diagnosis and treatment of LD is hindered by several factors. First, the majority of individuals who develop *erythema migrans* are unaware that they have been bitten by a tick [151]. Additionally, erythema migrans only occurs in 70-80% of LD cases and may go unnoticed in other cases [151,152]. Furthermore, the two-tiered diagnostic testing method is thought to have an accuracy of only 50-60% during early infection due to low bacterial burdens and high strain variation [153–155]. Finally, many cases of LD are initially misdiagnosed due to the non-specific nature of the accompanying symptoms [156,157].

If left untreated, patients can develop early disseminated LD (< 3 months post-infection) as the bacteria spread via the skin, lymphatic system, and circulation to cause multi-system inflammation [158]. The resulting tissue damage can yield a wide array of clinical symptoms, including cognitive difficulties, meningitis, neuropathy, fatigue, and Lyme carditis that most commonly presents as atrioventricular block. If infection persists for over three months, severe manifestations of late disseminated LD can include arthritis, Baker's cyst, myelitis, encephalopathy, radiculopathy, and in rare cases, diaphragmatic paralysis [144,159–162].

### 1.1.5. Post-treatment Lyme disease syndrome (PTLDS)

Post-treatment Lyme disease syndrome (PTLDS) is defined as experiencing persistent disabling symptoms for at least six months after standard treatment of diagnosed Lyme disease [163]. It is a more specific classification of the previously termed “chronic Lyme disease”, which did not require previous diagnosis with *B. burgdorferi* infection, and is not a recognized diagnosis

by major Canadian and American medical authorities [164]. More recently, the term Lyme infection-associated chronic illnesses (Lyme IACI) has been introduced as a general term that includes PTLDS, persistent Lyme disease, and long Lyme, in an effort to relate PTLDS to other infection-associated chronic illnesses, such as long COVID [165]. The most common symptoms of PTLDS include fatigue, headache, musculoskeletal pain, cognitive dysfunction, and difficulty sleeping [163]. Rate estimates suggest that between 10% and 20% of LD patients will develop PTLDS [166]. Furthermore, it is estimated that currently 2 million people are experiencing PTLDS, which is associated with a significant reduction in quality of life [153,167].

The etiology of PTLDS remains poorly understood. Several causes have been suggested, which can be divided into two major categories: 1) persistent bacterial infection or components and 2) aberrant host immune responses. First, it has been suggested that PTLDS may be caused by hidden reservoirs of *B. burgdorferi* or residual bacterial components following treatment. This hypothesis is supported by the fact that PTLDS symptoms are correlated with more severe disseminated disease at presentation and delayed antibiotic treatment [168,169]. Additionally, *B. burgdorferi* has been shown to enter a persister state under antibiotic treatment that is similar to biofilm formation [170–172]. Counter to this theory, *B. burgdorferi* DNA was not found in the joints of patients with post-treatment Lyme arthritis despite being detected prior to antibiotic treatment [173]. However, other studies suggest that chronic arthritis may be due to prolonged peptidoglycan exposure in the joints [164,174].

As an alternative theory, some groups have suggested that PTLDS is caused by persistent host immune responses following infection despite complete clearance of the bacteria. Continued symptoms may be due to immune-mediated sequelae or the development of cross-reactive autoimmunity. In support of this theory, several aspects of humoral and cell-mediated immune

responses have been correlated with PTLDS symptoms. For example, treatment-resistant chronic Lyme arthritis is associated with an increased frequency of the human leukocyte antigen (HLA)-DR4 and HLA-DR2 specificities [175]. Additionally, significant levels of anti-OspA and anti-OspB antibodies, as well as OspA-reactive T cells, have been identified in these patients but not in treatment-responsive groups [176,177]. Recent research has also identified fewer circulating CXCR5<sup>+</sup> CD4<sup>+</sup> naïve T cells and more CXCR3<sup>+</sup> CCR4<sup>-</sup> CCR6<sup>-</sup> CD8<sup>+</sup> T cells in PTLDS cohorts compared to healthy controls [178]. Taken together, these results suggest that an aberrant T cell or antibody response may be implicated in the etiology of PTLDS, however, further research is required to establish a causal link and better understand the factors that contribute to infection-associated chronic illnesses in general.

Currently, there is no approved treatment for PTLDS. There is no evidence supporting the use of prolonged antibiotic treatment for PTLDS, especially considering the accompanying side effects, including multiple cases of fatal secondary infections [179–181]. A meta-analysis of PTLDS treatments has only found evidence to support that Kundalini yoga may effectively improve symptoms of PTLDS with limited adverse reactions [182,183]. As such, further research is certainly needed to better understand the etiology of PTLDS and develop effective treatment strategies.

### 1.1.6. Host immune response to *B. burgdorferi*

#### 1.1.6.1. Innate immunity

The innate immune response to *B. burgdorferi* infection involves a complex network of innate immune cells, pathogen recognition receptors (PRRs), and soluble mediators working in tandem to limit bacterial loads. Spirochetes are initially deposited into the skin where the first immune cells they encounter are Langerhans cells (LCs), a specialized type of dendritic cell (DC)

found at the epidermal/dermal interface [184]. LCs act as a critical mediator between the innate and adaptive immune system, first sensing the spirochetes in a Toll-like receptor 2 (TLR2)-dependent manner and then upregulating MHC class II and migrating to the draining lymph nodes to perform antigen presentation [185]. Notably, adoptive transfer of LCs exposed to *B. burgdorferi* elicits an antibody response in naive mice that protects against subsequent tick challenge [186].

As infection progresses, the spirochetes encounter phagocytic cells, such as tissue-resident macrophages and DCs, as well as neutrophils that infiltrate the skin within four hours of initial infection [187]. Macrophages, DCs, and neutrophils efficiently phagocytose *B. burgdorferi* in vitro and this phagocytic activity can be enhanced by opsonization of spirochetes via complement and/or anti-*B. burgdorferi* antibodies [188–194]. These cells are essential to reduce the amount of initial bacterial infection in the skin but the response is transient and neutrophil infiltration does not persist as the infection progresses [185,187]. Furthermore, tick saliva contains protease inhibitors that impair neutrophil activity [195,196]. Phagocytosis of *B. burgdorferi* ultimately elicits a pro-inflammatory response, including the release of cytokines, chemokines, leukotrienes, and eicosanoids, that promotes *B. burgdorferi* clearance but simultaneously enhances pathological tissue inflammation [185,197–200].

Extracellular pathogens, including *B. burgdorferi*, are primarily recognized by PRRs on the surfaces of tissue-resident immune cells. Both LC and macrophage activation occurs through TLR2 recognition of *B. burgdorferi* outer membrane lipoproteins, which act as the primary pathogen-associated molecular patterns (PAMPs) [201–203]. TLR1/TLR2 heterodimers are also particularly suited to recognize the triacylated lipoproteins found on the spirochete surface [185,204]. In prototypical extracellular infection, TLR5 plays a critical role in sensing bacterial flagella, however the spirochete periplasmic flagellum is largely shielded from immune detection

[205]. Macrophages and endothelial cells are also activated by CD14, a PRR that has been shown to directly bind OspA and OspC [206,207]. CD14-mediated signaling enhances phagocytosis and neutrophil activity, ultimately reducing bacterial burden in a variety of tissues and reducing pathologies associated with *B. burgdorferi* infection [185,208].

Finally, *B. burgdorferi* can activate the complement system, a group of proteins that are critical for the control of extracellular pathogens, via all three pathways: classical (antibody-mediated), mannose-binding lectin, and alternative (C3-mediated). However, *B. burgdorferi* produces many proteins, including OspC, that bind regulators of the complement system to prevent immune activation [185,209,210]. Consequently, in the absence of spirochete-specific antibodies, *B. burgdorferi* is largely resistant to complement-mediated lysis [185,211–213]. The complement pathway primarily controls *B. burgdorferi* infection by acting as an opsonin to promote phagocytosis, rather than via formation of the membrane attack complex (MAC) [211]. Together, these innate immune mechanisms provide an essential first line of defense against *B. burgdorferi* and shape the ensuing adaptive immune response.

#### 1.1.6.2. Adaptive immunity

The human adaptive immune response to *B. burgdorferi* is capable of limiting, but not resolving, ongoing infection and can provide protection against reinfection with homologous strains. In mice lacking T and B cells, *B. burgdorferi* infection is lethal due to uncontrolled bacterial proliferation in the blood and tissues [214,215]. The B cell and antibody responses are thought to be the most critical aspects of the adaptive immune response to *B. burgdorferi*, as B cells are required for preventing and resolving inflammatory pathologies associated with infection and limiting spirochete burdens [216]. Passive transfer of immune sera containing anti-*B. Burgdorferi* antibodies is sufficient to protect naive mice from infection with homologous strains



and, in some cases, resolve ongoing infection [76,217,218]. Antibodies generated against *B. burgdorferi* are bactericidal and can lyse spirochetes *in vitro* through both complement-dependent and -independent mechanisms [116,118–121,219–221]. *B. Burgdorferi*-specific antibodies can also act as opsonins to engage FcγR receptors on macrophages and neutrophils, inducing antibody-dependent cellular phagocytosis [222,223]. Although OspC is a highly immunogenic surface lipoprotein, its expression is rapidly downregulated, limiting the efficacy of an anti-OspC antibody response [81,87,92,93,224]. VlsE expression is then upregulated by the spirochetes and elicits a strong antibody response from the host. However, the continual recombination of the VlsE locus generates substantial antigenic variation that renders anti-VlsE antibodies ineffectual [92,142,143].

The humoral immune response to *B. burgdorferi* is also uniquely characterized by persistent T cell-independent IgM antibody production as a result of impaired class-switching [185,225]. IgM is induced within 7 days of infection and plays a critical role in controlling bacteremia. However, due to its large size, anti-*B. burgdorferi* IgM antibodies cannot effectively diffuse from the bloodstream into the tissue to reduce bacterial burdens [225–227]. Additionally, IgG responses begin as early as 10 days after infection and IgA responses can be detected during early infection of patients presenting with *erythema migrans* [185,227,228]. The antibody response is a critical aspect of adaptive immunity to *B. burgdorferi*, however, its efficacy is limited by the immune evasion strategies of the spirochetes.

*B. burgdorferi* infection impairs germinal center (GC) responses in the host. GC formation does not occur until 15 days after infection and only persists until day 30 [229]. Additionally, *B. burgdorferi* directly infect the lymph nodes, causing extensive B cell proliferation in extrafollicular foci, where B cells differentiate into plasmablasts in the absence of T cells [158,230,231].

Although a large quantity of anti-spirochete antibodies are produced, the B cells do not undergo affinity maturation or differentiation into long-lived plasma cells or memory B cells [185,232,233]. These findings highlight a deficit in long-lived immune responses to *B. burgdorferi* infection.

The role of T cells in controlling *B. burgdorferi* infection remains unclear. Adoptive transfer of T cells from infected donor mice in the absence of B cells does not prevent infection in naive recipient mice [234]. However, adoptive transfer of T helper 1 (Th1) cells into T cell deficient mice reduces carditis [235]. Additionally, T cell deficient mice demonstrate increased spirochete burden and arthritis [232,236–238]. While Th17 cells typically mediate immune responses to extracellular bacterial infections and enhance the recruitment of neutrophils to infected tissues, IL-17 deficient mice do not display increased disease severity or impaired antibody responses in the context of *B. burgdorferi* infection [239]. Th2 responses are thought to help resolve inflammation later in infection but are not beneficial for acute pathogen clearance and have been correlated with reduced anti-*Borrelia* IgG2a levels [240,241]. Finally, CD8<sup>+</sup> T cells are generally not considered a critical aspect of the immune response to an extracellular bacterial infection. In fact, IFN- $\gamma$  production by CD8<sup>+</sup> T cells has been associated with greater severity of arthritis during *B. burgdorferi* infection [242].

## 1.2. Lyme disease vaccines

Many different vaccine platforms have been considered and employed for the development of LD vaccines. These platforms range from traditional methods, such as whole-cell and recombinant protein vaccines, to “next-generation” modern vaccine technologies, including viral-vector, DNA, and mRNA vaccines. Each of these platforms is associated with distinct advantages and limitations that must be carefully considered to best design a safe, broadly-protective, and long-lasting LD vaccine.

First, protein vaccines are a well-established and widely adopted vaccine platform. This approach administers peptides or proteins as vaccine antigens that are generated using recombinant cell-based expression systems. They are generally considered one of the safest vaccine types and have been successfully used in the clinic for the prevention of hepatitis B, shingles, and influenza [243–245]. A major limitation of protein vaccines is that they require time-consuming and costly manufacturing processes. Additionally, they have relatively low immunogenicity compared to whole-organism inactivated or attenuated vaccines that contain endogenous immunostimulatory molecules [246]. Protein vaccines consequently require the addition of adjuvants and/or repeated doses, which may increase the likelihood of experiencing adverse reactions.

Second, viral vector vaccines consist of a modified live virus engineered to express a recombinant target antigen. The viral vector infects host cells to directly deliver the target gene. To improve the safety of these vaccines, the vector is typically genetically modified to be non-pathogenic and replication-deficient. Still, the virus retains its intrinsic PAMPs that stimulate the innate immune system and induce robust cellular and humoral adaptive immune responses [247,248]. Therefore, viral vectors typically do not require the addition of exogenous adjuvants. Additionally, common viral vectors - such as Newcastle disease virus, adenoviruses, and poxviruses - have the capacity to incorporate large quantities of heterologous genetic material [248,249]. Several viral vector vaccines have been approved clinically, including the Oxford–AstraZeneca and Johnson & Johnson COVID-19 vaccines [250,251]. Limitations of viral vectors include pre-existing vector immunity that may impair vaccine immunogenicity, complex manufacturing processes, and cold chain storage and transport requirements [248,249]. Furthermore, vaccine-induced immune thrombotic thrombocytopenia is a rare but serious adverse effect that occurred in 1 case per 26,000 to 67,000 individuals receiving their first dose of the

AstraZeneca–Oxford adenovirus-based COVID-19 vaccine [252]. It is therefore essential that in-depth safety testing is performed for new viral vectors.

Third, DNA vaccination involves the delivery of DNA directly into host cells, often in the form of a plasmid engineered to encode a target antigen. Plasmid DNA (pDNA) vaccines offer several advantages over other vaccine platforms including that they are non-infectious, offer a good safety profile, and are stable at a wide range of temperatures [253,254]. Perhaps the greatest advantage of DNA vaccines is the ease of production, with a relatively low cost of manufacturing and simple purification process compared to viral vector or protein-based vaccines [254]. DNA vaccines can also be designed to encode multiple antigens for multivalent protection or to co-express adjuvants, such as cytokines, for enhanced immunogenicity [255–258]. Furthermore, DNA vaccines have been shown to induce both cellular and humoral immunity [253,254]. For most of their development, DNA vaccines have been largely limited by poor cellular uptake and protein expression, as well as degradation by nucleases. To circumvent these challenges, several delivery mechanisms have been employed, with the most popular techniques including electroporation, gene guns, and intradermal tattooing, however, these administration methods are not easily translated into the clinic [253]. Additionally, DNA-based vaccination has previously raised concerns of theoretical genetic integration, however, to-date, no evidence of integration has been detected when administering pDNA vaccines [259–261]. In 2021, a DNA vaccine administered intradermally by a needle-free jet injector, ZyCoV-D, was approved in India for the prevention of COVID-19 [262]. DNA vaccines represent a promising next-generation vaccine technology with substantial potential for clinical deployment and considerable opportunity for optimization.

Finally, mRNA vaccines have achieved unprecedented global success for the prevention of infectious disease since they were first approved for use during the COVID-19 pandemic. Over 100 million doses of mRNA vaccines have been administered in Canada alone since December 2020 [263]. mRNA vaccines are produced by *in vitro* transcription of mRNA encoding an antigen of interest, followed by encapsulation in an LNP to prevent nucleic acid degradation and facilitate entry into the host cells. They are rapidly synthesized at large-scales, highly immunogenic, and have an excellent safety profile [264,265]. The major limitation of mRNA vaccines is the need for ultra-cold storage conditions, which especially hinders delivery to remote locations [266]. Nevertheless, mRNA vaccines have revolutionized modern vaccinology and several mRNA vaccines are currently undergoing clinical and preclinical testing for a wide variety of viral and bacterial diseases.

### 1.2.1. Human clinical vaccines

#### 1.2.1.1. LYMERix™

Currently, only one vaccine is approved by Health Canada and the United States Food and Drug Administration (FDA) for the prevention of LD in humans. LYMERix™, a chemically lipidated full length recombinant OspA ST1 protein vaccine adjuvanted with aluminum hydroxide, was approved for use in 1998 following a successful Phase 3 clinical trial [267]. Two intramuscular injections one month apart demonstrated 49% efficacy in preventing infection. A third dose administered one year later improved the efficacy to 76%, however the duration of protection remains unknown. LYMERix™ was determined to have an acceptable safety profile with the most common adverse reactions including mild-to-moderate local and systemic reactions that lasted 3-7 days [267]. Consequently, LYMERix™ was recommended for persons between the ages of 15 and 70 living in endemic regions with frequent tick exposure [19,268].

Media reports following the approval of LYMERix™ were initially positive. However, as more doses were administered, a variety of musculoskeletal complaints, including arthritis, were presented by individuals who received the vaccine, eventually leading to a class-action lawsuit against the manufacturer. The FDA then re-examined the results of the Phase 3 clinical trial, which followed patients for one year after vaccination. When comparing the LYMERix™ recipients to the placebo controls, only expected local and systemic adverse reactions – such as soreness, swelling, and myalgias within 48 hours and lasting an average of 3 days – were significantly increased. Importantly, no difference in joint issues were detected between recipients and controls [19,268]. A Center for Disease Control (CDC) review of the Vaccine Adverse Event Reporting System (VAERS) also did not find an increased incidence of arthritis in vaccinated individuals compared to the unvaccinated population and consequently, the FDA never withdrew their approval of the LYMERix™ vaccine [269]. However, amidst declining sales and costly lawsuits, GlaxoSmithKline voluntarily removed LYMERix™ from the market in 2002.

A concern that persisted regarding LYMERix™ and other OspA-based vaccines was potential cross-reactivity between the OspA<sub>165–173</sub> peptide and the highly similar human leukocyte function-associated antigen (LFA)-1 $\alpha$ <sub>L332–340</sub> peptide. This LFA epitope was shown to strongly bind the DRB1\*0401 HLA major histocompatibility complex class (MHC) II receptor that is associated with treatment resistant Lyme arthritis [176,270]. T cells from patients with treatment-resistant Lyme arthritis patients that responded to OspA peptide stimulation also produced IFN- $\gamma$  when stimulated with the LFA peptide of interest [270]. These findings gave rise to potential autoimmune concerns for genetically susceptible individuals but were not sufficient to suggest that LYMERix™ and other OspA vaccines cause arthritis or other autoimmune concerns. Notably, the DRB1\*0401 allele is also associated with rheumatoid arthritis unrelated to *B. burgdorferi* infection

[268]. In addition to these concerns, the LYMERix™ vaccine was limited by its low efficacy after two doses, probable need for annual boosters, and lack of testing in children, highlighting the need for improved LD vaccine strategies [19].

#### 1.2.1.2. VLA15

VLA15 is a recombinant OspA vaccine developed by Valneva and Pfizer that is currently undergoing Phase 3 clinical trials in the United States. It consists of three fusion proteins containing pairs of the modified C-termini of the six most common OspA serotypes present in North America and Europe (ST1-6) [72]. As a precaution, VLA15 was designed to exclude the potentially cross-reactive OspA epitope. In phase 1 (Registration #NCT03010228), both adjuvanted and non-adjuvanted formulations were tested at three different dose concentrations (12 µg, 48 µg, and 90 µg) by intramuscular injection on days 1, 29, and 57. The study was completed in January 2019 and both formulations were found to be safe for up to 85 days post-injection and immunogenic, with higher immune responses in adjuvanted groups [271]. In the first two Phase 2 trials (Registration #NCT03769194 and #NCT03970733), the vaccine dose and administration schedule was optimized in adults and a third phase 2 trial (Registration #NCT04801420) is currently ongoing to investigate the safety and immunogenicity of a two-dose (0 and 6 months) or three-dose (0, 2, and 6 months) regimen in both adults and children [272,273]. The primary study objectives have been completed, demonstrating that the highest antibody titres were generated by the three-dose regimen. Vaccinations in both schedules produced only mild or moderate solicited adverse events during the initial 12 month monitoring period [272,273]. A Phase 3 clinical trial (Registration #NCT05477524) evaluating the efficacy of VLA15 in outdoor recreationalists began in August 2022 and is expected to reach completion in December 2025. The primary vaccination series, including injections at 0, 2, and 5 to 9 months, has been completed and the efficacy and

safety are being monitored over the 2025 LD season. Thus far, an independent Data Safety Monitoring Board has not detected any safety concerns in the treatment groups. A simultaneous second Phase 3 trial evaluating the efficacy of VLA15 in individuals ages 5-17 is also ongoing (Registration #NCT05634811). VLA15 represents a novel LD vaccine that may afford broader protection than the single serotype LYMERix™ vaccine.

#### 1.2.1.3. mRNA-1975 (ST1-7) and mRNA-1982 (ST1)

Moderna has developed an mRNA vaccine encoding all seven known OspA serotypes. The heptavalent vaccine (mRNA-1975), as well as a monovalent OspA ST1 mRNA vaccine (mRNA-1982), are currently undergoing a Phase 1/2 clinical trial across 20 locations in the eastern United States (Registration #NCT05975099). The trial has enrolled 807 adults, both males and females, ages 18-70 who have been administered three intramuscular injections on days 1, 57, and 169 of different doses of mRNA-1975, mRNA-1982, or a placebo. The study aims to investigate the safety, immunogenicity, and appropriate dosing of the candidate vaccines. While the study was expected to conclude by the end of July 2025, the results have yet to be released.

#### 1.2.2. Veterinary vaccines

Vanguard® crLyme, manufactured by Zoetis, was approved for veterinary use in canines in 2016. This recombinant protein vaccine consists of both OspA and an OspC chimeritope referred to as Ch14, which is composed of the immunodominant L5 and H5 epitopes of seven different OspC types. This chimeritope construct has been shown to elicit antibodies that bind diverse OspC types [118]. In a placebo controlled study, canines vaccinated subcutaneously on day 0 and 21 with Vanguard® crLyme were protected against challenge with wild ticks and Lyme



borreliosis-associated pathologies [125]. Vanguard® crLyme provides protection for at least 15 months after vaccination.

Another commercially available canine vaccine is Recombitek® Lyme manufactured by Boehringer Ingelheim. It is a monovalent lipidated recombinant OspA (rOspA) that does not require adjuvantation [274]. Lipidation of peptide vaccines, including rOspA, has previously been shown to enhance immunogenicity through stimulation of TLR2 [275–277]. In fact, this non-adjuvanted lipidated formulation has been shown to be more immunogenic and induce greater borreliacidal antibody titres than the adjuvanted and non-lipidated Vanguard® crLyme vaccine [278]. Recombitek® Lyme is delivered as a 1 mL subcutaneous injection followed by a booster injection 2-3 weeks later, with additional recommended boosters each year. Although the need for annual booster injections has not been confirmed, the duration of immunity has been established to be at least 12 months.

Nobivac® Lyme, manufactured by Merck Animal Health, is also commercially available for the prevention of LD in canines [279]. Unlike, Recombitek® Lyme and Vanguard crLyme, Nobivac Lyme is a bacterin vaccine composed of both a wild-type *B. burgdorferi* strain (S-1-10) and a *B. burgdorferi* strain (50772) that does not express OspA or OspB but does express high levels of OspC. This vaccine has been shown to induce both anti-OspA and anti-OspC borreliacidal antibodies, including anti-OspC antibodies that are specific for the well-conserved C7 epitope of OspC [279]. The S-1-10 and 50772 strains, grown at 35°C and inactivated using binary ethylenimine, were neutralized with sodium thiosulfate and used to vaccinate laboratory beagles on day 0 and again 2-4 weeks later. Vaccinated beagles were completely protected from challenge with ticks collected from an endemic region in Wisconsin. They were also protected from the development of sub-clinical arthritis caused by infection.

### 1.2.3. Preclinical vaccines

#### 1.2.3.1. Protein vaccines

##### 1.2.3.1.1. OspA protein vaccines

In addition to LYMERix™, VLA15, Recombitek® Lyme, and Vanguard® crLyme, several rOspA protein vaccines have generated protective immunity against *B. burgdorferi* infection in preclinical studies. Filkrig et al. first demonstrated that *Escherichia coli* expressing OspA from *B. burgdorferi* N40 or purified recombinant OspA were immunogenic in mice and afforded complete protection against challenge with *B. burgdorferi* N40, B31, or CD16 [69]. Passive immunization of naive mice with this immune sera was also completely protective against subsequent infection with N40 or B31. Similarly, Probert et al. demonstrated complete protection of C3N/HeN mice vaccinated with rOspA against *B. burgdorferi* SON 188 challenge, as well as induction of borreliacidal antibodies [280]. Following the removal of LYMERix™ and suggestions of potential cross-reactivity with human LFA-1 $\alpha$ , Willett et al. generated a rOspA vaccine, in which the cross-reactive epitope was mutated [71]. This modified vaccine afforded the same level of protection as the wild-type rOspA in C3H/HeJ and BALB/c mice, suggesting that this is a viable strategy to improve the safety of OspA-targeting vaccines.

Since seven different OspA serotypes have been identified, more recent studies have examined the efficacy of multivalent OspA vaccines. Nayak et al. developed a variety of chimeric OspA vaccines consisting of the six most clinically relevant OspA STs [72]. Most constructs afforded significant protection against needle or tick challenge with different OspA ST strains, while a fusion of two of these chimeras containing OspA ST1-6 protected completely against needle challenge with an ST1 strain and tick challenge with an ST2 strain. These findings led to the generation of the VLA15 vaccine described above.

Another study by Kamp et al. evaluated a ferritin nanoparticle formulated OspA vaccine with six different OspA serotypes [281]. The vaccine was found to induce antibodies that recognize rOspA of all six included serotypes with titres lasting for a duration of at least 6 months in non-human primates. The multivalent vaccine was found to induce higher antibody titres than Recombitek® Lyme and interestingly, the hexavalent vaccine induced higher antibody titres than the matching monovalent vaccine for most OspA STs. Furthermore, the vaccine afforded near complete protection against *B. burgdorferi* N40 (ST1) tick-challenge and complete protection against *B. afzelii* CB43 (ST2) tick-challenge.

#### 1.2.3.1.2. OspC protein vaccines

Protection against Bb infection by active immunization with rOspC was first demonstrated in 1992 using a gerbil model of needle infection with strain PKo [282]. Probert et al. then further confirmed the protective efficacy of rOspC fused to the C-terminal end of maltose-binding protein in affording homologous protection against *B. burgdorferi* SON 188 needle challenge in mice [280]. rOspC vaccination has also demonstrated complete protection against *B. burgdorferi* B31 infection in a tick-challenge mouse model [283]. In a subsequent study, Bockenstedt et al. highlighted strain-specific differences in rOspC-induced protection [99]. While immunization with rOspC from *B. afzelii* strain PKo afforded complete protection against homologous infection in mice, both active immunization with rOspC from *B. burgdorferi* strain N40 or passive immunization with the corresponding immune sera failed to protect mice against needle or tick-borne infection. These results suggest possible strain-specific differences in OspC expression levels or immunogenicity *in vivo*.

Many groups have demonstrated that OspC protection is type-specific and that rOspC vaccination fails to cross-protect against heterologous isolates [70,98–101]. In an attempt to afford

broad protection against *B. burgdorferi*, Marconi and colleagues developed several chimeric rOspC (crOspC) vaccines consisting of the immunodominant L5 and H5 epitopes from multiple OspC types. These designs included tetravalent, octavalent, and nonavalent constructs and eventually led to the development of the heptavalent Vanguard CrLyme® canine vaccine [116–118,125]. These chimeritope vaccines are capable of generating antibodies that recognize over 20 different OspC proteins spanning multiple *B. burgdorferi* genospecies and demonstrate borreliacidal activity *in vitro*. However, the breadth of protection afforded by these constructs without the inclusion of OspA has never been systematically tested by tick- or needle-challenge with a variety of known *B. burgdorferi* strains [121].

#### 1.2.3.1.3. Other protein vaccines

rOspA and rOspC have been tested in combination with other antigens. Brown 2005 et al. developed a tri-component LD vaccine containing DbpA, BBK32, and OspC that was more effective than single or double component formulations of any combination [140]. Recently, O'Bier et al. developed a multi-chimeric vaccine composed of two chimera, one containing two OspA epitopes inserted into an OspB backbone with the N-terminus of FltA joined at the C-terminus and the other, a crOspC, containing the L5 and H5 epitopes from 10 different OspC types [139]. Three doses of this vaccine elicited complement-dependent and -independent borreliacidal antibodies and afforded 80-90% protection against needle challenge with *B. burgdorferi* B31. However, the breadth of protection against different challenge strains was not assessed.

Other *B. burgdorferi* outer surface proteins have also been successfully administered as recombinant protein vaccines. Subcutaneous injection of outer surface protein BBI39 induced high levels of antigen-specific antibodies with *in vitro* borreliacidal activity that greatly reduced spirochete burden and Lyme arthritis following needle- or tick-challenge, when administered alone

or in combination with recombinant BB136 [137]. Similarly, a peptide derived from outer membrane protein BB0172 (PepB) induced high levels of antigen specific IgG antibodies with *in vitro* borreliacidal activity when conjugated to tetanus toxoid heavy chain molecules, providing 70% protection from needle challenge and significantly reducing bacterial burden in all tissues following tick challenge [284]. Recently, Brangulis et al. also showed that a mutated version of the CspZ surface protein generated high antigen-specific antibody titres with *in vitro* borreliacidal activity that protected mice from disseminated infection and associated joint inflammation [138]. Despite their preclinical success, none of these alternative antigens have been evaluated as vaccine targets in human clinical trials.

#### 1.2.3.2. Viral vector vaccines

Multiple viral vector vaccines have been tested preclinically for the prevention of LD. In 2003, Gipson et al. demonstrated that Venezuelan Equine Encephalitis (VEE) virus replicon particles (VRPs) encoding OspA with a tPA sequence elicited a Th1 immune response and afforded 83% protection against tick infection [285]. Similarly, Palmer et al. demonstrated that a nonproliferating parvovirus vaccine engineered to express OspA was highly immunogenic, eliciting a Th1 antibody and CD4<sup>+</sup> T cell responses, and afforded protection against tick challenge after one intranasal or intravenous vaccination [286]. Recently, Gingerich et al. developed two parainfluenza virus 5 (PIV5)-vectored OspA vaccines for LD [287]. The first vaccine construct was engineered to express OspA (ST1) while the second one expressed a chimeric ST1/ST2 OspA sequence. C3H/HeN mice were vaccinated intranasally with live PIV5-OspA(ST1) or PIV5-OspA(ST1-2) or subcutaneously with recombinant OspA (ST1) protein with alum. Both live vector vaccines induced higher anti-OspA antibody titres at 6 and 12 months post-prime vaccination than the recombinant protein vaccine and the neutralization activity was maintained for up to 18

months. Only the PIV-OspA(ST1) vaccine afforded complete protection against tick challenge at 1, 9 and 15 months post-immunization, with breakthrough infections observed in the PIV5-OspA(ST1-2) vaccine group at 15 months only, and breakthrough infections observed in the rOspA group at 9 and 15 months.

In 2011, Xiao et al. evaluated the immunogenicity and protective efficacy of a new castle disease (NDV) virus engineered to express intracellular or secreted BmpA or OspC [141]. None of the vaccine constructs elicited antibody responses in mice, however the NDV-BmpA vaccine elicited antibodies and reduced spirochete burden in the joints after intraperitoneal challenge in a guinea pig model. In the guinea pig model, both NDV-OspC constructs elicited antibodies but did not afford protection against the spirochete challenge, raising questions about the efficacy of OspC-based viral vector vaccines.

Finally, Rios et al. developed live recombinant Rabies virus (RABV)-vectored vaccine delivering BBI39 or a chimeric BBI39 antigen fused to the Rabies virus glycoprotein [136]. They compared these vaccines to an inactivated version of the chimeric fused vaccine with and without adjuvant as well as to an adjuvanted recombinant BBI39 protein vaccine. All constructs except for the live RABV-BBI39 vaccine induced significant anti-BBI39 antibody titres, with the greatest *in vitro* bactericidal activity observed in the RABV-vectored groups. Furthermore, the adjuvanted inactivated chimeric vaccine was protective against short-term tick challenge and long-term needle challenge, with significant antibody titres maintained for at least 8 months after vaccination. Both Rios' and Gingerich's studies suggest that viral vector vaccines may induce longer lasting antibody responses, increased bactericidal activity, and longer lasting protection from infection than conventional recombinant protein vaccines [136,287].

### 1.2.3.3. DNA Vaccines

Several DNA vaccines have been evaluated preclinically for the prevention of LD. Two studies have demonstrated the efficacy of a plasmid DNA (pDNA) vaccine encoding OspA. In 1997, Luke et al. demonstrated the immunogenicity and protective efficacy of a “naked” DNA OspA ST1 vaccine against needle challenge with two different *B. burgdorferi* strains expressing OspA ST1 (Sh-2-82 and N40) [288]. In 2020, Guibinga et al. demonstrated that a plasmid vaccine encoding OspA delivered by in vivo electroporation elicited cellular and humoral immune responses in mice and guinea pigs, with antibody responses that persist up to one year [289]. The DNA vaccine elicited stronger antibody and cell-mediated responses than the canine Vanguard® crLyme vaccine and conferred complete protection against homologous challenge in mice after a single dose.

There is an extensive history of OspC-targeting DNA vaccines yielding mixed results. In 1999, Weiss et al. first demonstrated that intramuscular administration of a plasmid encoding OspC of *B. burgdorferi* BITS or ZS7, without the native signal peptide, induced weak antibody and cell-mediated immune responses that were greatly enhanced by the addition of a human tissue plasminogen activator (hTPA) signal sequence [290]. Addition of this sequence is thought to enhance trafficking to the endoplasmic reticulum and decrease intracellular degradation of translated antigens, resulting in higher antigen expression after vaccination [291,292]. In line with these findings, Scheiblhofer et al. compared a pCMV vaccine encoding OspC from *B. burgdorferi* strain ZS7 with the hTPA signal sequence delivered by gene gun or intradermal needle injection with or without CpG motifs. Gene gun delivery was found to induce a Th2-biased response while needle injection induced a Th1-biased response, consistent with previous studies [293–296]. After three doses, the pDNA vaccines were found to be 80-100% protective with or without CpG

addition when delivered by gene gun or needle [83]. On the contrary, Wallich et al. had previously found that all pEF DNA constructs encoding OspC alone, with or without the OspC or hTPA leader sequence, were not immunogenic in mice when administered intramuscularly [297]. Only an OspA-OspC fusion DNA vaccine induced anti-OspC antibodies, as well as anti-OspA antibodies, and prevented infection of naive mice passively transferred with the immune sera.

Two more studies were then published in 2014 and 2021 supporting the use of OspC-targeting DNA vaccines for LD. Both studies employed tattoo intradermal administration of a pVAX plasmid vector, which was codon-optimized and included the hTPA signal sequence [298,299]. Wagemakers et al. demonstrated in 2014 that abdomen tattoo of an OspC-encoding DNA vaccine induced a strong anti-OspC antibody response with Th1-bias and was fully protective against homologous *B. afzelii* needle challenge [298]. Similarly, Klouwens et al. in 2021, demonstrated that their DNA vaccine encoding OspC administered via tail tattoo was immunogenic and protected 87.5% of mice against homologous tick challenge [299]. LD DNA vaccines targeting other antigens have not proven to be successful, including the *B. burgdorferi* antigen BB0405, or the tick proteins TSLPI, Salp15, tHRF, and Tix-5 [134,299]

#### 1.2.3.4. mRNA Vaccines

Three mRNA vaccines for LD are currently in preclinical development. In 2021, Sajid et al. published a study demonstrating the efficacy of a nucleoside-modified mRNA vaccine encoding 19 different *Ixodes* tick salivary proteins (19ISP) [300]. Ticks fed on guinea pigs two weeks after the vaccination with three 50 µg doses of 19ISP demonstrated early detachment and decreased engorgement. Moreover, vaccinated guinea pigs were completely protected from *B. burgdorferi* N40 infection. Notably, the breadth of protection conferred by 19ISP against a variety of *B. burgdorferi* strains has not been evaluated.



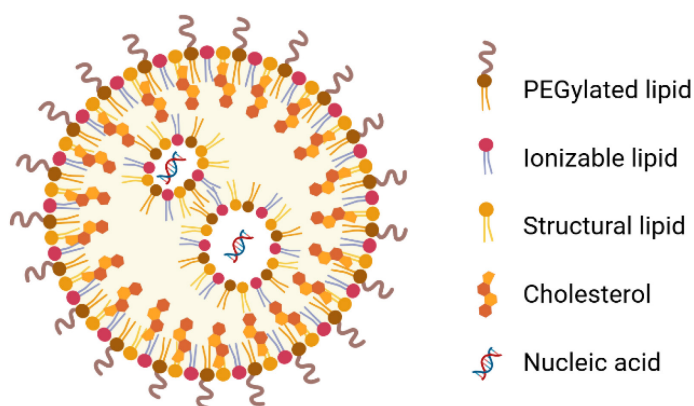
Two preclinical mRNA vaccines targeting OspA were published in 2023 and 2025. Pine et al. developed a nucleoside-modified mRNA vaccine encoding OspA from *B. burgdorferi* B31, which afforded 80% protection against needle challenge with *B. burgdorferi* N40, a strain with the same OspA serotype (ST1), after one dose in a Balb/c mouse model [301]. In comparison, an alum-adjuvanted rOspA vaccine (rOspA+alum) only afforded 50% protection after one dose in this study. Additionally, the OspA mRNA vaccine induced antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell, Tfh cells, and antigen-specific GC B cells responses that were significantly higher than the rOspA+alum vaccine. Furthermore, the OspA mRNA vaccine elicited antigen-specific memory B cell and long-lived plasma cell responses, as well as a durable antibody response for up to 24 weeks.

Tahir et al. further confirmed the utility of an mRNA vaccine targeting OspA in a study comparing different OspA mRNA constructs and ferritin-complexed recombinant OspA proteins against tick challenge in a C3H/HeNRj mouse model [302]. The candidate vaccines were administered on day 0 and 21 prior to challenge on day 35. The mRNA vaccine encoding OspA lacking the secretion signal or transmembrane domain conferred 100% protection against tick infection and transmission and elicited significantly higher borreliacidal antibodies than all other tested vaccines. IgG levels from all OspA vaccines tested in this study were reduced at the study's conclusion eight weeks post-prime, when compared to day 45, although the titres were still substantial. Notably, despite the success of OspA-based mRNA vaccines for LD, mRNA vaccines targeting OspC have yet to be tested preclinically.

## 1.3. Lipid Nanoparticles

A critical component of mRNA vaccines is the lipid nanoparticle (LNP) delivery system. Encapsulation of nucleic acids in LNPs serves multiple purposes for improving the delivery of nucleic acids *in vivo*. First, LNP encapsulation protects nucleic acids against degradation by nucleases and improves stability in biological fluids [303–306]. Second, LNPs increase *in vivo* transfection efficiency, leading to increased antigen expression [306–310]. Finally, LNPs have inherent immunostimulatory effects, driven by the ionizable lipid component, that removes the need for exogenous adjuvant compounds [311,312].

Modern LNPs are composed of four key components: ionizable lipids, structural lipids, cholesterol, and polyethylene glycol (PEG)-lipid conjugates (Figure 1.3). Helper lipids, including structural lipids and cholesterol, play an important role in stabilizing the LNP structure and enhancing cellular delivery [313]. The current commercial LNP systems utilize 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) as a structural lipid due to its efficiency in stabilizing liposomes and improving encapsulation efficiency [313]. The addition of PEG-lipids reduces aggregation of the particles, improving particle stability and sizing consistency [313–315]. Finally, the ionizable lipid constitutes the largest molar percentage in an LNP formulation and its acid-dissociation constant and shape significantly influence the delivery efficiency [316–318].



**Figure 1.3.** Components of a lipid nanoparticle encapsulating a nucleic acid payload. Created using BioRender software.

The ionizable lipids employed in modern LNPs were optimized over decades to increase cellular uptake and reduce cytotoxicity. The first generation of LNPs for nucleic acid delivery primarily consisted of cationic lipids that contained head groups with permanent positive charges [319,320]. The cationic nature of these lipids, such as 1,2-Dioleoyl-3-trimethylammonium (DOTAP) and N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) used for mRNA delivery, resulted in poor biocompatibility and weaker endosomal escape [321,322]. To increase potency and minimize toxicity, second generation LNPs instead use lipids with a pKa between 6 and 7, such as DLin-DMA [316–318]. These ionizable lipids are positively charged in acidic environments, including during encapsulation of negatively charged nucleic acids and in the cellular endosome to promote endosomal escape, but remain neutral at physiological pH. DLin-DMA was originally designed for siRNA delivery and modification of the linker and hydrophobic regions to form DLin-KC2-DMA (KC2) was found to improve cellular uptake [323]. Further modification to create DLin-MC3-DMA (MC3) resulted in the first approved siRNA treatment, Onpattro®, using MC3-LNP delivery for the treatment of hereditary transthyretin-mediated amyloidosis [324,325]. Third generation LNPs were then developed from

MC3, by including ester bonds in the linker and lipidic tails. These ionizable lipids, such as Lipid H (SM-102), used in the Moderna Spikevax COVID-19 vaccine, and ALC-0315, used in the Pfizer Comirnaty COVID-19 vaccine, have a prominent cone-like shape that enhances endosomal escape, increases potency, and improves biodegradability compared to second-generation LNPs [326–329]. Since the approval of these two COVID-19 mRNA vaccines, these LNP formulations have been extensively examined in clinical and preclinical studies for the development of mRNA vaccines for other infectious diseases, including an approved vaccine for respiratory syncytial virus (RSV) using the SM-102 lipid [330]. While the majority of third-generation LNPs have been employed for siRNA or mRNA delivery, this technology can be applied to the delivery of other nucleic acids, such as pDNA.

## Rationale, hypothesis, and objectives

### Rationale

Vaccines are the most promising public health measure to combat the rising incidence of LD in Canada. While effective antibiotics are available for the treatment of LD, their utility is limited by several factors. First, delayed diagnosis and misdiagnosis of LD can occur due to unrecognized tick exposure and non-specific symptoms [145,151,156,157]. Additionally, *erythema migrans* does not occur or is not noticed in 20-30% of cases [151,152]. Even with correct diagnosis and standard antibiotic treatment, 10-20% of patients experience PTLDS, which dramatically reduces their quality of life and has no known treatment [166]. Finally, antibiotics do not prevent reinfection, leaving patients once again susceptible to developing LD. Together, these limitations emphasize the importance of preventative measures, especially vaccination.

The majority of LD vaccine development efforts have focused on targeting the well-conserved OspA surface lipoprotein. While highly expressed during colonization of the tick-

vector, OspA is downregulated immediately following the tick bloodmeal and throughout infection of the vertebrate host [50,58,59,331]. Consequently, vaccines that target OspA must induce and maintain high circulating antibody titres that can pass into the tick vector during feeding to eliminate spirochetes within the tick midgut, likely necessitating frequent booster injections [268,332,333]. Alternatively, expression of OspC, which is required for vertebrate infection, is upregulated in response to the tick blood meal and persists throughout early infection [87,331]. Vaccines targeting OspC may therefore require less frequent booster immunizations to maintain protection.

The efficacy of OspC as an antigen delivered by next-generation vaccine platforms has been understudied. While recombinant protein vaccines have demonstrated protection against LD, modern vaccine technologies present several advantages over conventional approaches, including ease of production and greater immunogenicity, which could enhance both protection and clinical applicability [247,248,254,264,265]. Next-generation vaccines can include viral-vector vaccines and nucleic acid vaccines, such as pDNA- and mRNA-based platforms. A previous attempt at an OspC-based viral vector vaccine failed to confer protection against subsequent infection, suggesting that alterations to the vaccine design, including the use of a different viral vector, should be evaluated to determine whether viral-vectors are a viable strategy for LD vaccine design [141].

With respect to DNA-based vaccines, several preclinical OspC-targeting vaccines have been evaluated, however, the delivery methods employed by these studies are not easily translated to the clinic [83,290,297–299]. To address this limitation, modern LNP formulations that are highly effective for RNA delivery should be evaluated in the context of pDNA vaccine delivery. In addition, the kinetics, potency, biodistribution and immunogenicity of LNP-based nucleic acid vaccines should be fully characterized to inform the design of future LD nucleic acid vaccines.

Finally, despite the immense success of the mRNA vaccine platform, the efficacy of an mRNA vaccine encoding OspC has never been investigated. Therefore, the efficacy of viral vector, pDNA-LNP, and mRNA-LNP OspC vaccines should be evaluated against *B. burgdorferi* challenge. For each platform, it is also essential to characterize both the humoral immune response and the previously understudied cell-mediated immune response elicited by OspC-targeting vaccines to better inform the development and regulation of LD vaccines.

Finally, a major limitation of OspC vaccines is the extensive genetic diversity of the OspC antigen with limited cross-protection between major groups [70,94–96,98–101,290]. To afford protection against the wide array of OspC antigens, multivalent vaccines can be designed to contain multiple OspC types, especially those most frequently detected in human disseminated infection. To date, polyvalent OspC vaccines have only been studied using recombinant protein technology [116,219,334]. Furthermore, the protective efficacy has only been measured in conjunction with OspA, limiting our understanding of OspC-based immunity [125]. Finally, the breadth of protection afforded by these polyvalent vaccines has been evaluated using wild ticks infected with *B. burgdorferi* of uncharacterized OspC types rather than being systematically challenged with individual strains expressing defined OspC types. Taken together, these knowledge gaps highlight the critical need to evaluate the immunity induced by next-generation OspC-based vaccines across diverse platforms and against diverse OspC types.

## Hypothesis

I hypothesize that next-generation monovalent vaccines targeting OspC can afford homologous protection against *B. burgdorferi* infection through the induction of OspC-specific antibody and T cell responses. Furthermore, through the characterization of multiple *B. burgdorferi* sensu stricto strain infections and optimization of nucleic acid vaccines delivered by

LNPs, polyvalent OspC vaccines can be developed to afford multi-strain protection against Lyme borreliosis in mice.

## Objectives

1. Evaluate the protective efficacy and underlying humoral and cell-mediated immune responses of monovalent next-generation vaccines targeting OspC against homologous *B. burgdorferi* challenge.
2. Characterize the potency, kinetics, biodistribution, and immunogenicity of pDNA and mRNA vaccines delivered by modern LNP formulations.
3. Determine the breadth of protection conferred by a polyvalent OspC mRNA vaccine against challenge with characterized *B. burgdorferi* strains.

## Research progression

The ultimate goal of this thesis was to investigate the breadth of protection conferred by a next-generation polyvalent vaccine targeting OspC against *B. burgdorferi* infection. To select the optimal vaccine design, I investigated the protection and immune mechanisms induced by three different next-generation vaccine platforms: DNA, viral-vector, and mRNA. Prior to the evaluation of these vaccines, I first characterized the infection profiles of five different *B. burgdorferi* strains expressing a variety of common OspC types. Using a C3H/HeN mouse model, I investigated strain-specific differences in infectivity, tissue tropism, pathologies, and immunogenicity (**Chapter 2**). Each vaccine platform of interest was then tested as a monovalent construct targeting OspC type A against homologous challenge with strain B31-A3. First, the humoral immune response and protection against challenge were evaluated following vaccination with a pDNA construct delivered by LNPs (pVAX1-OspC) (**Chapter 3**). Second, a monovalent viral-vector vaccine (VV-OspC-A) was evaluated against challenge, alongside in-depth investigation of cellular and humoral immune responses (**Chapter 4**). I then tested three different LNP formulations delivering either pDNA or mRNA to determine if they improved upon the potency, expression kinetics, biodistribution, or immunogenicity of the formulation employed in Chapter 3 (**Chapter 5**). The optimal formulation, SM102-RNA, was then tested as a monovalent vaccine with characterization of humoral and cell-mediated immune responses (**Chapter 6**). Finally, this formulation was used for the generation of a polyvalent vaccine encoding five different OspC types (A, C, I, K, N) (OspC-ACIKN mRNA) and evaluated for protection against all five of the strains characterized in **Chapter 2**.



# Chapter 2: *Borrelia burgdorferi* Strain-Specific Differences in Mouse Infectivity and Pathology

## Preface

This article was previously published as detailed below.

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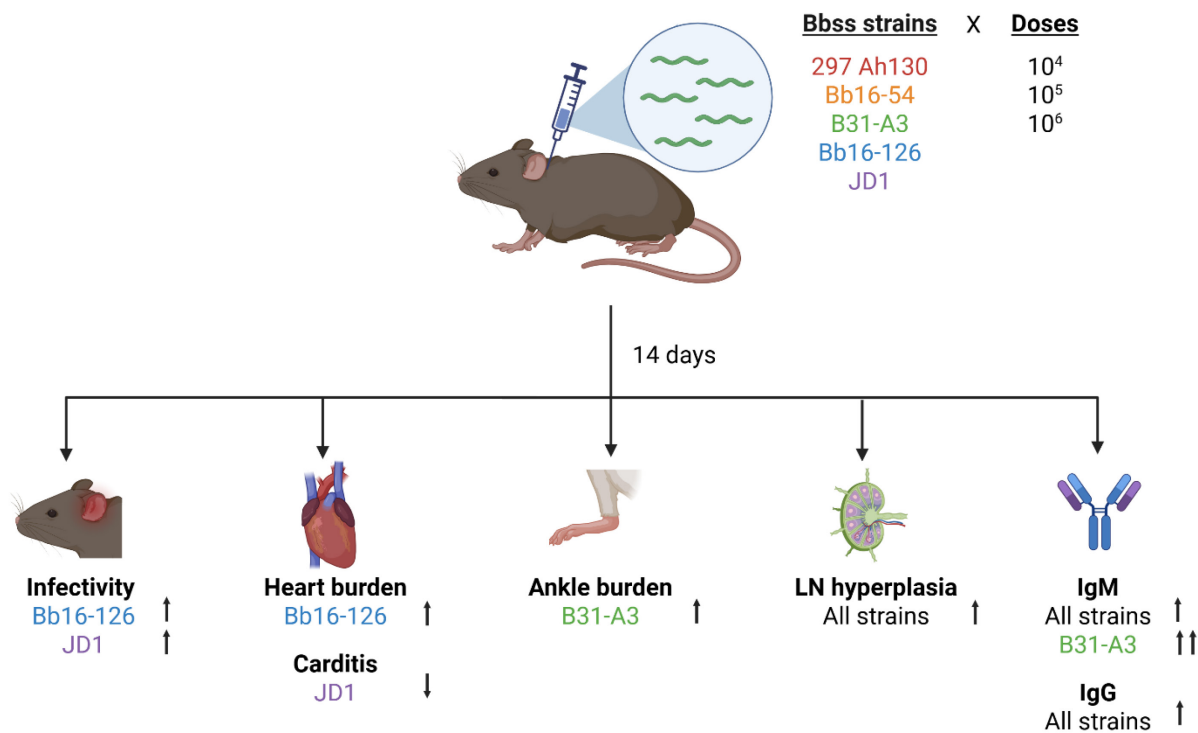
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## Abstract

Lyme disease (LD), caused by infection with the tick-borne bacteria, *Borrelia burgdorferi*, is associated with a wide array of symptoms in human patients. Variations in clinical manifestations are thought to be influenced by genetic differences among *B. burgdorferi* strains. In this study, we evaluated the infectivity, tissue bacterial load, pathology, and immunogenicity of five strains of *B. burgdorferi* sensu stricto (297 Ah130, Bb16-54, B31-A3, Bb16-126, JD1) in female C3H/HeN mice at three infectious doses ( $10^4$ ,  $10^5$ ,  $10^6$  spirochetes). We found that strains Bb16-126 and JD1 were the most infectious, resulting in 100% infection across all the tested doses. Strain Bb16-126 caused the highest bacterial burden in the heart tissue and significant carditis, whereas JD1 exhibited the lowest spirochete load in the heart and minimal carditis. In comparison, strain B31-A3 demonstrated the highest abundance in the tibiotarsal joint. Infection with all the strains induced severe lymph node hyperplasia, with JD1 producing the greatest increase in cellularity. Using a diagnostic C6 peptide ELISA, all the strains induced significant anti-C6 IgM and IgG antibody titers at 14 days post-infection; however, strain B31-A3 elicited the highest anti-C6 IgM

titers. Our findings demonstrate the importance of strain diversity in shaping *B. burgdorferi* pathogenesis in a mouse model and provide insights for developing strain-specific diagnostic, therapeutic, and vaccine strategies.

### Graphical Abstract



**Figure 2.1.** Graphical abstract of the study described in Chapter 2. Created using BioRender software.

### 2.1. Introduction

Lyme disease (LD) is the most common vector-borne disease in the Northern Hemisphere with an annual incidence of nearly 500,000 cases in North America and 650,000 to 850,000 cases in Europe [2,46,335]. LD is caused by spirochete bacteria of the *Borrelia burgdorferi* sensu lato (sl) genospecies complex [33]. In North America, the genospecies that causes the most LD cases

is *B. burgdorferi* sensu stricto (Bbss), whereas in Europe, the main genospecies are *Borrelia garinii* in central Europe and *Borrelia afzelii* in North America and Mediterranean Europe [336,337]. These spirochetes are primarily transmitted by ticks of the genus *Ixodes*. The initial infection is localized to the skin, resulting in the characteristic *erythema migrans* skin rash in 75% of cases [160,338]. If left untreated, the spirochetes can disseminate to colonize the heart, lymph nodes, joints, bladder, and nervous system. Consequently, infection with *B. burgdorferi* can produce a range of severe clinical manifestations in human Lyme disease patients, including arthritis, carditis, and ocular and neurological disorders [28,159,339,340]. Furthermore, *B. burgdorferi* s.l. genospecies are associated with different disease manifestations: Bbss is associated with arthritis; *B. mayonii* is associated with high spirochaetemia; and *B. bavarensis* and *B. garinii* are more likely to be associated with neuroborreliosis, whereas *B. afzelii* is associated with cutaneous manifestations such as acrodermatitis chronica atrophicans [46,270,337,341–345].

Bacterial pathogens are known to exhibit strain-based differences in infectivity and host responses [346–349]. Each of the previously mentioned *Borrelia* genospecies can be further subdivided into genetically distinct strains [350–352]. In North America, variations in LD symptoms are partly caused by genetic differences among infecting strains of Bbss and from a limited number of *B. bissettii* and *B. mayonii* infections [353–357]. Experimental infections with different strains of *B. burgdorferi* in rodent models have demonstrated variations in pathology, bacterial burden in host tissues, and immunogenicity [358–364]. The strain diversity within the Bbss genospecies has been characterized using different strain typing systems including multi-locus sequence typing (MST), the highly polymorphic outer surface protein C gene (*ospC*), or the ribosomal RNA intergenic spacer type (RST) [38,39,41–43,365]. Both *ospC* allele type and RST have been found to influence the infectivity, spirochete dissemination, and resulting arthritis and

carditis of *B. burgdorferi* infection [363,366,367]. Furthermore, the *ospC* allele type was shown to influence joint invasion in a mouse model, through the binding of extracellular matrix components [368]. Other studies evaluating strains of Bbss or other genospecies have also demonstrated differences in spirochete loads in the blood, brain, heart, skin, and ankle during infection of both humans and mice [354,362,366,369,370].

Laboratory strains of the house mouse, *Mus musculus*, are the most common animal model for studying Lyme disease [185,359,363,371,372]. The C3H/HeN strain of *M. musculus* is often used because *B. burgdorferi* infection causes pathology, including arthritis and carditis [373–377]. This mouse model is also used to study host immune responses, antimicrobial testing, preclinical vaccines, and post-treatment Lyme disease syndrome [355,378–381]. Given the pathogenicity differences between *B. burgdorferi* strains, it is essential to characterize the infectivity and resulting pathology for a variety of strains and at a range of infectious doses in the C3H/HeN mouse model. This remains especially true as new *B. burgdorferi* isolates are collected from the field and novel clones are generated in the lab for use in preclinical research.

In this study, we infected C3H/HeN mice with one of five strains of Bbss at three different doses via needle inoculation. These five diverse strains were isolated from humans and ticks found across the United States in the 1980s or in Canada in the year 2016, and they represent a variety of RST and *ospC* types (Table 2.1). First, we determined the infectivity of each strain at each dose. We measured the bacterial burden in the heart and tibiotarsal joint via qPCR and characterized the resulting carditis and lymph node hyperplasia. Finally, we investigated the host antibody response to each strain. Our findings help shed light on strain-specific differences in *B. burgdorferi* infection and pathogenesis.

| Strain ID                | Isolate source                                                                                                    | Year of Original Isolation | Geographic region                | RST [39] | OspC type | Reference(s) |
|--------------------------|-------------------------------------------------------------------------------------------------------------------|----------------------------|----------------------------------|----------|-----------|--------------|
| <b>297 Ah130</b>         | Laboratory isolate derived from strain 297, originally obtained from Lyme borreliosis patient cerebrospinal fluid | 1982                       | Connecticut, U.S.                | 2        | K         | [60,382]     |
| <b>B31-A3</b>            | Laboratory isolate of strain B31, originally obtained from <i>Ixodes dammini</i>                                  | 1981                       | New York, U.S.                   | 1        | A         | [383]        |
| <b>Bb16-54</b>           | <i>Ixodes scapularis</i>                                                                                          | 2016                       | Buffalo Point, MB, CA            | Unknown  | I         | [105,358]    |
| <b>Bb16-126</b>          | <i>Ixodes scapularis</i>                                                                                          | 2016                       | Big Grassy, ON, CA               | Unknown  | N         | [105,358]    |
| <b>JD1 (Clone SK143)</b> | Passaged in C3H/HeN mouse, originally obtained from <i>Ixodes dammini</i>                                         | 1986                       | Crane's Beach, Ipswich, MA, U.S. | 3        | C         | [384,385]    |

**Table 2.1.** Origins and ospC types of *B. burgdorferi* strains used in this study with associated references. RST: ribosomal RNA intergenic spacer type. OspC: outer surface protein C.

## 2.2. Materials and Methods

### 2.2.1. Bacterial Cell Culture

We used five different strains of Bbss including B31-A3, JD1, 297, Bb16-54, and Bb16-126. Strain B31-A3 was kindly provided by Dr. Patricia Rosa (National Institute of Allergy and Infectious Disease, MD, USA) [383]. Strain B31 was the first isolation strain, filed with the CDC as “ATCC 35210”, where B3 represents the three names of the researchers Barbour, Burgdorfer, and Benach, and the number “1” refers to the first isolation. The suffix “-A3” indicates the clonal derivative of the passage three B31 MI clone. Strain JD1 and strain 297 Ah130 were kindly provided by Dr. George Chaconas (University of Calgary, AB, Canada) [60,382,384,385]. Strain Bb16-54 and Bb16-126 were obtained from the isolate collection of the Public Health Agency of Canada [105,358]. All the *B. burgdorferi* strains were cultured from frozen glycerol stocks at 35 °C and

1.5% CO<sub>2</sub> in complete Barbour–Stoenner–Kelly (BSK)-H media containing 6% rabbit serum (Millipore Sigma, Burlington, ON, Canada) supplemented with 5 µg/mL of amphotericin B, 100 µg/mL of phosphomycin, and 50 µg/mL of rifampicin. The BSK-H medium is a glutamine-free derivative of CMRL media, which was found to increase spirochete yields of both human and tick isolates [386]. The bacterial cultures were propagated until the mid-log phase, and their density was estimated using a Petroff-Hausser counting chamber under darkfield microscopy. *B. burgdorferi* strains were not sub-cultured to avoid plasmid loss.

### 2.2.2. Animal Care and Infectious Challenge

Mice were purchased from Charles River, Senneville, Quebec, Canada. All the animal procedures were approved and performed in accordance with institutional guidelines by the Animal Care Committee at Health Canada (Study #2023-003). Groups of 14-week-old female mice were injected subcutaneously between the shoulder blades with 100 µL of one *B. burgdorferi* strain at one of three infection doses, 10<sup>4</sup>, 10<sup>5</sup>, or 10<sup>6</sup> spirochetes ( $n = 5$  mice per dose per strain). Additionally, five mice were injected with PBS instead of bacterial culture as a negative control ( $n = 5 \text{ mice} \times 3 \text{ doses} \times 5 \text{ strains} + 5 \text{ PBS} = 80 \text{ mice total}$ ). Prior to injection, the bacterial cultures were grown to the mid-log phase, centrifuged at 5000× *g* for 15 min, and diluted in incomplete BSK-H media without rabbit serum. Fourteen days after spirochete injection, the mice were euthanized for the collection of blood and tissues.

### 2.2.3. Tissue Culture for Confirmation of Infection

Cultures of live *B. burgdorferi* from the host tissues confirmed that the spirochetes were viable. The right ear was collected at euthanasia and cultured at 35 °C and 1.5% CO<sub>2</sub> in complete BSK-H media containing 6% rabbit serum and supplemented with 5 µg/mL of amphotericin B, 100 µg/mL

of phosphomycin, and 50 µg/mL of rifampicin. After 28 days, the samples were examined by darkfield microscopy. Cultures were considered positive for infection if at least one motile spirochete was identified across five fields of view.

#### 2.2.4. Quantitative PCR (qPCR) for Spirochete Burden

We used quantitative PCR (qPCR) to determine the spirochete burden in the mouse tissues infected with each of the three doses of the five *B. burgdorferi* strains. The left tibiotarsal joint and inferior half of the heart were collected at euthanasia and frozen in liquid nitrogen. DNA extraction and qPCR analysis was performed as previously described [387].

#### 2.2.5. Determination of Lymphadenopathy

Infection with *B. burgdorferi* in mice results in lymph node hyperplasia, which is the proliferation of lymphocytic cells in the lymph nodes. The left axillary lymph node was collected at euthanasia and placed in cold RPMI media supplemented with 0.15% (w/v) sodium bicarbonate, 1 mM sodium pyruvate, 5 mM HEPES, 20 U/mL of penicillin, 0.02 mg/mL of streptomycin, 0.1% (v/v) 2-mercaptoethanol, and 10% (v/v) heat-inactivated fetal bovine serum (FBS). Single-cell suspensions and white blood cell counts were generated as previously described [381,387].

#### 2.2.6. Histopathology

At euthanasia, the heart and right axillary lymph node of each mouse were collected in formalin for histopathology analysis. The tissue processing, hematoxylin and eosin staining, and scoring were performed as previously described [381,387]. In brief, each heart was assigned a numeric grade (0–5), and each lymph node was assigned three numeric grades (0–5) each indicating the severity of follicular hyperplasia, paracortical hyperplasia, or medullary cord plasma cell



hyperplasia, respectively. The numeric grade was 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.

### 2.2.7. Enzyme-Linked Immunosorbent Assay (ELISA)

We used ELISA to determine the strength of the mouse IgM and IgG antibody response to the variable major protein (VMP)-like sequence E (VlsE) C6 peptide, which is a commonly used *B. burgdorferi* antigen [388,389]. Blood was collected 14 days after spirochete infection by cardiac puncture exsanguination and centrifuged for 2 min at 10,000× *g* to collect the serum. The wells of 96-well Nunc Maxisorp™ flat bottom plates (ThermoFisher) were coated with 0.1 µg/mL of VlsE C6 peptide (Alpha Diagnostics International, San Antonio, TX, USA) in carbonate buffer pH 9.6 overnight at 4 °C. The plates were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-T). For the IgG ELISA, the plates were blocked for 1 h with 1% (*w/v*) bovine serum albumin (IgG-Free, Protease-Free, Jackson Immuno Research, West Grove, PA, USA) in PBS-T. For the IgM ELISA, the plates were blocked for 1 h with 5% (*w/v*) milk powder (Rockland Immunochemicals, Limerick, PA, USA) in PBS-T. Mouse serum obtained 14 days after bacterial challenge was two-fold serial diluted in blocking buffer from 1:10 to 1:20,480 and added to the plate. The plates were washed six times with PBS-T and then incubated with HRP-conjugated goat anti-mouse IgG (Cytiva, Marlborough, MA, USA) diluted 1:5000 or HRP-conjugated goat anti-mouse IgM (ThermoFisher) diluted 1:3000 in blocking buffer for 1 h at 37 °C. The plates were washed six more times in PBS-T, incubated for 5 min with 100 µL tetramethylbenzidine (TMB) substrate (Cell Signaling Technology, Danvers, MA, USA), and the reaction was stopped with 0.16 M sulfuric acid. Absorbance was read at 450 nm using a spectrophotometer. Endpoint titers were calculated 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 control mice injected with PBS plus three times the standard deviation.

### 2.2.8. Multiplex Cytokine ELISA

Blood was collected 14 days after spirochete infection by cardiac puncture exsanguination and centrifuged for 2 min at  $10,000\times g$  to collect the serum. Cytokine concentrations in the serum were determined using a ProcartaPlex™ Mouse Th1/Th2 11-plex Cytokine Panel (EPX110-20820-901, ThermoFisher) according to the manufacturer's instructions. A Luminex 200 system (Millipore-Sigma) was used to read the plate, and data analysis was performed using MILLIPLEX Analyst software (v5.1) (Merck Millipore, Burlington, MA, USA).

### 2.2.9. Statistical Analysis

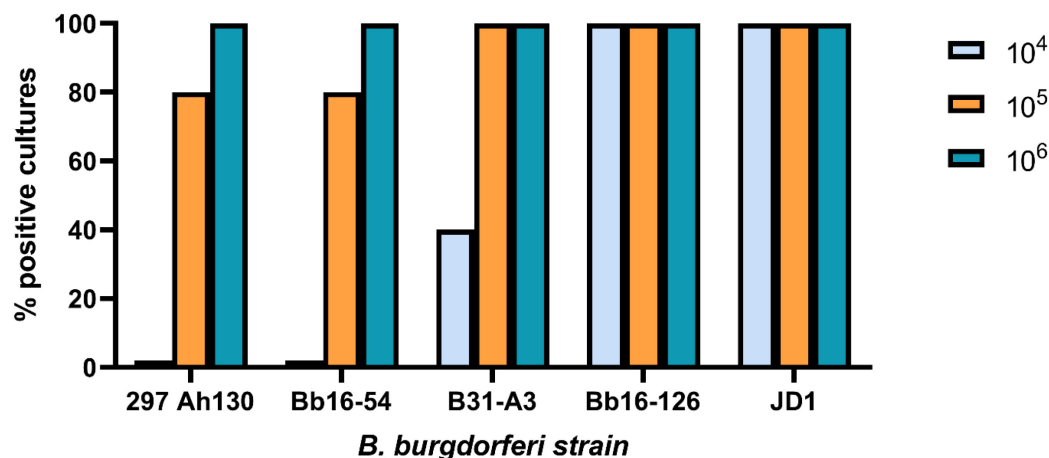
Statistical analysis was only performed for the groups injected with  $10^6$  spirochetes in which all the strains were found to be 100% infectious. Statistical significance was calculated using an ordinary one-way ANOVA with Tukey's multiple comparison test. For ELISA data, values were log<sub>10</sub>-transformed prior to statistical analysis. All the statistical analyses were performed using GraphPad Prism 9 (Dotmatics Inc, Boston, MA, USA) at  $\alpha = 0.05$ ; \*  $p$ -value < 0.05, \*\*  $p$ -value < 0.01, \*\*\*  $p$ -value < 0.001, \*\*\*\*  $p$ -value < 0.0001.

## 2.3. Results

### 2.3.1. Strains Bb16-126 and JD1 are highly infectious in C3H/HeN mice

To evaluate the infectivity of the *B. burgdorferi* strains, mice were injected subcutaneously with  $10^4$ ,  $10^5$ , or  $10^6$  spirochetes of one of five strains: 297 Ah130, Bb16-54, B31-A3, Bb16-126, and JD1. Two weeks after injection, the mice were euthanized, and a range of tissues were

collected to evaluate the presence and burden of *B. burgdorferi* infection. We have previously shown that cultivating spirochetes from the ear is a reliable measure of infection at this time point [381,387]. Cultures of live spirochetes from the right ear indicated that Bb16-126 and JD1 were the most infectious strains, both causing 100% infection at all the tested doses (Figure 2.2). The B31-A3 strain was only 40% infectious at the  $10^4$  dose; however, both the  $10^5$  and  $10^6$  doses were 100% infectious. Finally, the Bb16-54 and 297 Ah130 strains were the least infectious with no infection at the  $10^4$  dose, 80% infection at the  $10^5$  dose, and 100% infection at the  $10^6$  dose. No spirochetes were observed in the ear biopsies from the control mice inoculated with PBS.

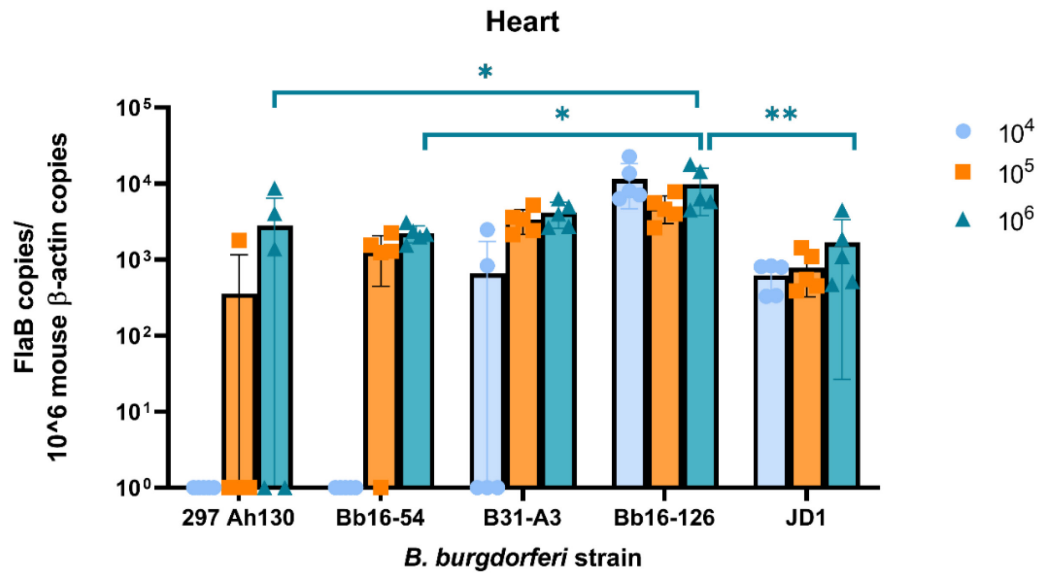


**Figure 2.2.** The five *B. burgdorferi* strains differed in their infectiousness in female C3H/HeN mice at 14 days post-challenge. Mice were infected via subcutaneous needle injection with an infectious dose of either  $10^4$ ,  $10^5$ , or  $10^6$  spirochetes. Strains Bb16-126 and JD1 were the most infectious. Mouse ears were collected 14 days after the infectious challenge and incubated for four weeks in BSK-H growth media. Cultures were considered positive if at least one spirochete was identified by darkfield microscopy across five fields of view. Five mice were tested for each of the 15 combinations of strain and dose.

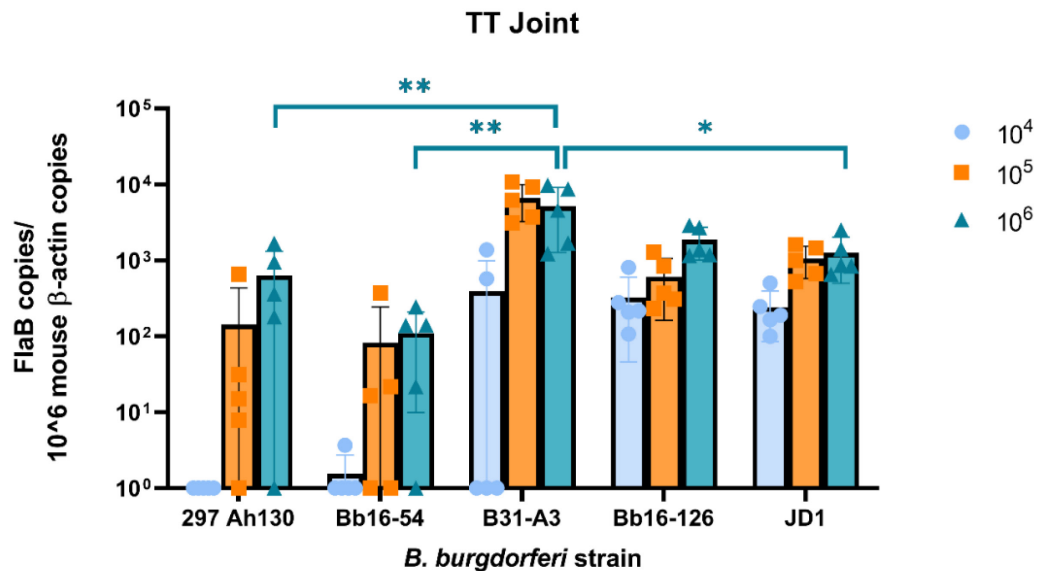
### 2.3.2. *B. burgdorferi* strains differ in spirochete load in mouse tissues

Next, we quantified the bacterial burden in the heart and tibiotarsal joints of the mice using qPCR. The spirochete loads were calculated as the number of *Borrelia FlaB* gene copies per million mouse  $\beta$ -actin gene copies. At the  $10^6$  dose, at which all the strains were 100% infectious, all the groups had significant spirochete burdens in both the heart and joint (Figure 2.3). One of the highly infectious strains, Bb16-126, had the highest mean spirochete load in the heart tissue (Figure 2.3A). In comparison, the other highly infectious strain, JD1, had the lowest mean spirochete load in the heart tissue. When evaluating the tibiotarsal joint, the highest spirochete loads were observed in mice infected with the B31-A3 strain, while the Bb16-54 strain had the lowest spirochete loads (Figure 2.3B). None of the control mice inoculated with PBS tested positive for *B. burgdorferi*. Taken together, these data support previous reports that *B. burgdorferi* strains differ in their abundance in host tissues [358,362,364].

**A**



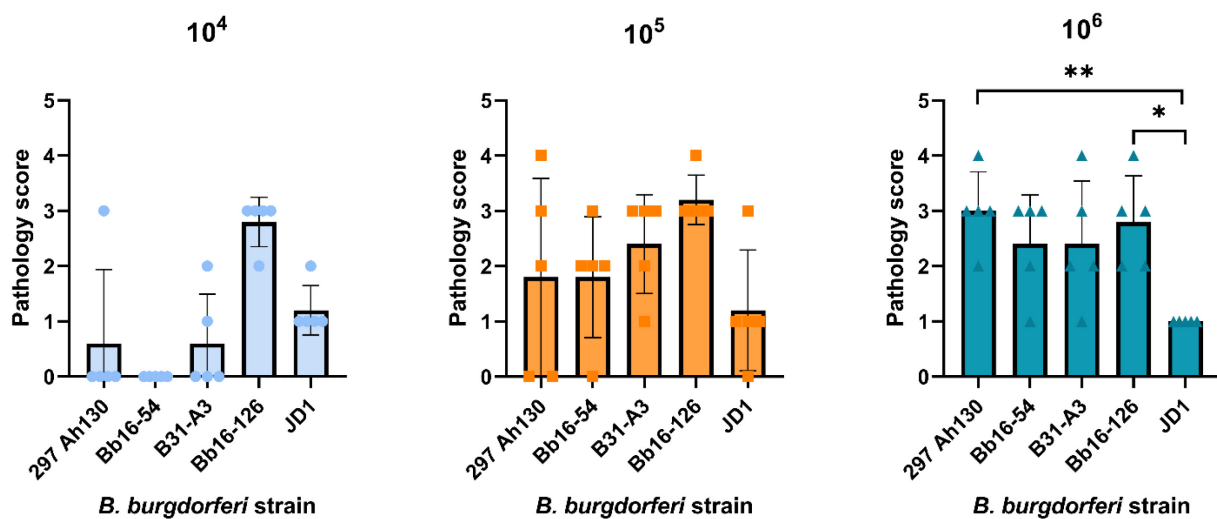
**B**



**Figure 2.3.** The five *B. burgdorferi* strains differed in their tissue spirochete loads in the (A) heart and (B) tibiotarsal joint at 14 days post-challenge. Mice were infected via subcutaneous needle injection with an infectious dose of either 10<sup>4</sup>, 10<sup>5</sup>, or 10<sup>6</sup> spirochetes. Spirochete load was determined by qPCR and has units of the number of copies of the *B. burgdorferi* FlaB gene per million mouse β-actin gene copies. Samples below the threshold of amplification were assigned a value of one. TT joint, tibiotarsal joint. Error bars represent the standard deviation; \* *p*-value < 0.05, \*\* *p*-value < 0.01.

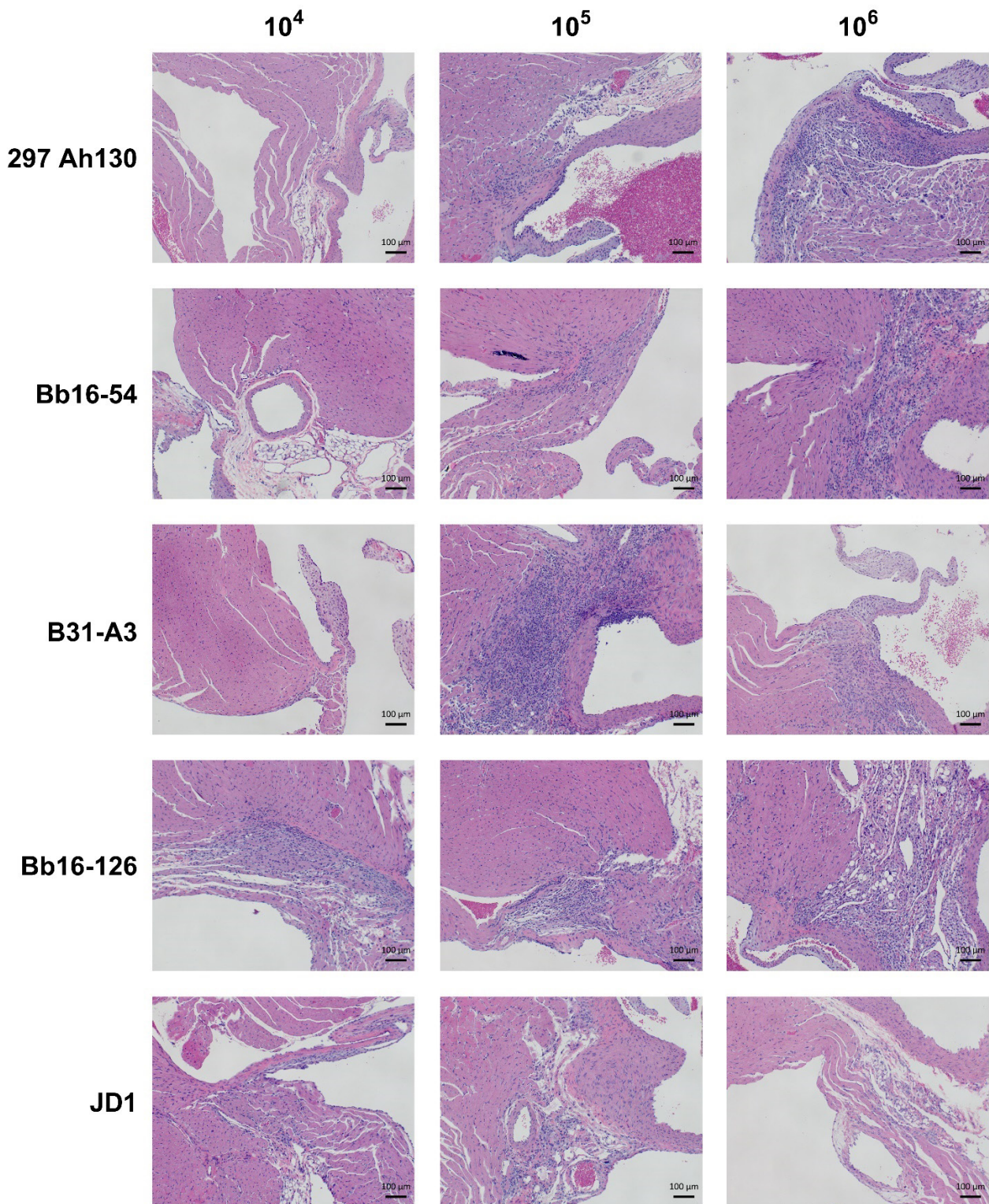
### 2.3.3. Strain JD1 causes minimal carditis in C3H/HeN mice

Based on the differences among strains in *B. burgdorferi* burdens in the heart tissue, we evaluated the carditis resulting from infection with each strain. Consistent with the spirochete load in the heart tissue, the Bb16-126 strain caused moderate carditis at all three infectious doses, while the JD1 strain yielded minimal carditis at all doses (Figures 2.4 and 2.5). At the  $10^6$  dose, at which all the strains were 100% infectious, the 297 Ah130, Bb16-54, and B31-A3 strains all induced moderate carditis. None of the control mice inoculated with PBS had significant levels of carditis. These results suggest that although carditis is a consistent manifestation of *B. burgdorferi* infection, the severity of the inflammation may be strain dependent.



**Figure 2.4.** The *B. burgdorferi* strain and infectious dose influenced carditis in the mouse heart. Strain JD1 caused the lowest levels of carditis of all tested strains at the highest infectious doses ( $10^5$  and  $10^6$  spirochetes). Summaries of the carditis pathology scores from heart tissue collected at 14 days post-challenge are shown. Scoring criteria are described in the Materials and Methods Section of this article. Error bars represent the standard deviation; \*  $p$ -value  $< 0.05$ , \*\*  $p$ -value  $< 0.01$ .



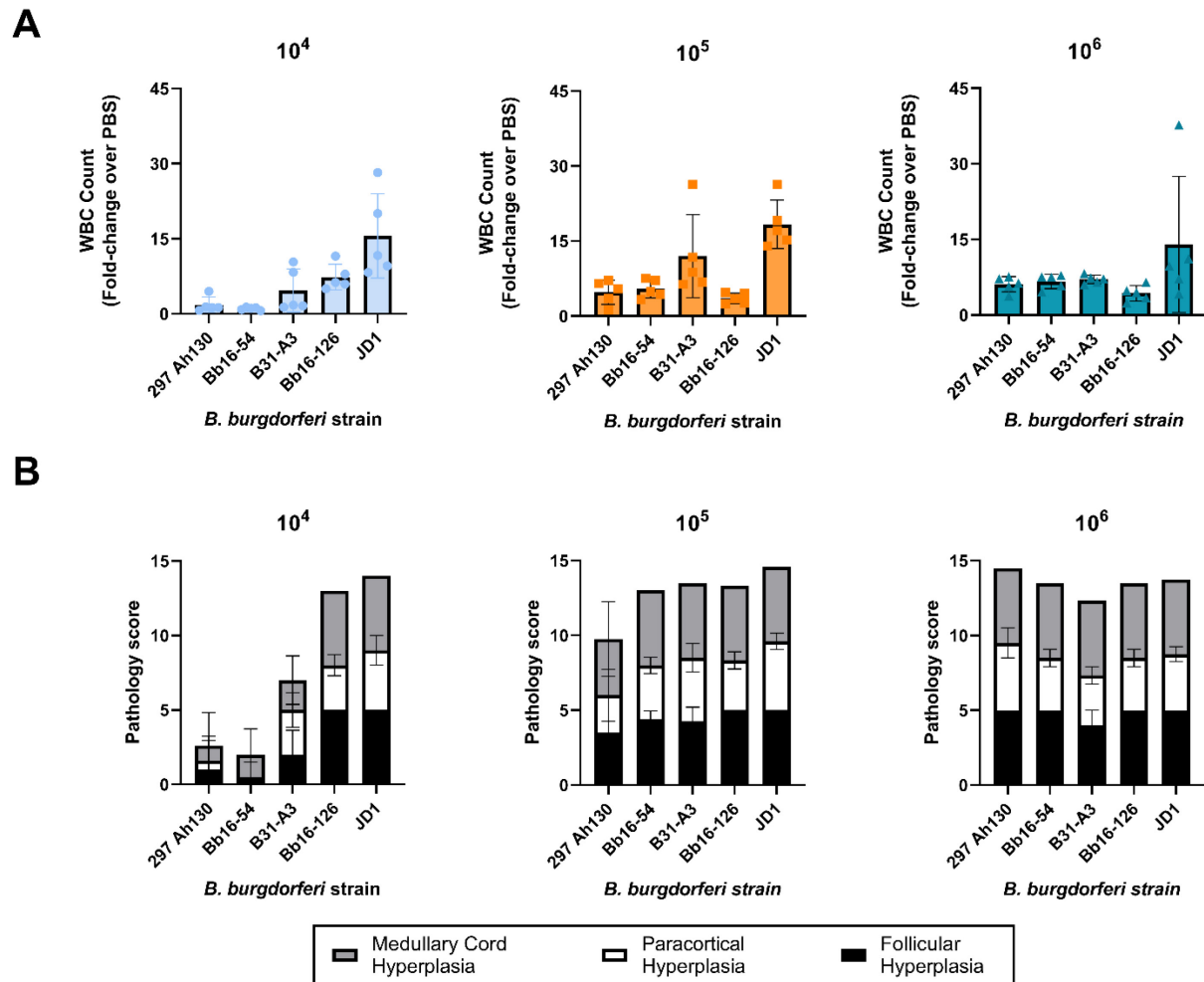


**Figure 2.5.** Infection with *B. burgdorferi* induced carditis in the mouse heart. Representative images of H&E-stained heart tissue from mice injected with each of the five *B. burgdorferi* strains at infectious doses of  $10^4$ ,  $10^5$ , or  $10^6$  spirochetes. Scale bar represents 100  $\mu\text{m}$ .

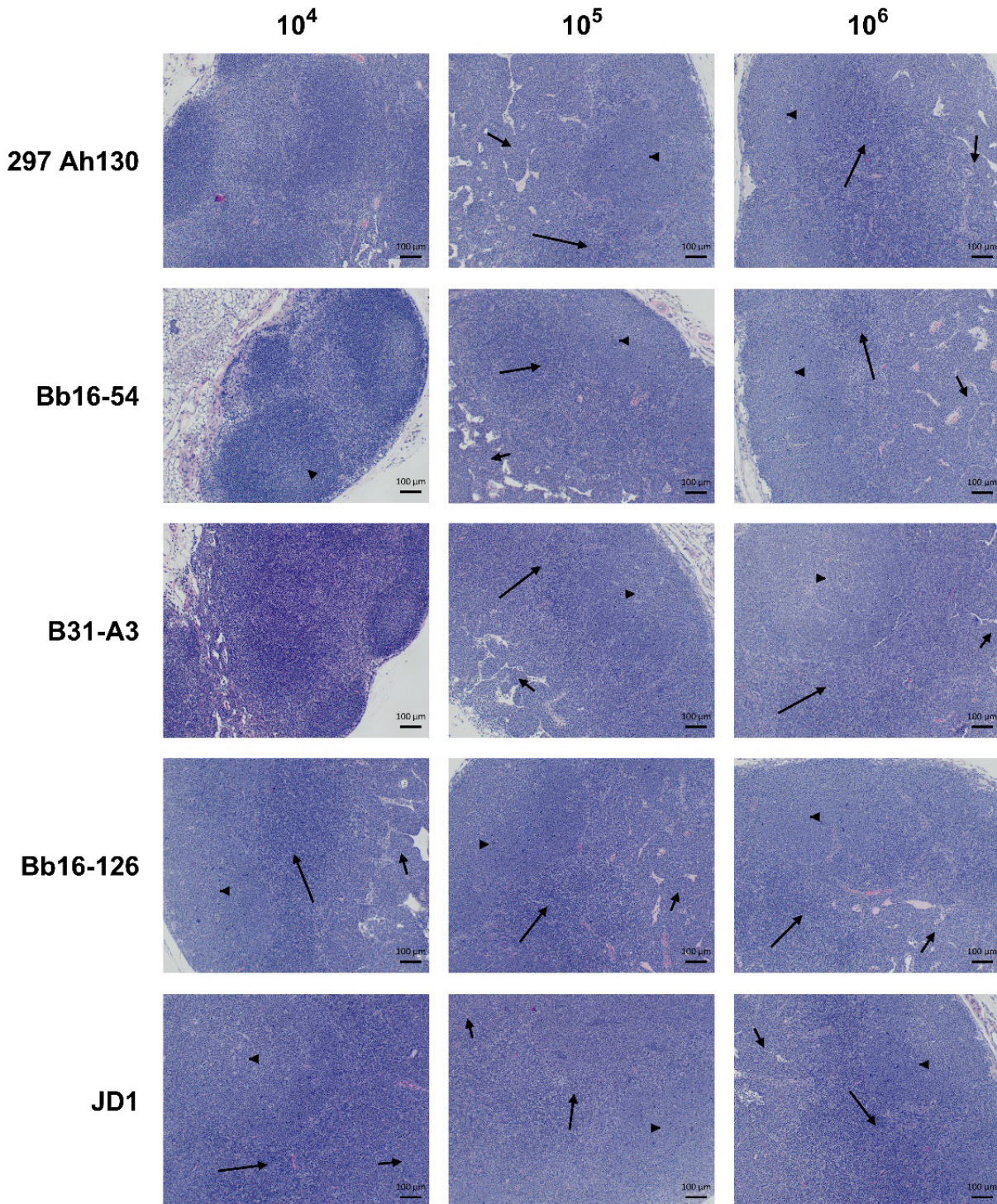
#### 2.3.4. All *B. burgdorferi* Strains Cause Severe Lymph Node Hyperplasia

Infection with *B. burgdorferi* is known to cause an accumulation of B cells in the lymph nodes, especially those proximal to the site of infection [30,158,390]. Therefore, we performed white blood cell counts of the axillary lymph nodes at 14 days post-infection for each strain. All the strains induced severe lymph node swelling at the  $10^6$  dose, with greater than 4-fold increases in WBC counts compared with mice injected with PBS (Figure 2.6A). Strain JD1 induced the most severe lymph node hyperplasia, with an average fold change of 14 compared with control mice injected with PBS. Histopathology was also performed on the axillary lymph nodes to further characterize the follicular, paracortical, and medullary cord hyperplasia. At the  $10^6$  dose, all the strains induced severe medullary cord hyperplasia marked by dense populations of plasma cells. All the strains also induced severe follicular hyperplasia and moderate-to-severe paracortical hyperplasia marked by dense populations of lymphoid cells in these compartments (Figures 2.6B and 2.7). These results suggest that lymphadenopathy is a consistent feature of Bbss infection.





**Figure 2.6.** All five *B. burgdorferi* strains induced severe lymph node hyperplasia at 14 days post-challenge. **(A)** White blood cell (WBC) counts from left axillary lymph nodes shown as fold change compared with uninfected control mice inoculated with PBS. **(B)** Summaries of lymph node hyperplasia pathology scores are shown. Scoring criteria are described in the Materials and Methods Section of this article. Error bars represent standard deviation.



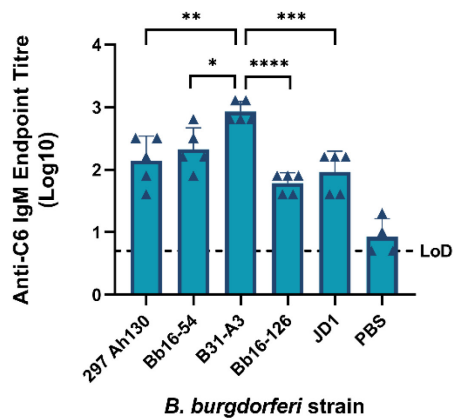
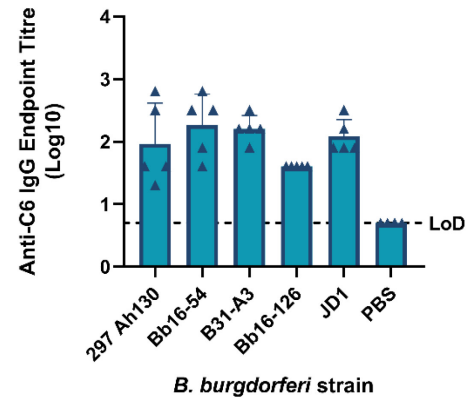
**Figure 2.7.** Representative images of H&E-stained axillary lymph nodes from mice inoculated with each of the five *B. burgdorferi* strains at infectious doses of  $10^4$ ,  $10^5$ , or  $10^6$  spirochetes. Arrowheads indicate follicular hyperplasia; short arrows indicate medullary cord hyperplasia; and long arrows indicate paracortical hyperplasia. Scale bar represents 100  $\mu$ m.

### 2.3.5. All *B. burgdorferi* Strains Induce Significant Host Immune Responses

To characterize the host immune response to infection with each strain, we measured the concentration of 11 different cytokines in the mouse serum at 14 days post-infection. High-dose infection with all the strains elicited significant increases in GM-CSF, IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, and IL-12p70 when compared with the PBS control (Figure S2.1). All strains, except Bb16-126, induced IL-18, while none of the strains induced significant IL-13 or TNF- $\alpha$  levels. One of the least infectious strains, 297 Ah130, was the only strain that induced significant increases in serum IFN- $\gamma$ . It also induced the highest levels of GM-CSF, IFN- $\gamma$ , IL-6, and IL-18, when compared with the other tested strains. This finding may suggest that certain less infectious strains better stimulate the early host immune response.

Finally, diagnosis of *B. burgdorferi* infection in humans and canines can be performed by measuring the combined IgG and IgM antibody response to the C6 peptide of the variable major protein (VMP)-like sequence E (VlsE) lipoprotein of *B. burgdorferi*. Convalescent human and canine serum samples can be collected for testing as early as two weeks after the onset of symptoms [144]. Therefore, we measured the anti-C6 IgM and IgG serum antibody titers 14 days after the high-dose infection with each strain. Infection with all the strains induced C6-specific IgM and IgG serum antibody titers that were significantly higher than those in the PBS control group (all  $p$ -values < 0.01) (Figure 2.8). This finding suggests that infection would be identifiable using the C6-diagnostic assay as early as 14 days after high-dose infection with all the tested strains. In addition, mice infected with the B31-A3 strain had significantly higher C6-specific IgM titers than those with the other four strains, whereas no differences between strains were observed for anti-C6 IgG titers at 14 days post-infection. Therefore, C6-based assays may more easily detect early infection with specific strains, such as B31-A3.



**A****B**

**Figure 2.8.** Serum antibody responses to high-dose *B. burgdorferi* infection by strain. ELISA determination of C6-specific (A) IgM or (B) IgG antibody titers in serum of mice at 14 days post-challenge with a dose of  $10^6$  spirochetes per strain. Dashed line indicates the limit of detection (LoD). Samples with data points on the dashed line have antibody titers below the limit of detection. All infected groups had antibody titers that were significantly higher than those in the PBS control group (all  $p$ -values  $< 0.01$ ); \*  $p$ -value  $< 0.05$ , \*\*  $p$ -value  $< 0.01$ , \*\*\*  $p$ -value  $< 0.001$ , \*\*\*\*  $p$ -value  $< 0.0001$ .

## 2.4. Discussion

In this study, we compared five *B. burgdorferi* strains based on the following phenotypes: (1) infectivity, (2) spirochete load in the tibiotarsal joint and heart, (3) carditis, (4) lymph node hyperplasia, and (5) host antibody response. We evaluated Bbss from two Canadian tick isolates (Bb16-54 and Bb16-126) and three derivatives of common laboratory strains (297 Ah130, B31-A3, JD1 Clone SK143) that represent a variety of RST and *ospC* types. Furthermore, the infectivity and tropisms of many of these strains have not been comprehensively characterized in the literature to date.

Previous studies examining the pathogenicity of the JD1 and Bb16-126 strains are limited. JD1 was found to be 100% infectious in rhesus macaques, causing *erythema migrans*, conjunctivitis, and inducing IgM and IgG antibodies [391]. Bb16-126 was determined to be 100%

infective in mice at a dose of  $10^4$  or  $10^5$  spirochetes and resulted in similar tissue loads to other isolates from the same collection [358]. Here, we found that JD1 and Bb16-126 were the most infectious of the five strains we tested, causing 100% infection in female C3H/HeN mice at the lowest tested dose (Figure 2.2). Infection with Bb16-126 also resulted in the highest spirochete burden in the heart (Figure 2.3A). Clinically, two different strains of Bbss have been isolated in culture from a patient with myocarditis: Myo I and Myo II [392]. All the strains in our study were found to cause mild-to-moderate carditis at the highest dose, except JD1, which consistently produced the lowest carditis scores (Figures 2.4 and 2.5). These results are in line with JD1 infection generating the lowest spirochete load in the heart tissue. In contrast, JD1 was found to induce the highest-fold increase in lymph node cellularity. However, all the strains consistently induced severe lymphadenopathy (compared with the uninfected control mice) characterized by hyperplasia of the lymph node paracortex, medulla, and follicles (Figures 2.6 and 2.7).

Strains B31-A3 and Bb16-54 had the highest and lowest spirochete load in the tibiotarsal joint, respectively (Figure 2.3B). The preferential joint tropism of strain B31-A3 is consistent with the findings of Sertour and colleagues, which demonstrated that B31 was among the most prevalent strains in the ankle joint and skin at 38 days post-infection via needle inoculation when compared with other *B. burgdorferi* strains [362]. Despite the high abundance of spirochetal DNA in the tibiotarsal joint, we did not detect any significant arthritis at 14 days post-infection for any of the five strains by both measurement of the tibiotarsal joint diameter and histology analysis. Numerous studies have shown that infection with *B. burgdorferi* induces ankle swelling in mice, but peak ankle swelling generally occurs between weeks four and six post-infection [363,372,375–377,393–395]. As we euthanized the mice in this study at two weeks post-infection, it may have been too early to observe the development of arthritis.

Finally, we found that infection with all the five strains of *Bbss* induced significant host IgM and IgG antibody responses to the conserved C6 peptide at 14 days post-infection (Figure 2.8). The C6 immunoassay is frequently used for the diagnosis of LD in canines and previously comprised the first tier of the two-tiered LD diagnostic test for humans [396–398]. We found that strain B31-A3 elicited a significantly higher anti-C6 IgM response than all the other tested strains, suggesting it may be more easily detected during early stages of infection using the C6 diagnostic method than the other strains.

In this study, we have demonstrated key differences among strains of *B. burgdorferi* in female C3H/HeN mice following subcutaneous needle inoculation. Studies of variation in infection phenotype among *B. burgdorferi* strains have also been performed using a tick-infection model for enhanced ecological relevance [358,359,399,400]. It is important to note that infection phenotypes, including infectivity and tissue dissemination, differ when comparing needle inoculation and tick bite [362,363,399]. Nevertheless, needle infection remains the most common infection model of LD since it requires fewer resources to perform than tick infection. Another limitation of this study is that all the data were collected 14 days post-infection. As strains may grow at different rates, the observed discrepancies in spirochete load and pathology may be reduced or enhanced at later timepoints. Despite this consideration, examining an early timepoint provides insight into the initial dynamics of *B. burgdorferi* infection, including early spirochete dissemination, pathology, and immune activation. Considering the rising incidence of LD in the human population and the corresponding increases in research efforts, it is essential to characterize infections with model research strains and known circulating strains of *B. burgdorferi*.

**Supplementary Materials:** Appendix - Figure S2.1: Serum cytokine levels following infection with *B. burgdorferi* strains.

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**Data Availability Statement:** The raw data supporting the conclusions of this article will be made available by the authors on request.

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# Chapter 3: DNA lipid nanoparticle vaccine targeting outer surface protein C affords protection against homologous *Borrelia burgdorferi* needle challenge in mice

## Preface

This article was previously published as detailed below.

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## Contributions:

AP, SNTR, WZ, LT, JW, CG, and XL contributed to the conceptualization of this project. AP, CL, JC, EL, DP, GF, and JG performed data generation and analysis. WC, GC, SS, MR-M, LW, MJWJ and XL assisted with funding acquisition, supervision, and project administration. AP, SNTR, WZ, and LT were involved in data interpretation and manuscript preparation. The first draft of the manuscript was written by AP and the final submission was approved by all authors.

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## Abstract

The incidence of Lyme disease (LD) in Canada and the United States has risen over the last decade, nearing 480,000 cases each year. *Borrelia burgdorferi* sensu lato, the causative agent of LD, is transmitted to humans through the bite of an infected tick, resulting in flu-like symptoms and often a characteristic bull's-eye rash. In more severe cases, disseminated bacterial infection can cause arthritis, carditis and neurological impairments. Currently, no vaccine is available for the prevention of LD in humans. In this study, we developed a lipid nanoparticle (LNP)-encapsulated DNA vaccine encoding outer surface protein C type A (OspC-type A) of *B. burgdorferi*. Vaccination of C3H/HeN mice with two doses of the candidate vaccine induced significant OspC-type A-specific antibody titres and borreliacidal activity. Analysis of the bacterial burden following needle challenge with *B. burgdorferi* (OspC-type A) revealed that the candidate vaccine afforded effective protection against homologous infection across a range of susceptible tissues. Notably, vaccinated mice were protected against carditis and lymphadenopathy associated with

Lyme borreliosis. Overall, the results of this study provide support for the use of a DNA-LNP platform for the development of LD vaccines.

### 3.1. Introduction

Nearly half a million people in North America are estimated to develop Lyme disease (LD) each year [2,401]. Since the implementation of standardized surveillance, the number of reported cases has increased 2- and 20-fold in the United States and Canada, respectively [4,401]. Lyme disease is caused by bacteria of the *Borrelia burgdorferi* sensu lato complex, which are transmitted to humans via ticks of the *Ixodes* genus. Infection with *B. burgdorferi* often results in *erythema migrans*, more commonly known as a “bull’s-eye” rash, and flu-like symptoms. Moreover, bacterial dissemination can lead to more severe clinical manifestations such as arthritis, carditis, and neurological impairments [28,145,159]. Given the current challenges in diagnosing and treating LD, novel prevention strategies are critical in reducing the LD health care burden.

Currently, there is no vaccine available for the prevention of LD in humans. The development of LD vaccines has primarily focused on two immunogenic lipoproteins located on the surface of *B. burgdorferi*, outer surface protein A (OspA) and outer surface protein C (OspC). OspA is responsible for colonization of the tick vector, while OspC is required to initiate and maintain early infection in the host [60,87]. OspA and OspC are reciprocally expressed throughout the tick feeding cycle, with expression of OspA highest while the bacteria reside in the tick midgut [50]. Following a blood meal, the spirochete downregulates OspA expression to facilitate migration into the salivary glands and expression remains low throughout infection of the mammalian host [58,331]. Consequently, to prevent transmission during feeding, vaccines targeting OspA must induce high titres of antibodies in the host prior to encountering the pathogen. Furthermore, vaccines targeting OspA are thought to require frequent boosters to maintain high

circulating antibody titres. In contrast, OspC is highly expressed by the bacteria in response to hematophagy and during initial infection of the mammalian host [50,58]. Importantly, antibodies targeting both OspC and OspA are capable of borreliacidal activity, an important mechanism of spirochete clearance [118,119,130]. However, only OspC-containing vaccines may confer host-based protection. While some licensed veterinary vaccines contain OspC antigens in addition to OspA, there are currently no approved vaccines that target OspC alone.

The majority of past LD vaccine research has centered on recombinant subunit vaccines. Next-generation vaccine platforms, such as DNA vaccines, offer clear advantages over protein subunit vaccines, including improved cost-efficiency and ease of production [402,403]. Previous studies investigating the use of DNA vaccines for the delivery of OspA or OspC antigens, have demonstrated strong immunogenicity and protection against challenge [83,288,289,298,299]. However, these studies employed the use of gene guns, tail-tattoo, or intradermal injection for DNA delivery. Despite the observed efficacy, these administration techniques may not be easily translated to human vaccination programs. In comparison, liposomes and lipid nanoparticles have been shown to be a safe and reliable method of delivering DNA by intramuscular injection [307,404,405]. While these particles have been explored in detail for mRNA delivery, including their use in the Comirnaty (Pfizer-BioNTech) and Spikevax (Moderna) COVID-19 vaccines, further research is required to evaluate their potential for DNA-based vaccination against a breadth of infectious agents [406,407].

In this study, we present a plasmid DNA vaccine encoding OspC-type A encapsulated in a lipid nanoparticle (pVAX1-OspC:LNP). Following the injection of two doses of the candidate or control vaccine in mice, we evaluated the serum antibody response and corresponding *in vitro* borreliacidal activity. We also used qPCR and culture microscopy to assess the bacterial burden

following challenge with *B. burgdorferi* expressing OspC-type A. Finally, histopathology was performed to observe the development of carditis and lymphadenopathy following challenge.

## 3.2. Materials and Methods

### 3.2.1. Bacterial cell culture

*Borrelia burgdorferi* strain B31-A3 used for challenge and strain B31-A3 constitutively expressing OspC-type A (*B. burgdorferi*-OspC-con) used for the borreliacidal assay were kindly provided by Dr. Patricia Rosa (National Institute of Allergy and Infectious Disease, Montana, US). The derivation of strain B31-A3 from strain B31 MI and engineering of *B. burgdorferi*-OspC-con were previously described [383,408]. *B. burgdorferi* strains were cultured from glycerol stocks in complete Barbour-Stonner-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 were not sub-cultured. Enumeration was performed using a Petroff-Hausser counting chamber under dark-field microscopy. Plasmid content of the wild-type B31-A3 strain was validated by multiplex PCR and OspC expression was confirmed by the induction of OspC-specific anti-sera following infection in naïve mice.

### 3.2.2. 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 in Health Canada, Ottawa and performed in accordance with institutional guidelines.

### 3.2.3. DNA vaccine generation

The type A outer surface protein C (OspC) gene of *Borrelia burgdorferi* B31 (GenBank accession #AAC66329.1) was designed to include the human tyrosinase signal peptide (MLLAVLYCLLWSFQTSAGHFPR; GenBank accession #AH003020) at the N-terminus [409,410]. The coding region was optimized for expression in mice and commercially synthesized in a pUC57 plasmid vector by Bio Basic Inc. (Markham, ON). The OspC gene containing the signal sequence was sub-cloned into a pVAX1 plasmid vector (ThermoFisher, Ottawa, ON), using NotI-HF and EcoRI-HF restriction enzymes (New England Biolabs, Whitby, ON). Large-scale amplifications of the pVAX1-OspC plasmid were generated using the QIAGEN Plasmid Giga Kit (Montreal, QC) according to the manufacturer's instructions. The genetic sequences were validated by Sanger Sequencing prior to nanoparticle encapsulation.

### 3.2.4. Lipid Nanoparticle (LNP) Synthesis

LNPs were synthesized by rapid microfluidic mixing of an aqueous phase containing the plasmid DNA with an ethanol phase containing the lipids. Briefly, the aqueous phase was prepared in 25 mM acetate buffer, pH 4, containing pVAX1 or pVAX1-OspC-type A DNA. The ethanol phase was comprised of ionizable lipid : phospholipid : cholesterol : PEG-lipid at a ratio of 50 : 10 : 38.5 : 1.5 mole %, respectively. Lipids used were 2,2-dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (DLin-KC2-DMA, MedKoo Biosciences, Inc., Morrisville, North Carolina, USA), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, Millipore Sigma), ovine cholesterol (Cholesterol, Millipore Sigma), and 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG2000, Millipore Sigma). The two phases were mixed using a NanoAssemblr BT™ instrument (Precision Nanosystems, Inc., Vancouver, BC) with a microfluidics cartridge containing a staggered herringbone mixing unit (Total Volume 1.5 mL; Flow Rate Ratio 3:1 aqueous:organic; Total Flow

Rate 5 mL/min). The resulting LNPs were dialysed against phosphate-buffered saline (PBS, ThermoFisher) for 18 h at 4°C in a 10k MWCO cassette (ThermoFisher), then concentrated using an Amicon Ultra 4 10k MWCO centrifugal concentrator (Millipore Sigma). For both the prime and boost vaccinations, fresh batches of DNA-LNPs were prepared within three days of injection and stored at 4°C.

### 3.2.5. LNP characterization

Nanoparticle size was measured in PBS by nanoparticle tracking analysis (NTA) (NanoSight, Malvern Panalytical, Westborough, MA, USA) under static conditions. For each sample, a 1.2 mL dilution was prepared in PBS and five consecutive tracking videos (1 min each) were recorded, with the sample syringe being advanced by 100 µL between each video. NTA sizing data is the result of merging the five separate tracking videos (5 x 1 min videos).

Nucleic acid encapsulation efficiency in LNP samples was measured using Triton X-100 (Millipore Sigma) and SYBR<sup>TM</sup> Gold (Thermo Fisher). Briefly, LNPs were disrupted with 1% Triton X-100 in PBS in a 96-well plate. 1X SYBR<sup>TM</sup> Gold dye in PBS was added to the sample and the plate was read using a Synergy MX plate reader (Ex/Em: 495/537 nm) (BioTek, Winooski, Vermont, USA). Total nucleic acid in the sample was determined by applying the relative fluorescent units to a standard curve of nucleic acids with detergent. Non-disrupted LNPs were also measured concurrently under the same conditions, but without detergent, to detect un-encapsulated nucleic acid. The amount of DNA encapsulated in LNPs was determined by subtracting the un-encapsulated DNA from the total amount of DNA in a sample. Finally, the amount of encapsulated DNA was divided by the total amount of DNA in a sample to obtain the encapsulation efficiency.

### 3.2.6. Immunizations and bacterial challenge

Groups of five eight-week old mice were injected intramuscularly into the tibialis anterior with lipid nanoparticles containing 12 µg of pVAX1 (pVAX1:LNP) or pVAX1-OspC-type A (pVAX1-OspC:LNP). The injection volume was 50 µL distributed between two injections sites, one in each leg. A second identical vaccination was administered four weeks after the initial injection. Four weeks after the boost injection, mice were challenged subcutaneously between the shoulder blades with  $1 \times 10^5$  *B. burgdorferi*. For challenge, wild-type *B. burgdorferi* B31-A3 was grown to mid-log phase in complete BSK-H media as described above. Cultures were centrifuged at  $5000 \times g$  for 20 minutes and resuspended in incomplete BSK-H media without rabbit serum (Millipore Sigma). Cells were counted by dark-field microscopy and diluted to a final concentration of  $1 \times 10^6$  cells/mL. Finally, mice were sacrificed 14 days after challenge for the collection of blood and tissues.

This study was repeated once with an additional three mice per group following the same above-mentioned procedure. The data from both studies were combined for a total of eight mice per group, represented in each figure.

### 3.2.7. Enzyme linked immunosorbent assay (ELISA)

96-well Nunc Maxisorp™ flat bottom plates (ThermoFisher) were coated with 0.5 µg/mL of recombinant OspC-type A (Native Antigen, Oxfordshire, UK) in carbonate buffer, pH 9.6. Plates were incubated overnight at 4°C then washed three times with PBS containing 0.05% Tween-20 (PBS-T) prior to blocking with 1% (w/v) Bovine Serum Albumin (IgG-Free, Protease-Free) (Jackson Immuno Research, West Grove, PA). For total IgG analysis, two-fold serial dilutions of mouse serum ranging from 1:50 to 1:102,400 were added to the wells and plates were incubated

for 1 h at 37°C. For IgG2a and IgG1 analysis, serum was diluted three-fold between the range of 1:100 to 1:11,809,800. Subsequently, plates were washed six times with PBS-T and 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) diluted 1:5,000 in blocking buffer for 1 h at 37°C. The plates were washed six times with PBS-T prior to the addition of 100 µL of Tetramethylbenzidine (TMB) substrate (Cell Signaling Technology, Danvers, MA). After incubating for five minutes at room temperature, the reaction was terminated with 0.16 M sulfuric acid. Finally, the absorbance at 450 nm was measured using a spectrophotometer. Endpoint titres were defined as the reciprocal of the highest dilution that resulted in an OD greater than the average OD of all wells containing serum from mice vaccinated with the pVAX1:LNP vaccine plus three times the standard deviation.

### 3.2.8. Serum borreliacidal assay

Cultures of *B. burgdorferi* B31-A3 constitutively expressing OspC type A (*B. burgdorferi*-OspC-con) were grown to mid-log phase and diluted to  $5 \times 10^7$  cells/mL. Four microlitres of culture were then added to 0.2 mL microtubes, followed by 4 µL of mouse serum and 4 µL of guinea pig serum (GPS, Complement Technologies, Tyler, TX). Reaction volumes were topped to 20 µL with complete BSK-H media and incubated at 35°C and 1.5% CO<sub>2</sub> for 24 hours. Motile cells were counted using a Petroff-Hausser counting chamber under dark-field microscopy. A negative control consisting of serum from a naïve mouse heat-inactivated for 56°C for 30 min was also included. Percent live spirochetes was determined by dividing the cell count by the average cell count of negative control samples.



### 3.2.9. qPCR for bacterial burden

At necropsy, the bladder, left ear, left tibiotarsal joint, inferior half of the heart, and left popliteal 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. DNA samples were diluted to match the lowest concentration sample and 5 µL of DNA was added to each well of a 96-well MicroAmp™ Fast Optical 96-Well Reaction Plate (ThermoFisher) containing 10 µL TaqMan Fast Advanced MasterMix and 1 µL of either *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). Reaction volumes were topped to 20 µL with nuclease-free water. qPCR reactions were performed using an Applied Biosystems™ 7500 Fast Real-time PCR instrument. Standard curves of plasmids encoding mouse β-actin or extracted *B. burgdorferi* genomic DNA were used to determine the number of FlaB copies per  $1 \times 10^6$  β-actin copies.

### 3.2.10. Tissue culture for bacterial identification

At necropsy, the right ear and skin surrounding the bacterial injection site were collected and placed separately in complete BSK-H media containing 6% rabbit serum and supplemented with 5 µg/mL Amphotericin B, 100 µg/mL Phosphomycin, and 50 µg/mL Rifampicin. Cultures were incubated for fourteen days at 35°C and 1.5% CO<sub>2</sub> then examined by darkfield microscopy. Cultures were considered positive if at least one spirochete was identified across five fields of view.

### 3.2.11. Determination of lymphadenopathy

The left and right axillary lymph nodes were collected at necropsy and placed together 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). Single-cell suspensions were generated by gently pressing lymph nodes between two frosted microscope slides then filtering through a 70  $\mu$ m cell strainer. Cell suspensions were centrifuged at  $300 \times g$  for 5 minutes and resuspended in media. White blood cell counts were obtained using the Sysmex XT-2000iV hematology analyser and represent the counts for a combined suspension of the left and right axillary lymph nodes.

### 3.2.12. Histopathology

At necropsy, the heart was bisected horizontally and the superior half of the heart, including the origin of the great vessels, heartbase, and top halves of the ventricles and atria was collected. The tissue was immersed in 10% neutral buffered formalin for 48 h before proceeding with trimming and routine histologic processing. Tissues were embedded in paraffin and 4  $\mu$ m thick sections were stained with Harris hematoxylin (ThermoFisher) and Instant eosin (ThermoFisher) in an EpreDia™ Gemini™ AS Automated Slide Stainer (ThermoFisher). Inflammation in the heart sections was subjectively assessed by a certified veterinary pathologist and assigned a numeric grade (0-5) where 0 is normal, 1 is minimal, 2 is mild, 3 is moderate, 4 is marked, and 5 is severe.

### 3.2.13. Statistical Analysis

Statistical significance was calculated using a one-sided Wilcoxon rank-sum exact test at a 5% significance value. All statistical analyses were performed using SAS Enterprise Guide 7.1. \* p-value < 0.05, \*\* p-value < 0.01, \*\*\* p-value < 0.001, \*\*\*\* p-value < 0.0001.

### 3.3. Results

#### 3.3.1. DNA vaccine design and encapsulation

First, we engineered a pVAX1 vector to express the outer surface protein C (OspC) of *Borrelia burgdorferi* B31. To promote antigen secretion, a human tyrosinase signal sequence was added to the N-terminus of the OspC gene (Figure S3.1A). Next, the pVAX1-OspC plasmid was encapsulated into lipid nanoparticles (pVAX1-OspC:LNP) prior to immunization of mice to facilitate nucleic acid delivery. The pVAX1 plasmid, which does not express an antigen of interest, was also encapsulated in LNPs as a negative control (pVAX1:LNP). When analyzed by nanoparticle tracking analysis, both particle formulations resulted in similar size distributions and encapsulation efficiencies (Table 3.1, Figure S3.1B).

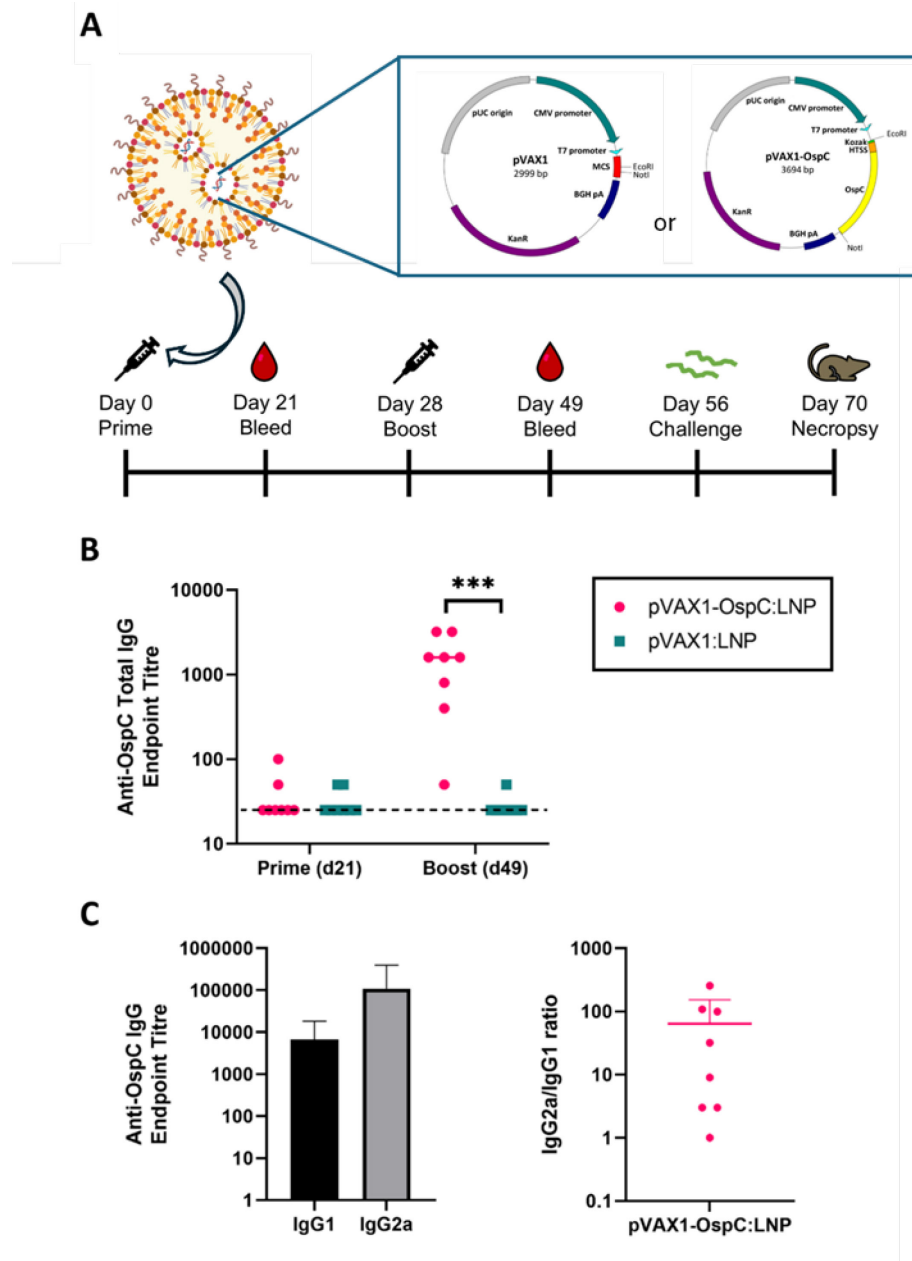
| Vaccine Group  | Dose  | Mean ± SD (nm) | Encapsulation Efficiency |
|----------------|-------|----------------|--------------------------|
| pVAX1:LNP      | Prime | 93 ± 30        | >97%                     |
| pVAX1:LNP      | Boost | 107 ± 38       | >99%                     |
| pVAX1-OspC:LNP | Prime | 92 ± 27        | >97%                     |
| pVAX1-OspC:LNP | Boost | 103 ± 36       | >99%                     |

**Table 3.1.** Characterization of DNA-LNP vaccine formulations. Mean sizes were measured by nanoparticle tracking analysis (NTA). Encapsulation efficiency was determined by SYBR™ Gold assay. SD: standard deviation.

#### 3.3.2. pVAX1-OspC:LNP induces robust antibody response with borreliacidal activity

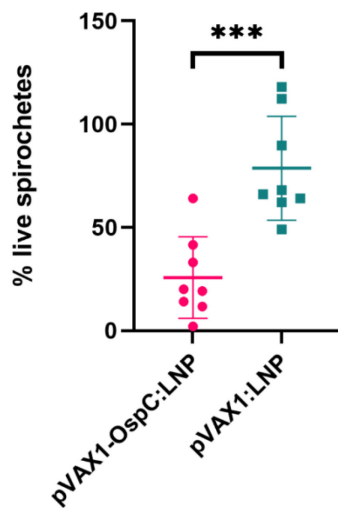
To evaluate the immunogenicity and protection conferred by our candidate vaccine, C3H/HeN mice were injected intramuscularly with 12 µg of either pVAX1-OspC:LNP or pVAX1:LNP (Figure 3.1A). A second dose of the same construct was administered 28 days after

the initial prime injection. Three weeks after the prime injection, OspC-type A-specific antibodies were undetectable in the serum of either vaccine group, however, significant anti-OspC-type A antibodies were detected in mice vaccinated with pVAX1-OspC:LNP three weeks after the boost immunization (Figure 3.1B). Analysis of the IgG2a/IgG1 antibody ratio revealed that the antibody response to pVAX1-OspC:LNP vaccination was predominated by the IgG2a subtype, indicating a Th1 immune bias (Figure 3.1C).



**Figure 3.1.** pVAX1-OspC:LNP vaccination is immunogenic in mice. **(A)** Schematic depicting the immunization and challenge schedule in C3H/HeN mice. Mice were bled on day 21 and day 49. Five mice per group were injected intramuscularly with pVAX1-OspC:LNP or pVAX1:LNP on day 0 and day 28. Mice were challenged subcutaneously with  $1 \times 10^5$  *Borrelia burgdorferi* B31 on day 56 and sacrificed on day 70 for tissue collection. **(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)** Titres and ratio of IgG2a and IgG1 OspC-specific serum antibodies three weeks after boost immunization (day 49). Error bars represent standard deviation. \*\*\* p-value < 0.001

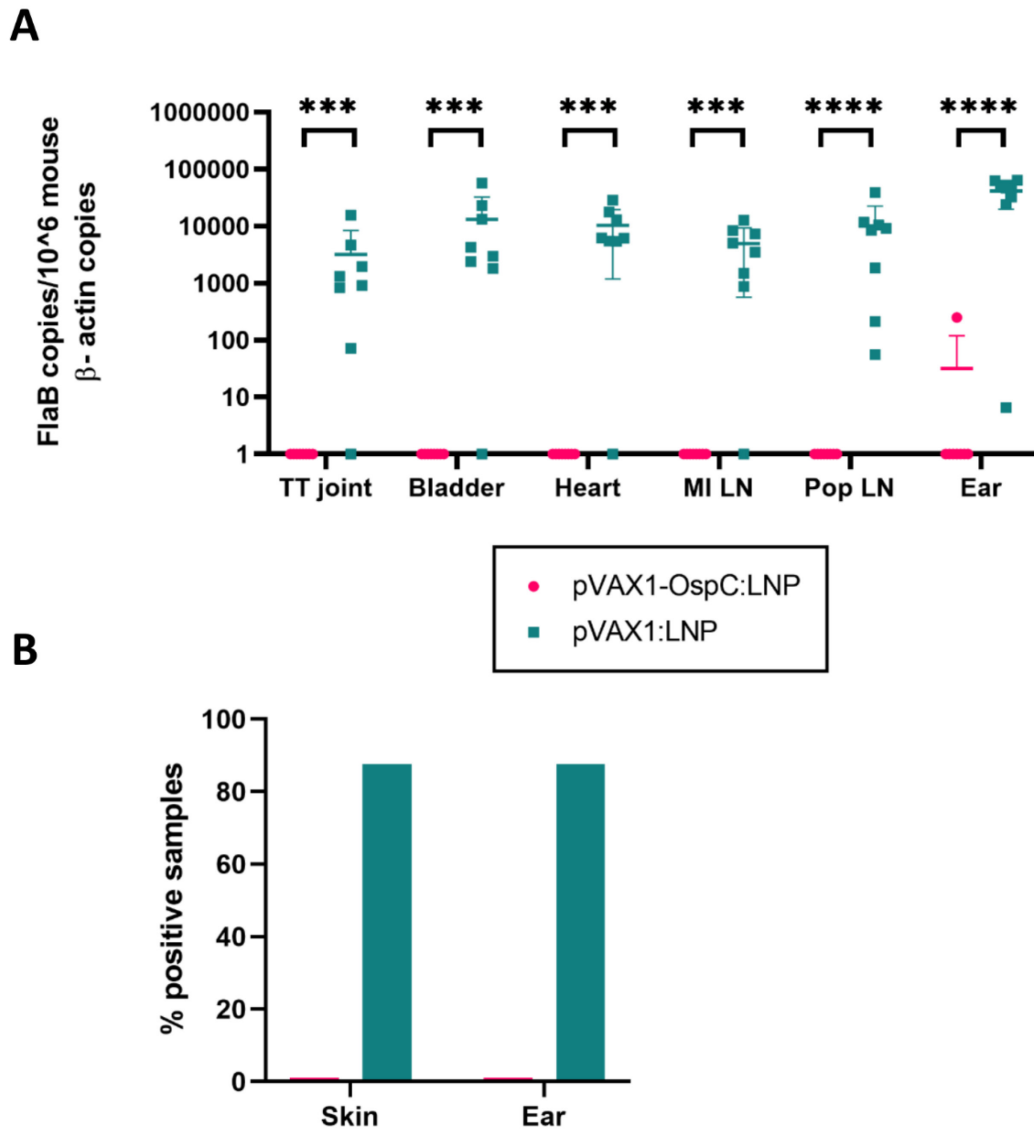
Next, we evaluated the bactericidal ability of the antibodies generated in response to vaccination. Investigating the mechanisms of OspC-based vaccine protection can be made difficult by the variability of OspC expression during *in vitro* culture. To best reflect the high OspC expression levels that occur *in vivo*, we conducted the *in vitro* borreliacidal assay using a *B. burgdorferi* strain engineered to constitutively express OspC-type A (*B. burgdorferi*-OspC-con). The bacteria was cultured with mouse serum collected three weeks after boost vaccination in the presence of guinea pig serum for 24 hours. When standardized to a naïve serum control, serum from mice vaccinated with the candidate pVAX1-OspC:LNP vaccine resulted in a 80% reduction in live spirochetes on average (Figure 3.2). In comparison, the serum from mice in the pVAX1:LNP group resulted in an average of only 33% fewer live spirochetes. These results indicate that the candidate vaccine generates functional OspC-specific antibodies that are capable of lysing the spirochete.



**Figure 3.2.** pVAX1-OspC:LNP vaccination generates borreliacidal antibodies. Serum from vaccinated or naïve mice was collected three weeks after boost injection (day 49) and incubated with *B. burgdorferi*-OspC-con for 24 hours. Motile *B. burgdorferi* cells were enumerated by dark field microscopy. Percentage live spirochetes represents the percentage of motile cells compared to naïve serum control. Error bars represent standard deviation. \*\*\* p-value < 0.001

### 3.3.3. pVAX1-OspC:LNP confers protection against homologous *B. burgdorferi* challenge

To evaluate the protection conferred by the candidate vaccine, immunized mice were challenged with  $1 \times 10^5$  *B. burgdorferi* B31-A3 four weeks after boost vaccination. Two weeks post-challenge, the mice were sacrificed and several tissues were collected to determine the bacterial burden. qPCR analysis of these organs revealed that vaccination with pVAX1-OspC:LNP resulted in *B. burgdorferi* DNA loads below the limit of detection in all tested organs (Figure 3.3A). In comparison, the challenge of mice vaccinated with the control pVAX1:LNP vaccine resulted in significant bacterial burden across all organs. Similarly, cultures of the ears and injection site skin of seven out of eight mice vaccinated with the control pVAX1:LNP were positive for *B. burgdorferi* when examined under dark-field microscopy (Figure 3.3B). In line with the qPCR results, all cultures of the skin and ears obtained from pVAX1-OspC:LNP vaccinated mice were negative for spirochetes when examined by dark-field microscopy. Together, these results demonstrate that vaccination with pVAX1-OspC:LNP fully prevented the establishment and dissemination of *B. burgdorferi* infection in C3H/HeN mice.



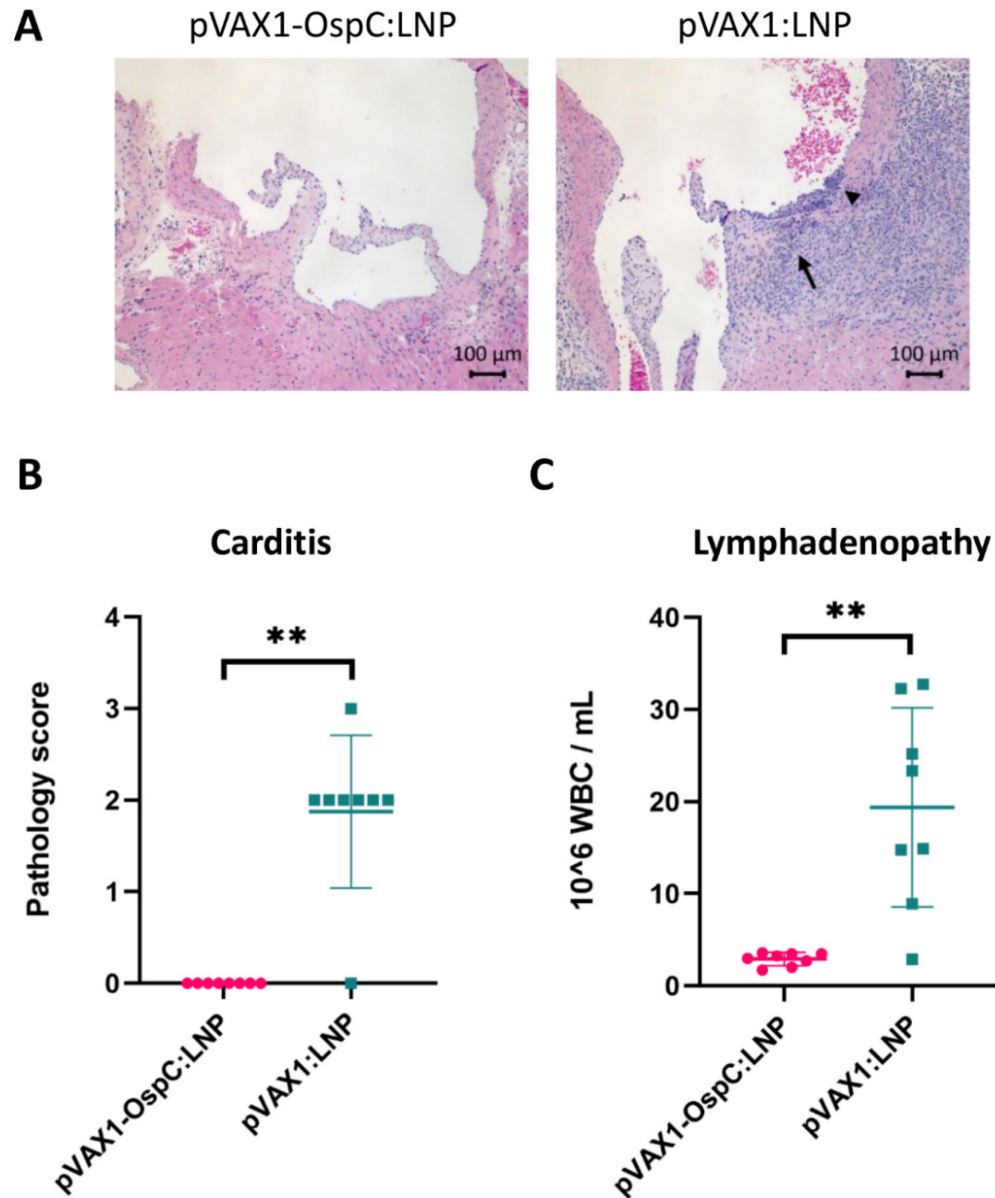
**Figure 3.3.** Vaccination with pVAX1-OspC prevents bacterial infection and dissemination. (A) qPCR was used to determine bacterial burden in a variety of mouse tissues 14 days after *B. burgdorferi* challenge. Bacterial burden is represented by copies of the *B. burgdorferi* FlaB gene per  $1 \times 10^6$  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. (B) The right ear and skin surrounding the bacterial injection site were collected at necropsy, placed in BSK-H growth media, and incubated for 14 days. Cultures were considered positive if at least one spirochete was identified by dark-field microscopy across five fields of view. Error bars represent standard deviation. \*\*\* p-value < 0.001, \*\*\*\* p-value < 0.0001.



### 3.3.4. pVAX1-OspC:LNP protects against carditis and lymphadenopathy

Disseminated infection with *B. burgdorferi* can lead to carditis in both humans and C3H/HeN mice [159,373,411]. To evaluate the inflammation resulting from *B. burgdorferi* challenge, histopathology analysis of the heart was performed following necropsy. As expected, mice vaccinated with the control pVAX1:LNP vaccine consistently developed mild-to-moderate mononuclear-neutrophilic inflammation of the heart base and aorta 14 days post-challenge (Figure 3.4A,B). In comparison, mice vaccinated with pVAX1-OspC:LNP were protected from developing any detectable carditis. These results are in line with our demonstration that the candidate vaccine prevents bacterial dissemination into the heart tissue.

Acute infection with *B. burgdorferi* can also result in systemic lymph node enlargement, termed lymphadenopathy [30,158,390]. The most severe lymphadenopathy is typically observed nearest to the site of infection [158,390]. For this reason, we collected the axillary lymph nodes at necropsy for enumeration of leukocytes. Control mice that were positive for *B. burgdorferi* infection based on the culture data above had on average eight-fold higher leukocyte counts in the lymph nodes than all *B. burgdorferi*-negative mice, including all mice vaccinated with pVAX1-OspC:LNP (Figure 3.4C). Taken together, these results demonstrate that the candidate pVAX1-OspC:LNP vaccine protects against the development of lymphadenopathy and carditis associated with Lyme disease and bacterial dissemination.



**Figure 3.4.** Vaccination with pVAX1-OspC:LNP prevents the development of carditis and lymphadenopathy. **(A)** Representative images of H&E stained heart tissue from mice vaccinated with pVAX1-OspC:LNP or pVAX1:LNP post-challenge. Arrows indicate moderate mononuclear-neutrophilic inflammation in the heart base (long arrow) and transmurally in the aortic origin (short arrowhead). **(B)** Summary of carditis pathology scores as determined by trained histopathologist. Scoring criteria is described in full in the methods section of this article. **(C)** White blood cell (WBC) counts from combined left and right axillary lymph nodes extracted from vaccinated mice two weeks after bacterial challenge. Error bars represent standard deviation. \*\* p-value < 0.01.

### 3.4. Discussion

OspC, an immunogenic lipoprotein found on the surface of *B. burgdorferi*, is an attractive target for the development of second-generation LD vaccines. Previous and current LD vaccines in clinical trials have focused on targeting OspA, however, OspC-based vaccines may offer a key advantage. Specifically, expression of OspC is induced by tick feeding and remains high during early infection of the host [50,58]. OspC expression persists for 1-3 weeks in mammals, allowing time for the secondary immune response to facilitate clearance of the infection [92,93,412]. Therefore, unlike vaccines that target OspA and only provide vector-based immunity, OspC-based vaccines have the potential to provide host-based immunity.

When vaccines targeting OspC alone have been evaluated in preclinical trials, variable results have been reported. Wallich et al. and Xiao et al. both found that OspC was not immunogenic in mice when employing a DNA and viral-vector platform, respectively [297,413]. Additionally, oral vaccination with rOspC was found to be immunogenic but not protective against homologous or heterologous tick-challenge [414]. In comparison, Scheiblhofer et al. and Klouwens et al. both demonstrated the immunogenicity and protective efficacy of OspC DNA vaccines delivered by gene gun, intradermal injection, or tail-tattoo [83,299]. It should be noted that due to the genetic diversity of the OspC protein both of these studies demonstrated protection only against homologous bacterial strains administered via needle- and tick-challenge, respectively. To our knowledge, no OspC-based vaccine has conferred protection against heterologous tick- or needle-challenge to date.

In this report, we demonstrate that vaccination of C3H/HeN mice with a DNA-LNP vaccine targeting OspC-type A afforded effective protection against homologous needle challenge with *B. burgdorferi* B31 (OspC-type A) and the resulting pathology associated with Lyme

borreliosis. First, candidate vaccine formulations for prime and boost injections were determined to have mean particle sizes of 92 nm and 103 nm with standard deviations of 27 nm and 36 nm, respectively. These size distributions are within the optimal nanoparticle size range of 20-200 nm for uptake by antigen presenting cells and induction of robust immunogenicity [415–417]. In addition, all particle formulations had encapsulation efficiencies >97%, which enables lower cost production at larger scales.

Second, we demonstrated that two doses of the pVAX1-OspC:LNP vaccine induced significant OspC-specific antibodies predominated by a Th1 bias. This finding is in line with that of Wagemakers and colleagues, who reported that vaccination with their DNA-based OspC vaccine by tail-tattoo induced an IgG2a-biased antibody response [298]. Additionally, a study by Scheiblhofer et al. suggested IgG1 antibodies may contribute more significantly to vaccine-induced protection, illustrating the need for further studies on the roles of IgG subtypes in the context of LD vaccination and infection [83]. In this study, we also demonstrate that antibodies generated in response to the candidate vaccine possess notable borreliacidal activity against a homologous bacterial strain *in vitro*. Clearance of *B. burgdorferi* is known to be largely mediated by the humoral immune response, specifically via the classical complement pathway and antibody-dependent cellular phagocytosis [418,419]. Mice deficient in the  $\alpha$ -chain of C1q, a component of the classical complement pathway, have demonstrated higher bacterial burdens and dissemination [418]. The induction of bactericidal antibodies is therefore a critical feature of LD vaccine development.

Finally, we performed qPCR and microscopy to determine the bacterial burden in a range of tissues following homologous bacterial challenge. All tissue samples from pVAX1-OspC:LNP vaccinated mice were found to be negative for *B. burgdorferi*, indicating that just two doses of the

candidate vaccine provided protection from infection and bacterial dissemination. In comparison, previous LD vaccines and the majority of vaccines undergoing preclinical and clinical testing, employ a regimen of at least three doses [83,268,288,289,298–300,420]. Furthermore, the candidate vaccine prevented the development of carditis and lymphadenopathy that were observed in the control group and are consistent with LD infection [30,158,373,390,411]. This lack of carditis and lymphadenopathy in vaccinated mice is in agreement with the qPCR data, which confirmed that *B. burgdorferi* was unable to disseminate to the heart tissue and lymph nodes of vaccinated mice.

In the United States, 1-10% of LD patients develop carditis [421]. Lyme carditis may be caused by multiple factors, including direct infection of the myocardium, excessive macrophagic and lymphocytic infiltration, and autoimmunity [422]. In humans, Lyme carditis most commonly affects the atrioventricular node [421,422]. In comparison, carditis in mice typically presents with inflammation of the connective tissue in the heart base and aorta [373]. The ability of LD vaccines to prevent carditis associated with infection has not been well documented. In the present study, we observed mild-to-moderate mononuclear-neutrophilic inflammation of the heart base and aorta in all infected mice and, most importantly, we report the complete absence of carditis in all mice vaccinated with pVAX1-OspC:LNP. Overall, these findings demonstrate that vaccines targeting OspC alone are suitable for the prevention of infection with homologous strains of *B. burgdorferi*.

A major limitation of vaccines that target OspC exclusively is the genetic heterogeneity of the OspC antigen. Over 20 different subtypes of OspC have been found among *B. burgdorferi* pathogens and cross-protection between subtypes is limited [423]. Prior infection with *B. burgdorferi* or vaccination with recombinant OspC protein has shown to protect against needle- or tick-challenge of bacterial strains only if they possess the homologous OspC allele [70,98,101].

For the greatest biological relevance, human LD vaccines should confer protection against heterologous challenge using a tick-infection model. Therefore, future studies may explore the efficacy of DNA vaccines encoding multiple OspC subtypes. In addition, OspC may be used in combination with tick salivary proteins, such as Salp15, or other *B. burgdorferi* surface proteins to improve the breadth of protection. Future studies should therefore evaluate the efficacy of mono- and polyvalent vaccines in larger populations featuring both sexes.

Due to their ease of production and cost-efficiency, DNA-LNP platforms are ideal for the administration of multiple antigens. Polyvalent DNA-LNP vaccines can be administered in a multitude of ways, including: a) a single plasmid expressing multiple antigens, b) multiple plasmids each encoding an antigen and encapsulated in separate LNPs and c) multiple plasmids each encoding an antigen of interest and encapsulated together in an LNP. Additionally, LNPs encapsulating different plasmids can be formulated and injected together or separately. The optimal administration method of polyvalent DNA vaccines delivered by LNPs requires further investigation.

To our knowledge, few studies have demonstrated the efficacy of LNPs for the delivery of DNA vaccines. DNA vaccines delivered by syringe without a carrier are generally considered to have low immunogenicity and may require the addition of CpG or other adjuvants [424]. Additionally, unformulated DNA vaccines are susceptible to DNase degradation. Several studies have demonstrated that encapsulation with LNPs protects the DNA payload against treatment with DNase enzymes [306,425,426]. Furthermore, we demonstrate that delivery of DNA by LNPs induces sufficient immune responses without the incorporation of an adjuvant or use of a specialized immunization device. The DNA-LNP platform offers an advantage over conventional LD vaccine platforms, such as recombinant protein and bacterin vaccines, which also require the

use of adjuvants. Together, the rapid production, ease of administration, and immunogenicity of DNA-LNP vaccines, render this platform an attractive candidate for LD vaccine development.

Overall, our study demonstrates the ability of LD vaccines targeting homologous OspC to confer sterile immunity in susceptible tissues and afford effective protection against Lyme carditis and lymphadenopathy. This study also provides support for the use of LNPs in the formulation of DNA vaccines in addition to their conventional use in mRNA vaccines. Given the increasing rates of LD in North America and the genetic diversity of the causative agent, the conclusions drawn from this study could benefit the generation and application of broadly protective human LD vaccines.

**Supplementary Materials:** Appendix – Figure S3.1 DNA lipid nanoparticle vaccine design and characterization.

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**Ethics statement:** The animal study was reviewed and approved by the Health Canada Institutional Animal Care Committee.

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**Conflict of interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.



# Chapter 4: Novel recombinant vaccinia virus-vectored vaccine affords complete protection against homologous *Borrelia burgdorferi* infection in mice

## Preface

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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.

## 4.1. 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 [2,335]. 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 [4,160,427,428]. 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 [28,429]. Despite the efficacy of commercially available canine LD vaccines, there has been no vaccine available for human use since the withdrawal of the LYMERix™ vaccine from the market in 2002 [430].

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™ vaccine, is primarily expressed by the bacterium

while it resides in the tick vector but is downregulated during transmission into the mammalian host [50]. Consequently, OspA-based vaccines may require multiple booster doses to achieve circulating antibody levels capable of preventing tick-to-host transmission [268]. In addition, molecular similarity between OspA and human leukocyte function-associated antigen (hLFA1) has evoked concerns of vaccine-induced autoimmunity [270,431]. 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 [272,420]. In contrast to OspA, OspC is induced during transmission to the mammalian host and plays a crucial role in early mammalian infection and bacterial dissemination [50,432]. Its sustained expression throughout host infection renders OspC an attractive target for vaccine development [92,268,412]. 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 [433]. It has been widely used for smallpox vaccination campaigns, eventually leading to the development of several attenuated strains that cause fewer adverse reactions [434]. 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 [435]. 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 [436]. A previous study by our lab demonstrated that

this modified vector conferred protection against respiratory syncytial virus after a single intramuscular injection [437]. 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 [437,438].

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.

## 4.2. Materials and Methods

### 4.2.1. 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 [383,387,408]. In brief, strains were cultured from glycerol stocks in complete Barbour-Stonner-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.

#### 4.2.2. 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.

#### 4.2.3. 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) [410,439,440]. 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'-TACGCTACTATAACCGGCATT-3', 5'-GGATCAGCATCTGGTACAAT-3'.

The recombinant vaccinia virus was generated as previously described [435]. In brief, the shuttle vector was transfected into HeLa/PKR-knockout cells infected with the parental VV $\Delta$ E3L $\Delta$ K3L. The virus was harvested 48 hours 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 [435].

Viral titres were determined using a standard plaque assay in which serial dilutions of the virus were incubated on RK13 cells for 2 hours 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.

#### 4.2.4. 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 hours at 37°C and then cells were collected by scraping into the media. Cells were freeze-thawed three times then centrifuged at  $250 \times g$  for 10 min. The cell pellet was lysed in sodium dodecyl sulfate (SDS) lysis buffer (50mM Tris-HCl pH 6.8, 2% (W/V) SDS, 0.1% (W/V) bromophenol blue, 10% (W/V) glycerol, 5% fresh  $\beta$ -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 hour 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 hour 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).

#### 4.2.5. Immunizations and bacterial challenge

Eight-week-old mice were randomly assigned to groups and injected subcutaneously with 50  $\mu$ L of the Vanguard® CrLyme vaccine (Zoetis, NJ) (n=13) or intramuscularly with 50  $\mu$ L 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  $\mu$ L 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 anesthesia.

#### 4.2.6. 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 [387]. Cultures were considered positive if at least one mobile spirochete was identified across five fields of view.

#### 4.2.7. 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  $\beta$ -actin gene assay (ID: Mm02619580\_g1, ThermoFisher) [387]. Standard curves of plasmids encoding mouse  $\beta$ -actin or extracted *B. burgdorferi* genomic DNA were used to determine the number of FlaB copies per  $10^6$   $\beta$ -actin copies.

#### 4.2.8. 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 hematoxylin and eosin staining was performed as previously described [387]. 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 [441,442]. 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.

#### 4.2.9. 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 hematology analyzer.

#### 4.2.10. Enzyme-linked immunosorbent assay (ELISA)

ELISAs were performed as previously described [387]. 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 sulfuric 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.

#### 4.2.11. 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 5,000 g for 15 min. The bacterial pellet was washed with PBS and resuspended in 200  $\mu$ L 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  $\mu$ L of the diluted and stained *B. burgdorferi*-OspC was then added to each well of 96-well plate. Next, 20  $\mu$ L 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  $\mu$ L 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 hour at 37°C without shaking. To arrest the phagocytosis, 100  $\mu$ L of cold PBS was added to each well and plates were analyzed immediately by flow cytometry using a BD *FACSymphony*<sup>™</sup> A1.

#### 4.2.12. 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  $\mu$ m 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 hematology 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 hours at 37°C in RPMI media. After incubation the plate was centrifuged at 1800 rpm 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 (MilliporeSigma) and data analysis was performed using MILLIPLEX Analyst version 5.1 software to determine the concentration of each cytokine.

#### 4.2.13. 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 hours 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 hour 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- $\gamma$  BUV737 (Clone XMGI.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 FACS Aria™ Fusion. Data were analyzed using FlowJo v10 software (Treestar). A representative gating strategy is depicted in Figure S4.1.

#### 4.2.14. 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.

### 4.3. Results

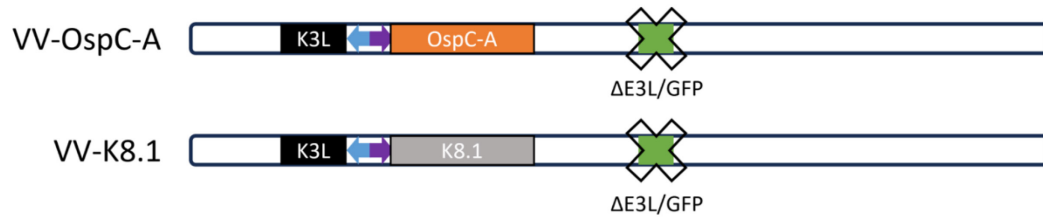
#### 4.3.1. VV-OspC-A protects against homologous *B. burgdorferi* infection and dissemination

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 4.1A). 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 [125]. Prior to vaccination, OspC expression was confirmed *in vitro* by western blotting of VV-OspC-A infected BHK21 cells (Figure S4.2). Four weeks after the boost vaccination (day 56), mice were challenged by

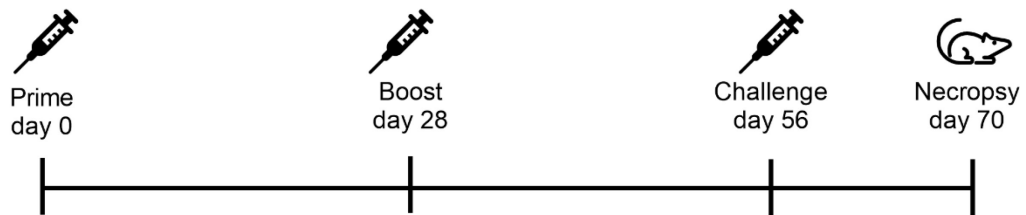
subcutaneous injection of  $10^6$  *B. burgdorferi sensu stricto* strain B31-A3. Finally, mice were sacrificed 14 days post-challenge (day 70) for tissue collection (Figure 4.1B).

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 4.1C). 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 4.1D). Importantly, spirochete 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.

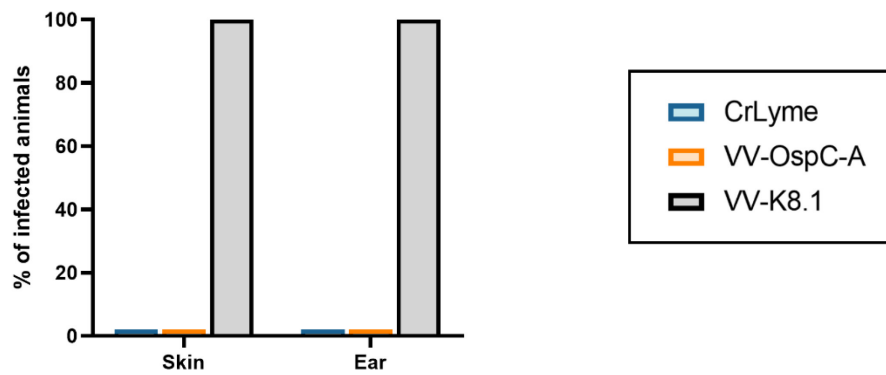
**A**



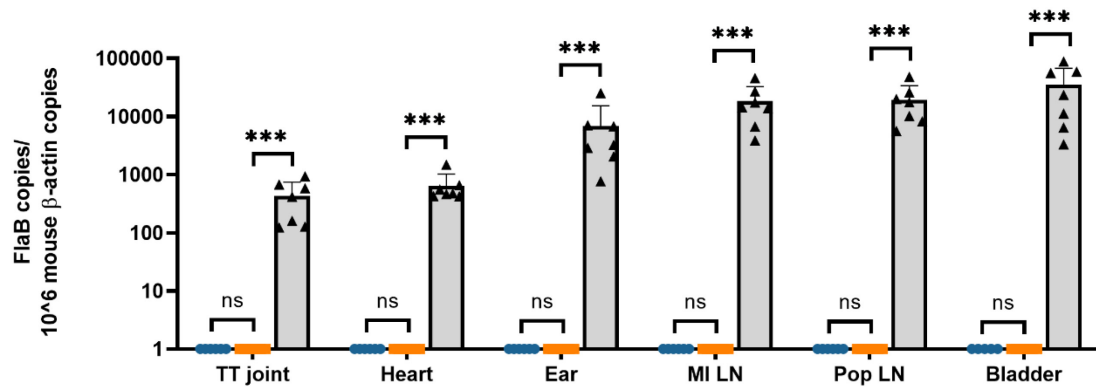
**B**



**C**



**D**

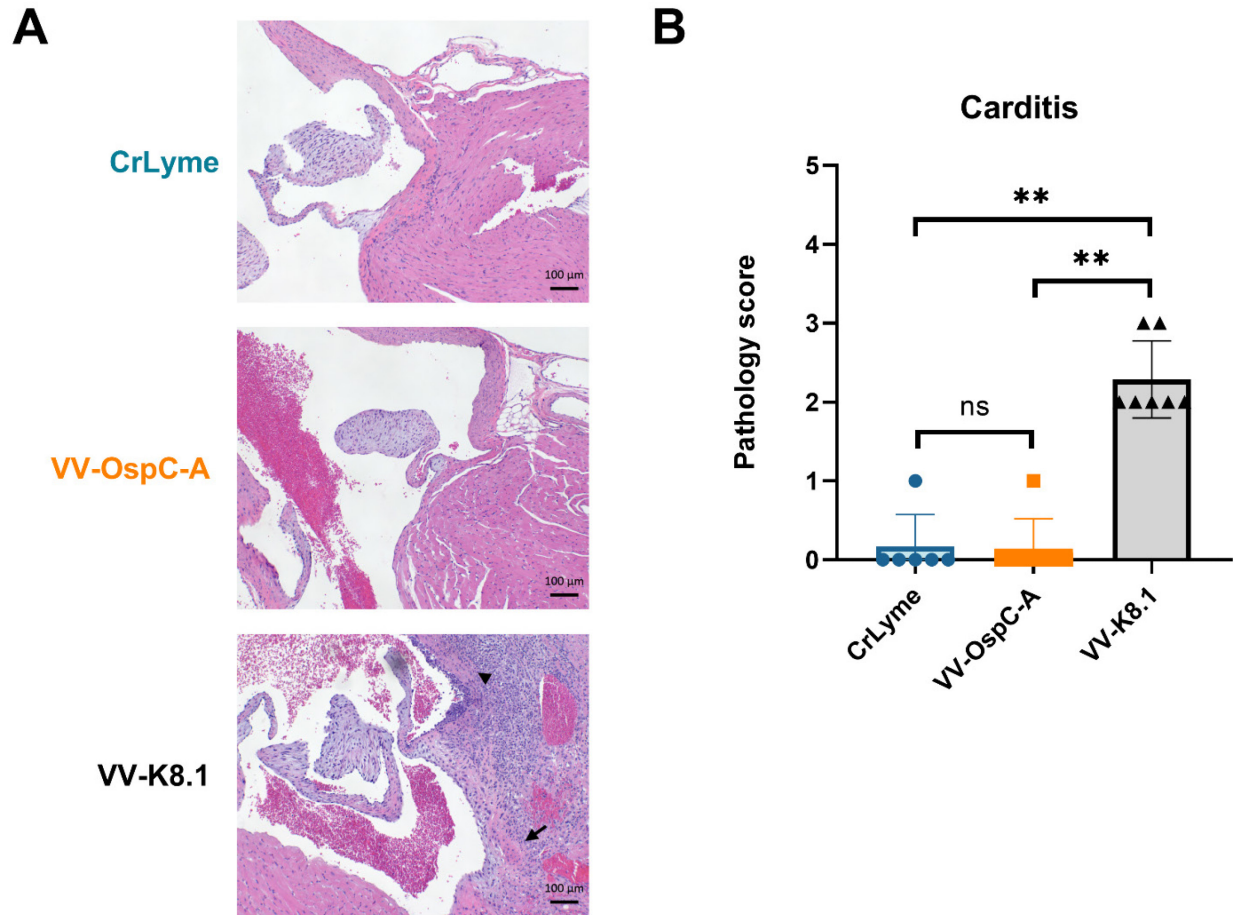


**Figure 4.1.** Vaccination with VV-OspC-A affords protection against *B. burgdorferi* challenge in mice. **A)** Schematics depicting the VV-vector vaccines encoding outer surface protein C type A (OspC-A) or an unrelated protein from herpes virus (VV-K8.1) showing the taterapox virus K3L ortholog (black 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 spirochete 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.

#### 4.3.2. 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 4.2A, 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.

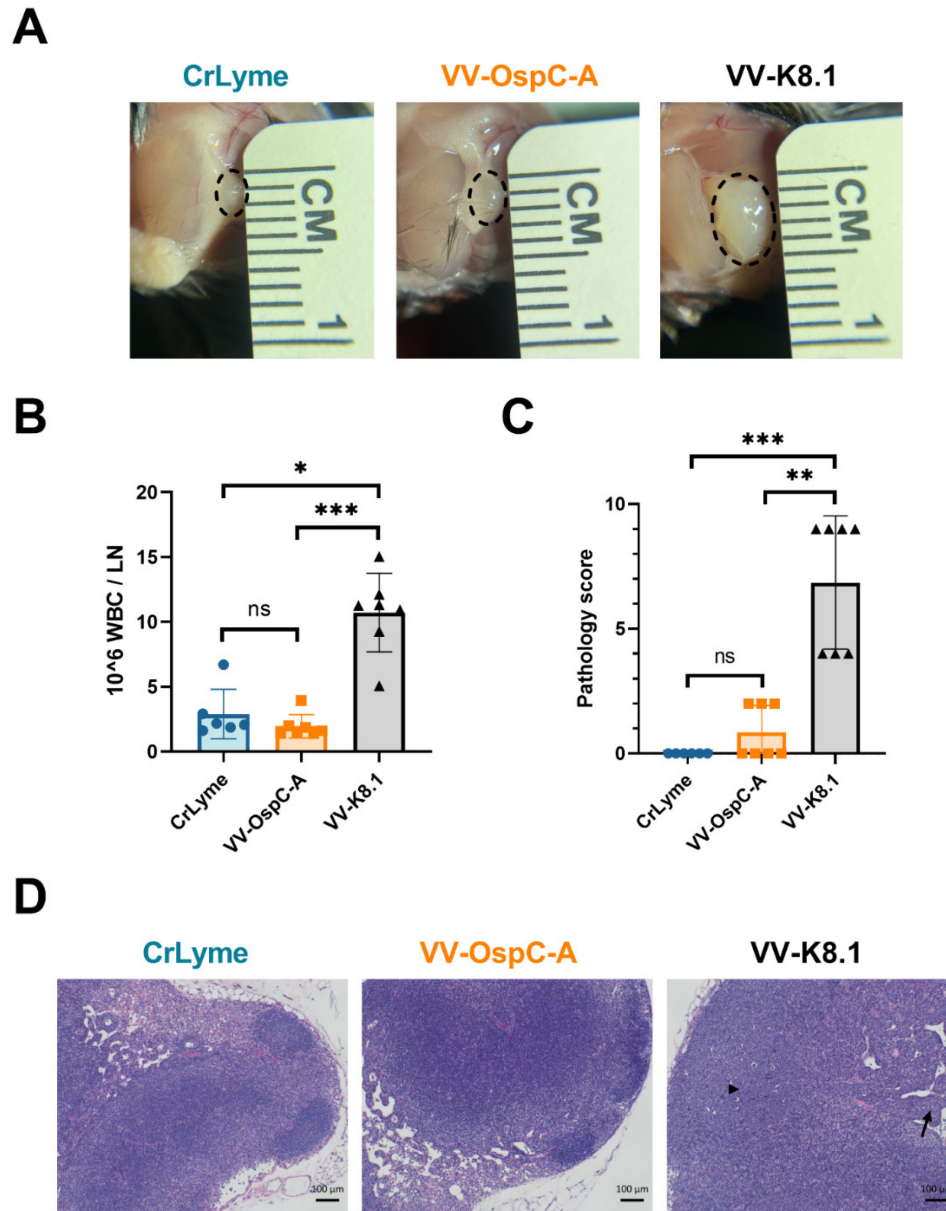




**Figure 4.2.** Vaccination with VV-OspC-A prevents the development of Lyme carditis. **A)** Representative images of H&E stained heart tissue collected from mice vaccinated with CrLyme, VV-OspC-A, or VV-K8.1 14 days post-challenge. Arrowhead indicates inflammation of the aorta wall. Arrow indicates subadjacent 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.

### 4.3.3. 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 [30,158,390]. At 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 4.3A). 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 4.3B). 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 4.3C,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.

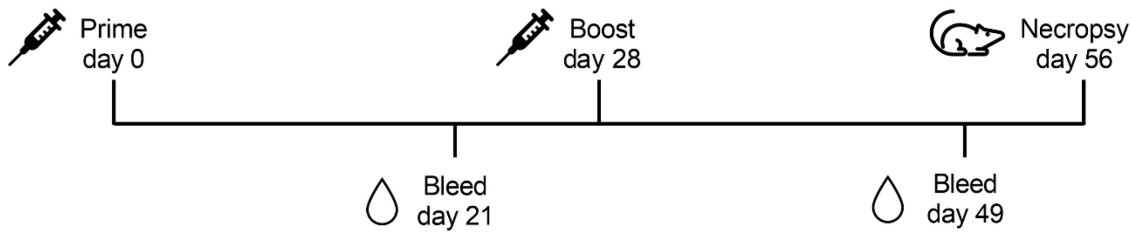
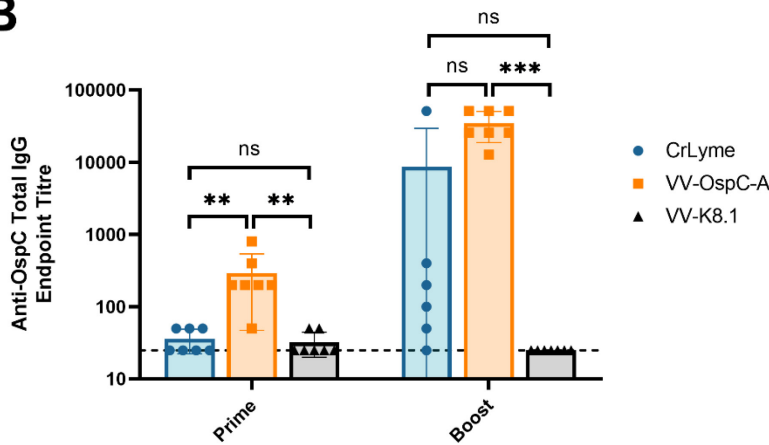
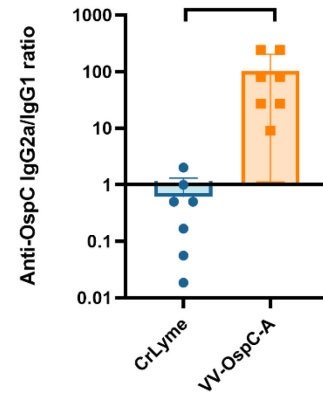


**Figure 4.3.** Vaccination with VV-OspC-A prevents the development of lymphadenopathy. **A)** In situ images of the left axillary lymph node from mice vaccinated with CrLyme, VV-OspC-A, or VV-K8.1 at the time of necropsy (day 70). The node is encircled by a dashed black line. **B)** White blood cell (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.

#### 4.3.4. 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 4.4A). 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 4.4B). 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 = 8,663), however, the titres were highly variable with some mice producing very low anti-OspC titres (Figure 4.4C). 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.

To further characterize the antibody response, the serum level 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 4.4D). 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.

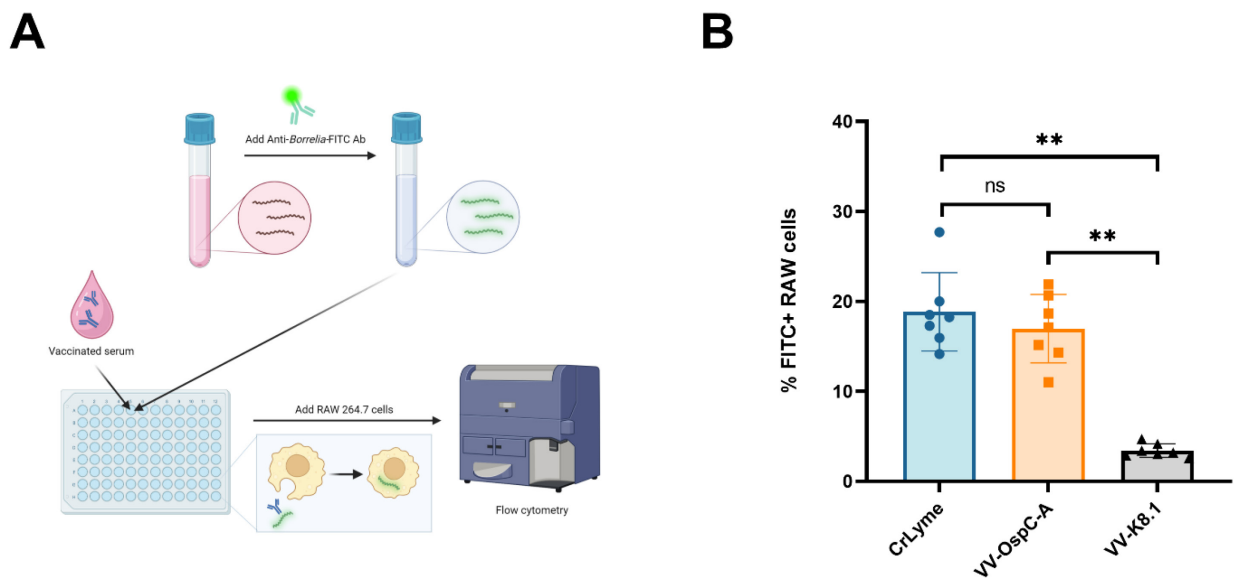
**A****B****C**

**Figure 4.4.** 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.

#### 4.3.5. 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 4.5A). Flow cytometry was used to measure the percentage of fluorescent macrophages as an indicator of 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 4.5B). 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.

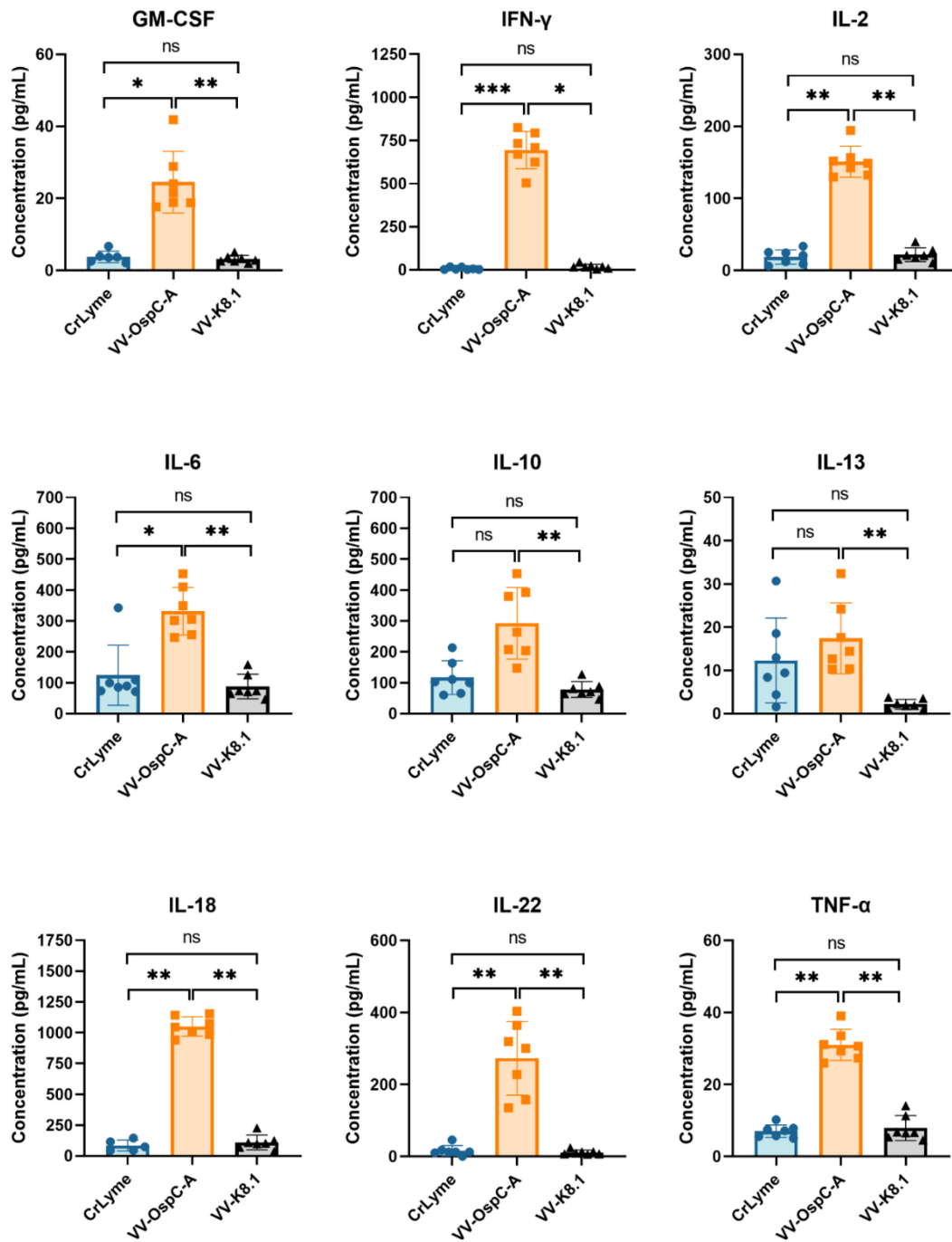


**Figure 4.5.** Vaccination with VV-OspC-A induces antibody-dependent phagocytosis (ADCP) *in vitro*. **A)** Schematic depicting the workflow for the ADCP assay created with BioRender.com. See methods section for details. **B)** Percentage of RAW 264.7 cells emitting FITC signal following incubation 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.

#### 4.3.6. 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 4.6). 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 [443–446]. 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.

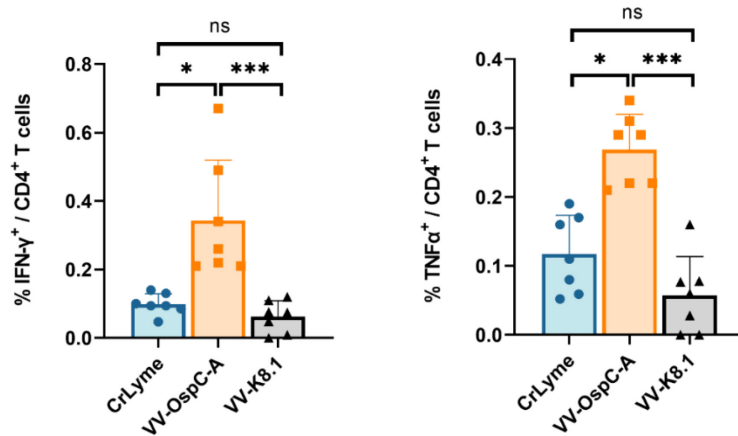
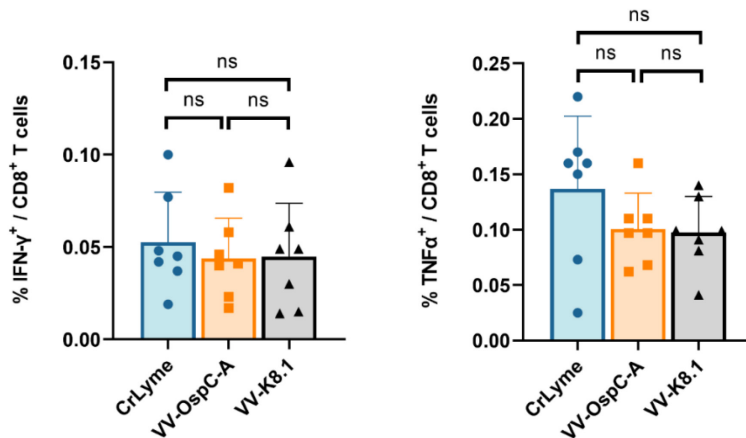




**Figure 4.6.** Vaccination with VV-OspC-A induces cytokine expression in splenocytes following stimulation with OspC. Splenocytes harvested from mice 28 days after vaccination with CrLyme, VV-OspC-A, or VV-K8.1 were stimulated with an overlapping peptide library of OspC for 24 hours. The concentration 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.



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 4.7A). Significant IFN- $\gamma$  and TNF- $\alpha$  expression was not observed in CD8<sup>+</sup> T cells isolated from the VV-OspC-A vaccinated mice (Figure 4.7B). 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.

**A****B**

**Figure 4.7.** VV-OspC-A induces antigen specific CD4<sup>+</sup> 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<sup>+</sup> and B) CD8<sup>+</sup> 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

## 4.4. 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 [447]. 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* spirochetes and *B. burgdorferi* DNA (Figure 4.1). 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 [30,158,373,411,429]. Carditis and lymph node hyperplasia were both significantly reduced in mice vaccinated with VV-OspC-A (Figure 4.2 & 4.3). 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 preclinical 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 [280,413,448].

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 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-OspC antibody titres predominated by the IgG2a subtype, indicating a Th1-skewed immune response (Figure 4.4). 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 [449–453]. In comparison, viral-vector vaccines, including VV, are known to induce Th1 responses [454–456]. Although it is not well understood whether Th1-biased or Th2-biased responses are preferred in the context of *B. burgdorferi* clearance, IgG2a antibodies have been implicated in the clearance of extracellular bacteria via Fc receptor binding [457–459].

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 (Figure 4.6 & 4.7). 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 4.6). 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) [460]. CD4<sup>+</sup> T cells expressing IFN- $\gamma$  or TNF- $\alpha$  were also increased in VV-OspC-A-vaccinated mice following stimulation (Figure 4.7). 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 [91,461–463]. 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 4.5). 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 [383,408].

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 [70]. 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 [125]. 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.

**Supplementary Materials:** Appendix – Figure S4.1. Gating strategy for flow cytometry analysis of antigen-specific T cells, Figure S4.2. Verification of OspC expression *in vitro*. BHK21 cells were infected with VV-OspC-A or the negative control VV-K8.1. OspC protein in whole cell lysates were detected by Western Blot.

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

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# Chapter 5: The Expression Kinetics and Immunogenicity of Lipid Nanoparticles Delivering Plasmid DNA and mRNA in Mice

## Preface

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and G.F.; writing—original draft preparation, W.Z. and A.P.; writing—review and editing, C.L., G.F., L.T., C.G., S.N.T.R., X.L. and M.J.W.J.; visualization, W.Z. and A.P.; supervision, L.W., X.L. and M.J.W.J.; project administration, W.Z., A.P., C.G., S.N.T.R., X.L. and M.J.W.J.; funding acquisition, L.W., S.S., M.R.-M., X.L. and M.J.W.J. All authors have read and agreed to the published version of the manuscript.

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## Abstract

In recent years, lipid nanoparticles (LNPs) have emerged as a revolutionary technology for vaccine delivery. LNPs serve as an integral component of mRNA vaccines by protecting and transporting the mRNA payload into host cells. Despite their prominence in mRNA vaccines, there remains a notable gap in our understanding of the potential application of LNPs for the delivery of DNA vaccines. In this study, we sought to investigate the suitability of leading LNP formulations for the delivery of plasmid DNA (pDNA). In addition, we aimed to explore key differences in the properties of popular LNP formulations when delivering either mRNA or DNA. To address these questions, we compared three leading LNP formulations encapsulating mRNA or pDNA encoding firefly luciferase based on potency, expression kinetics, biodistribution, and immunogenicity. Following intramuscular injection in mice, we determined that RNA-LNPs formulated with either SM-102 or ALC-0315 lipids were the most potent (all  $p$ -values  $< 0.01$ ) and immunogenic (all  $p$ -values  $< 0.05$ ), while DNA-LNPs formulated with SM-102 or ALC-0315 demonstrated the longest duration of signal. Additionally, all LNP formulations were found to induce expression in the liver that was proportional to the signal at the injection site (SM102:  $r = 0.8787$ ,  $p < 0.0001$ ; ALC0315:  $r = 0.9012$ ,  $p < 0.0001$ ; KC2:  $r = 0.9343$ ,  $p < 0.0001$ ). Overall, this study provides important

insights into the differences between leading LNP formulations and their applicability to DNA- and RNA-based vaccinations.

## 5.1 Introduction

Since the start of the COVID-19 pandemic, over 90 million doses of lipid nanoparticle (LNP)-based mRNA vaccines have been administered in Canada alone [464]. The success of these immunization programs has brought nucleic acid technologies to the forefront of vaccine research. LNPs are a critical component of these vaccine platforms, presenting several key advantages for the delivery of nucleic acids.

First, encapsulation in an LNP protects nucleic acids from degradation and improves stability in biological fluids [303,306,465,466]. Second, LNPs improve cellular uptake, leading to increased expression of the target antigen, which may contribute to increased immunogenicity [306–308,467,468]. Third, nucleic acid vaccines delivered by LNPs do not require additional adjuvants for immune activation [311,312]. Finally, when compared to delivery by viral vectors, LNP-based vaccines are more easily produced and do not induce anti-vector immunity that may hinder vaccine efficacy [469].

To date, all approved LNP-based vaccines have been designed to encapsulate modified mRNA. Despite the success of this technology, there are several limitations associated with mRNA vaccines, including poor stability, cold storage requirements, and high production costs [470–474]. In comparison, plasmid DNA (pDNA) vaccines are more thermostable and less susceptible to degradation [474–478]. We have observed that DNA-LNPs maintain transfection potency better than mRNA-LNPs after one week at 37 °C (Figure S5.1). Moreover, DNA vaccines are less expensive to produce, store, and transport than mRNA vaccines [479,480]. LNP-based DNA

vaccines therefore have the potential to alleviate a number of issues inherent to mRNA vaccine technology, which could improve suitability for more wide-spread use.

Recently, the first DNA vaccine was approved in India for the prevention of COVID-19 [481]. Many other DNA vaccines in clinical and preclinical development are administered using intradermal injection or specialized instruments such as gene guns or needle-free injectors [482,483]. Considering that these techniques may not be easily translated into human vaccination programs, LNPs offer a safe and reliable alternative for delivering DNA vaccines by intramuscular injection [307,404,405]. Despite these advantages, there are currently no DNA vaccines approved or undergoing clinical trials that are delivered by LNPs. One study by Mucker and colleagues demonstrated that LNP encapsulation increased the neutralizing antibody titres induced by DNA vaccines for Andes virus and Zika virus [307]. In addition, a study by Algarni et al. demonstrated that a DNA-LNP vaccine formulated with the ionizable lipid DLin-KC2-DMA (KC2), resulted in greater antigen expression than the leading DLin-MC3-DMA (MC3)-formulated particle when administered intramuscularly [484]. These studies provide promising insights into the potential of DNA-LNP vaccines as an alternative platform for vaccine development.

To our knowledge, the potency, expression kinetics, biodistribution, and immunogenicity conferred by DNA-LNP vaccines formulated with the ionizable lipid KC2 or the lipid formulations utilized in clinical COVID-19 vaccines (SM-102 and ALC-0315) have not yet been investigated [327,485]. This study aims to bridge a significant knowledge gap regarding the efficacy and characteristics of DNA-LNP vaccines formulated with different lipid components. Development of DNA vaccines can be accelerated by employing LNPs that have already demonstrated safety and efficacy in the context of mRNA vaccines. Therefore, comparisons of mRNA and DNA formulated with the same particle should be explored. To address these questions, we generated

three LNP formulations encapsulating mRNA- or pDNA-encoding firefly luciferase. We characterized each particle and then evaluated the potency of these particles in vitro. Furthermore, we evaluated the intensity and duration of the luminescence signal in vivo at both the injection site and distally, in the liver. Finally, the anti-luciferase antibody response induced by each formulation was evaluated as a surrogate for vaccine immunogenicity.

## 5.2. Materials and Methods

### 5.2.1. Preparation of pDNA- and mRNA-Encoding Luciferase

mRNA-encoding firefly luciferase (mRNA-Luc) was purchased from TriLink Biotechnologies (San Diego, CA, USA) with the substitution of N1-Methylpseudouridine for uridine.

To generate the pDNA-encoding firefly luciferase gene (pVAX1-Luc), the Luc gene from the pcDNA3-Luc plasmid (Addgene, Watertown, MA, USA) was cloned into the pVAX1 plasmid vector (ThermoFisher, Ottawa, ON, Canada) using HindIII-HF and XbaI restriction enzymes (New England Biolabs, Whitby, ON, Canada). pVAX1-Luc was transformed into NEB<sup>®</sup> 10-beta Competent *E. coli* (High Efficiency) (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocol. Large-scale amplifications of pVAX1-Luc were generated using the EndoFree QIAGEN Plasmid Giga Kit (Montreal, QC, Canada) according to the manufacturer's instructions.

### 5.2.2. Lipid Nanoparticle (LNP) Generation

To generate the LNPs, an aqueous phase containing pDNA, mRNA, or buffer alone was combined with an organic phase containing lipids in ethanol via rapid microfluidic mixing using a NanoAssemblr BT<sup>™</sup> (Precision Nanosystems, Inc., Vancouver, BC, Canada) as described previously [387]. Samples containing no nucleic acid or pDNA were prepared with 25 mM acetate

buffer (pH 4.0), while samples containing mRNA were prepared with 50 mM citrate buffer (pH 4.0). The ethanol phase was composed of ionizable lipid: phospholipid: cholesterol: PEG-lipid at the molar ratios listed in Table 5.1, using a concentration of 15 mM total lipid. After mixing, the particles were dialyzed against a 1000-fold volume of phosphate-buffered saline (PBS) using a 10 k MWCO cassette (Thermo Fisher, Ottawa, ON, Canada) at 4 °C for 18 h. LNPs were 0.22 µm filtered, then concentrated using an Amicon Ultra 4 10 k MWCO centrifugal concentrator (Millipore Sigma, Burlington, ON, Canada). All particles were made with a polymer amine (N = nitrogen) group to nucleic acid phosphate (P) group (N/P) ratio of 6:1.

| Particle Name | Encapsulated Nucleic Acid | Components                          | Molar Ratio                 | LNP Diameter *<br>(Mean ± SD, nm) | Encapsulation Efficiency * |
|---------------|---------------------------|-------------------------------------|-----------------------------|-----------------------------------|----------------------------|
| SM102-DNA     | DNA                       | SM-102 : DSPC : Chol : DMG-PEG 2000 | 50:10:38.5:1.5<br>[327,485] | 78 ± 24                           | 97%                        |
| ALC0315-DNA   | DNA                       | ALC-0315 : DSPC : Chol : ALC-0159   | 46.3:9.4:42.7:1.6<br>[329]  | 67 ± 19                           | 97%                        |
| KC2-DNA       | DNA                       | KC2 : DSPC : Chol : DMG-PEG 2000    | 50:10:38.5:1.5<br>[327,485] | 82 ± 36                           | 99%                        |
| SM102-RNA     | RNA                       | SM-102 : DSPC : Chol : DMG-PEG 2000 | 50:10:38.5:1.5<br>[327,485] | 74 ± 20                           | 88%                        |
| ALC0315-RNA   | RNA                       | ALC-0315 : DSPC : Chol : ALC-0159   | 46.3:9.4:42.7:1.6<br>[329]  | 71 ± 21                           | 92%                        |
| KC2-RNA       | RNA                       | KC2 : DSPC : Chol : DMG-PEG 2000    | 50:10:38.5:1.5<br>[327,485] | 83 ± 31                           | 97%                        |

**Table 5.1.** Lipid nanoparticle formulas and characterization. LNP size was determined by nanoparticle tracking analysis and encapsulation efficiency was determined by SYBR™ Gold assay. All particles were made with a polymer amine (N = nitrogen) group to nucleic acid phosphate (P) group (N/P) ratio of 6:1. \* Representative of multiple LNP fabrications.

Lipids purchased from MedKoo Biosciences, Inc. (Morrisville, NC, USA) include 2,2-dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (DLin-KC2-DMA), heptadecan-9-yl 8-((2-hydroxyethyl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate (SM-102), [(4-Hydroxybutyl)azanediyl]di(hexane-6,1-diyl) bis(2-hexyldecanoate) (ALC-0315) and 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide (ALC-0159). Lipids purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA) include 1,2-dioleoyl-sn-glycero-3-phosphocholine (18:1 ( $\Delta^9$ -Cis) PC (DOPC)), 1,2-distearoyl-sn-glycero-3-phosphocholine (18:0 PC (DSPC)), 1,2-Dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG 2000) and cholesterol (ovine).

### 5.2.3. Nanoparticle Characterization

Nanoparticle size characterization was performed as described previously [387]. In brief, the particle size was determined by nanoparticle tracking analysis (NTA) (NanoSight, Malvern Panalytical, Westborough, MA, USA) under static conditions. Five separate tracking videos were merged to generate the sizing data. Due to smaller particle size, dynamic light scattering (DLS) (Zetasizer Ultra, Malvern, Panalytical, Westborough, MA, USA) was used to measure LNPs without nucleic acid.

Nucleic acid encapsulation efficiency was measured as previously described [387]. In brief, LNPs were either untreated or disrupted with 1% Triton X-100 (Millipore Sigma, Burlington, ON, Canada) in a 96-well plate before the addition of SYBR<sup>TM</sup> Gold dye (Thermo Fisher, Ottawa, ON, Canada) to a final concentration of  $1\times$ . Nucleic acid detected in wells without triton was considered unencapsulated, while nucleic acid detected in wells where triton was added to disrupt the LNP represented total nucleic acid.

#### 5.2.4. In Vitro Transfection Assay

HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher, Ottawa, ON, Canada) supplemented with 10% fetal-bovine serum (FBS). In a 24-well plate, cells were transfected with 500 ng of pDNA or mRNA alone or encapsulated in LNPs, diluted in PBS (Gibco, Thermo Fisher, Ottawa, ON, Canada). RNA- and DNA-transfected cells were incubated at 37 °C, 5% CO<sub>2</sub> for 16 h or 48 h, respectively, according to the peak transfection time for each payload. The media was then replaced with 200 µL of 1× passive lysis buffer (Promega, Madison, WI, USA) and incubated for 30 min at room temperature on an orbital shaker at 50 rpm. The lysate was centrifuged at 15,000× g for 5 min at room temperature, and the cleared lysate was added in triplicate, 100 µL per well, to a white Costar 96-well plate (Corning, Glendale, AZ, USA) followed by 100 µL of room temperature Bright-Glo Reagent (Promega, Madison, WI, USA) prepared according to manufacturer instructions. The luminescence was read within 5 min and normalized to total protein content as measured by a Bicinchoninic Acid (BCA) Kit (MilliporeSigma, Oakville, ON, Canada). The in vitro potency was expressed as relative luminescence units/mg of protein (RLU/mg).

#### 5.2.5. Animal Study

Six-week-old female BALB/c mice were obtained from Charles River, Senneville, Quebec, Canada. All animal procedures were performed in accordance with institutional guidelines and ethical approval was granted by the Animal Care Committee at Health Canada, Ottawa, ON, Canada.

Mice were randomly divided into eight groups, with three to four in each group. At time zero, mice received intramuscular injection into the left and right tibialis anterior muscle, with a total injection

volume of 50  $\mu$ L. The injection contained either 1  $\mu$ g of mRNA or 25  $\mu$ g of pDNA encapsulated in LNPs diluted in PBS. An LNP containing no nucleic acid (empty particle) control group was also included in the study and dosed with the equivalent amount of lipid as the DNA-LNP groups. Depilatory cream was applied to the mouse legs under anesthesia to improve visualization prior to initial injection and again 17 days after injection.

Bioluminescence imaging was performed with IVIS Lumina XRMS imaging system (Perkin Elmer, Woodbridge, ON, Canada). At each of the 6 h, 24 h, 48 h, 72 h, 168 h, 336 h, 504 h, and 672 h timepoints after injection, mice were administered intraperitoneally with IVISbrite D-Luciferin Potassium Salt Bioluminescent Substrate (Perkin Elmer) dissolved in PBS at 150 mg/kg. After 15 min, the mice were anesthetized in a chamber with 5% isoflurane at 1.5 L/min flow rate. The mice were placed on the imaging platform while maintained on 2% isoflurane at 500 cc/min flow rate via a nose cone. The mice were imaged 20 min after receiving D-Luciferin using the automatic exposure setting. Bioluminescence values were quantified by measuring photon flux (photons/second) in the region of interest using Living Image software (version 4.7.3) provided by Perkin Elmer.

### 5.2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

High-binding Nunc Maxisorp™ flat bottom 96-well plates were coated with 100  $\mu$ L/well of 2  $\mu$ g/mL of recombinant luciferase (Promega) in PBS. After overnight incubation at 4 °C, plates were washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 3% (w/v) Bovine Serum Albumin (IgG-Free, Protease-Free) (Jackson Immuno Research, West Grove, PA, USA) in PBS-T. After washing, two-fold serial dilutions of the mouse serum in blocking buffer were added for one hour at 37 °C. Plates were washed again, then HRP-conjugated goat anti-mouse IgG (Cytiva, Marlborough, MA, USA), HRP-conjugated goat anti-mouse IgG1 (Jackson Immuno



Research, West Grove, PA, USA), or HRP-conjugated goat anti-mouse IgG2a (Jackson Immuno Research) were added at a 1:2000 dilution and incubated for one hour at 37 °C. 75 µL of tetramethylbenzidine (TMB) substrate (Cell Signaling Technology, Danvers, MA, USA) was added after washing and plates were incubated at room temperature for five minutes. The reaction was terminated by addition of 0.16 M sulfuric acid and absorbance was read at 450 nm on a spectrophotometer. Endpoint antibody titers were expressed as the reciprocals of the final detectable dilution, with a cut-off value defined as the mean of all wells containing serum from mice in the empty particle control group plus three times the standard deviation.

### 5.2.7. Mathematical and Statistical Analyses

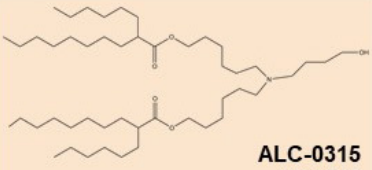
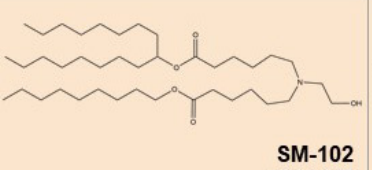
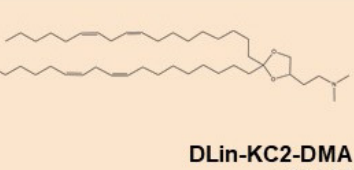
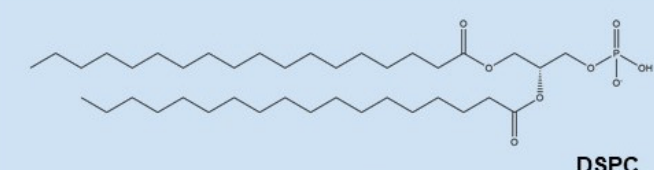
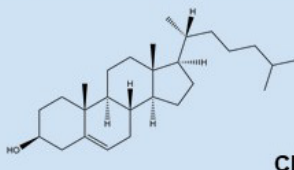
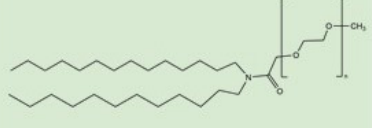
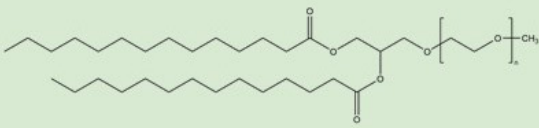
Statistical analyses were performed using GraphPad Prism 9 and SAS Enterprise Guide 7.1. The total in vivo signal (total flux<sup>2</sup>) was calculated as area under the curve (AUC) of the fitted signal curves minus the average AUC of the empty particle control group. Statistical differences between groups for in vitro signal, total in vivo signal, and liver and injection site ratio were analyzed using an unpaired two-tailed *t*-test with a significance threshold of  $p < 0.05$ . The cut-off value for in vivo signal duration was defined as the mean of the signal from the empty particle group plus three times the standard deviation. When comparing the same formulation delivering DNA versus RNA, comparisons between peak signal at the injection site and liver was calculated using a Welch's (Satterthwaite) *t*-test with a significance threshold of  $p < 0.05$ . The comparisons between different LNP formulations for DNA and RNA were carried out using analysis of variance (ANOVA). If the result of ANOVA was significant ( $p < 0.05$ ), then the pairwise comparisons of least square mean were carried out using two tailed *t*-test, and the obtained *p*-values were adjusted for multiple comparisons (Bonferroni method). Correlations were analyzed using Pearson correlation. The anti-luciferase IgG endpoint titres and IgG2a:IgG1 ratio were compared using Mann–Whitney two-

tailed U-test with a significance threshold of  $p < 0.05$ . \*  $p$ -value  $< 0.05$ , \*\*  $p$ -value  $< 0.01$ , \*\*\*  $p$ -value  $< 0.001$ , \*\*\*\*  $< 0.0001$ .

## 5.3. Results

### 5.3.1. Characterization of Nanoparticle Formulations

We first generated three LNP formulations containing the ionizable lipids SM-102, ALC-0315, or KC2 (Figure 5.1, Table 5.1). The formulations for the ALC-0315- and SM-102-based particles were identical to those used in the approved COVID-19 mRNA vaccines [327,329,485]. To evaluate the suitability of each formulation to deliver both DNA-based and RNA-based vaccines, either 25  $\mu$ g of pDNA- or 1  $\mu$ g of mRNA-encoding firefly luciferase was encapsulated in each LNP formulation. The size distributions of the resulting LNPs were analyzed by nanoparticle tracking analysis. The mean particle sizes for the formulations in the present study ranged from 67 to 83 nm (Table 5.1). Previous reports have demonstrated that the optimal size range for efficient uptake by antigen presenting cells and induction of a robust immune response is between 20 and 200 nm [415–417]. Furthermore, all formulations demonstrated high encapsulation efficiencies, ranging between 88 and 99% when evaluated by SYBR™ Gold assay.

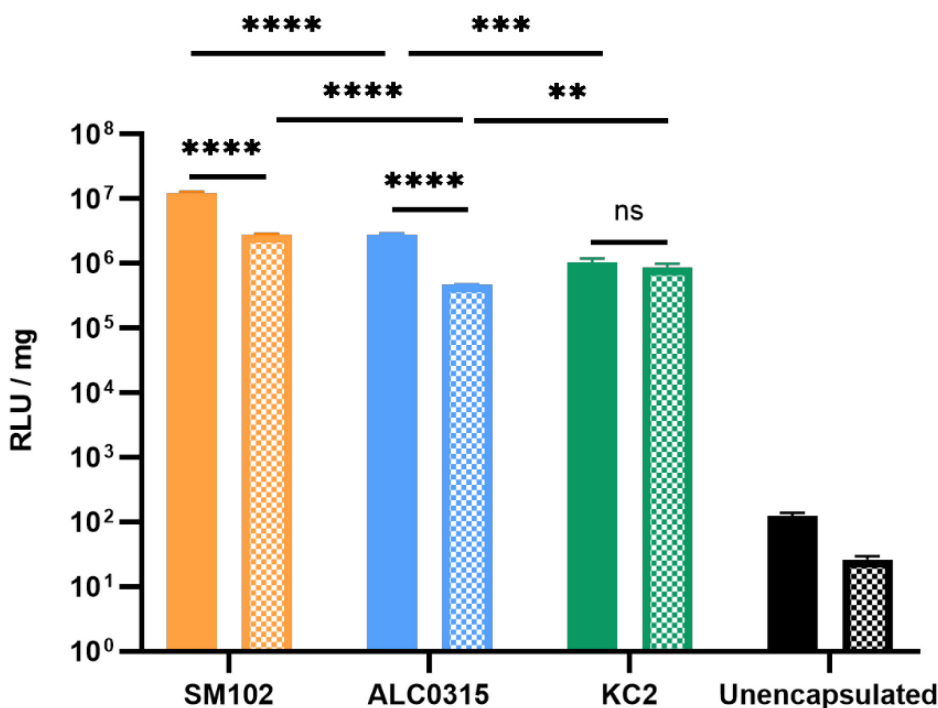
|                        | ALC0315                                                                                                                 | SM102                                                                                                                  | KC2                                                                                                                          |
|------------------------|-------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------|
| <b>Ionizable Lipid</b> |  <p><b>ALC-0315</b><br/>pKa = 6.09</p> |  <p><b>SM-102</b><br/>pKa = 6.75</p> |  <p><b>DLin-KC2-DMA</b><br/>pKa = 6.7</p> |
| <b>Helper Lipids</b>   |  <p><b>DSPC</b></p>                   |                                                                                                                        |  <p><b>Cholesterol</b></p>                |
| <b>PEG-lipid</b>       |  <p><b>ALC-0519</b></p>                |  <p><b>DMG-PEG 2000</b></p>          |                                                                                                                              |

**Figure 5.1.** Chemical structures of components in the tested lipid nanoparticle formulations. The header row indicates the particle names referred to in this study.

### 5.3.2. Potency of LNP Formulations

#### 5.3.2.1. SM102-RNA Particles Result in the Highest Luciferase Expression In Vitro

The potency of the RNA- and DNA-LNPs was first evaluated in vitro. HEK293T cells were incubated with LNPs containing firefly luciferase mRNA or pDNA for 16 or 48 h, respectively. For both RNA- and DNA-LNPs, the SM-102 formulation resulted in the highest luciferase expression, likely due to the unique structural characteristics of SM-102. For RNA-LNPs, the ALC-0315 formulation demonstrated the second-best transfection capability. However, when delivering DNA, the KC2 formulation performed better than the ALC-0315 formulation (Figure 5.2). For both the SM-102 and ALC-0315 formulations, transfection with RNA resulted in higher expression of luciferase than transfection with DNA. Peak transfection occurred at similar levels for the KC2-LNPs regardless of payload.



**Figure 5.2.** SM102-RNA results in the highest luciferase expression in vitro. HEK293T cells were transfected with 500 ng of mRNA-Luc or DNA-Luc unencapsulated or encapsulated in one of three LNP formulations. Relative luminescence units (RLU) were measured at 16 h for RNA (solid) and 48 h for DNA (stippled) and were standardized per mg of protein quantified by Bicinchoninic Acid (BCA) assay. Error bars represent standard deviation (SD). ns: not significant, \*\*  $p$ -value < 0.01, \*\*\*  $p$ -value < 0.001, \*\*\*\* < 0.0001.

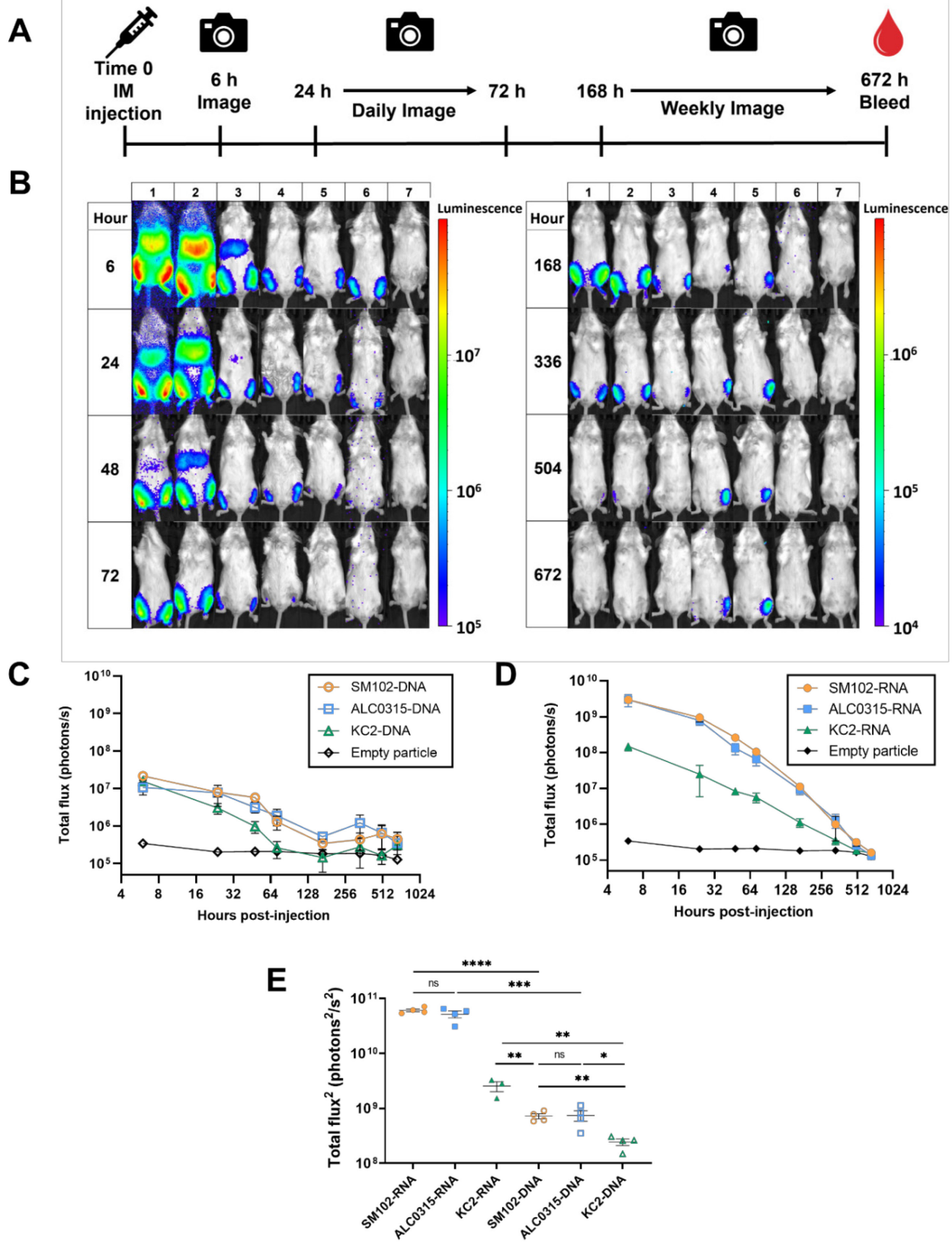
#### 5.3.2.2. SM102-RNA and ALC0315-RNA Particles Result in the Highest Luciferase Expression In Vivo

Next, BALB/c mice were injected intramuscularly with the RNA- or DNA-LNP formulations to evaluate the in vivo potency and expression kinetics. Six hours later, mice were injected intraperitoneally with luciferin substrate and imaged using an IVIS Lumina XRMS imaging system to quantify luciferase expression. The imaging was repeated 24 h, 48 h, 72 h, 168 h (7 days), 336 h (14 days), 504 h (21 days), and 672 h (28 days) after the initial LNP injection (Figure 5.3A).

For all RNA and DNA formulations, maximum luciferase expression was observed at six hours (Figure 5.3B–D). All three DNA particles yielded similar peak expression levels at the six-hour time point. In comparison, over the complete time course, the total luciferase expression for both the SM102-DNA and ALC0315-DNA groups was significantly higher than the KC2-DNA group. Importantly, no significant difference was observed between the SM102-DNA and ALC0315-DNA groups (Figure 5.3E).

When delivering an mRNA payload, both SM-102 and ALC-0315 particles resulted in the highest luciferase signal of all groups at the six-hour peak, with no significant difference between the two formulations (Figure 5.3D,E). The peak intensity of KC2-RNA particles was approximately 20-fold lower than ALC0315-RNA ( $p = 0.002$ ) and SM102-RNA ( $p = 0.0021$ ). This result is consistent with the in vitro results where SM102-RNA and ALC0315-RNA both outperformed KC2-RNA. Over the complete time course, the total luciferase expression was not significantly different between the SM102-RNA and ALC0315-RNA groups; however, both groups resulted in significantly higher expression than the KC2-RNA group (Figure 5.3E).

For each formulation, the peak luciferase expression was greater when encapsulating 1  $\mu$ g of mRNA than 25  $\mu$ g of pDNA. SM102-RNA and ALC0315-RNA resulted in greater total luciferase expression over the 28-day period. In addition, the total luciferase expression generated by the KC2-RNA group was significantly higher than the KC2-DNA group (Figure 5.3E). These results suggest that, when comparing mRNA and DNA as LNP payloads, significantly lower doses of mRNA can result in increased protein expression in vivo. Overall, all LNP formulations generated significant luciferase expression when delivering both DNA and mRNA; however, the mRNA payload resulted in more efficient protein expression.



**Figure 5.3.** SM102-RNA and ALC0315-RNA result in the highest luciferase expression in vivo. (A) Schematic depicting the injection and imaging schedule in BALB/c mice. Mice were injected intramuscularly (IM) with either 1 µg of RNA or 25 µg of pDNA encapsulated in one of the LNP formulations. Mice were imaged at 6 h, 24 h, 48 h, 72 h, 168 h, 336 h, 504 h, and 672 h using an IVIS Lumina XRMS imaging system. After the final imaging time point, mice were sacrificed and bled. (B) Representative whole-body bioluminescence imaging at different time points after IM administration. (1) SM102-RNA; (2) ALC0315-RNA; (3) KC2-RNA; (4) SM102-DNA; (5) ALC0315-DNA; (6) KC2-DNA; (7) empty particle control. Luciferase signal expression kinetics following intramuscular particle injection. Quantification of whole-body bioluminescent signal over time in BALB/c mice injected with luciferase encoded by (C) DNA or (D) RNA encapsulated in various particle formulations. (E) Total in vivo signal (total flux<sup>2</sup>) produced for each formulation over the complete time-course. Total in vivo signal was calculated as area under the curve (AUC) of the fitted signal curves minus the average AUC of the empty particle control. Error bars represent standard deviation (SD). ns: not significant, \* *p*-value < 0.05, \*\* *p*-value < 0.01, \*\*\* *p*-value < 0.001, \*\*\*\* < 0.0001.

### 5.3.3. Expression Kinetics: SM102-DNA and ALC0315-DNA Particles Result in the Longest Duration of Protein Expression

Following the peak luciferase expression observed at the six-hour time point, protein expression steadily decreased over the remaining time course for all groups. The KC2-DNA formulation resulted in signal significantly above baseline for the shortest duration of all groups, lasting only 48 h. In comparison, the SM102-DNA and ALC0315-DNA groups were found to have detectable luciferase signal at the final time point of 28 days (Figure 5.3C, Table 5.2). SM102-RNA and ALC0315-RNA groups yielded signal for 21 days, while the KC2-RNA formulation lasted a shorter duration of 14 days (Figure 5.3D, Table 5.2). Overall, the SM102-DNA and ALC0315-DNA particles resulted in the longest duration of protein expression when compared to all other groups.

| Particle Name | Signal Duration (Inj. Site) | Signal Duration (Liver) |
|---------------|-----------------------------|-------------------------|
| SM102-DNA     | >28 days                    | 48 h                    |
| ALC0315-DNA   | >28 days                    | 72 h                    |
| KC2-DNA       | 48 h                        | N/A                     |
| SM102-RNA     | 21 days                     | 7 days                  |
| ALC0315-RNA   | 21 days                     | 7 days                  |
| KC2-RNA       | 14 days                     | 72 h                    |

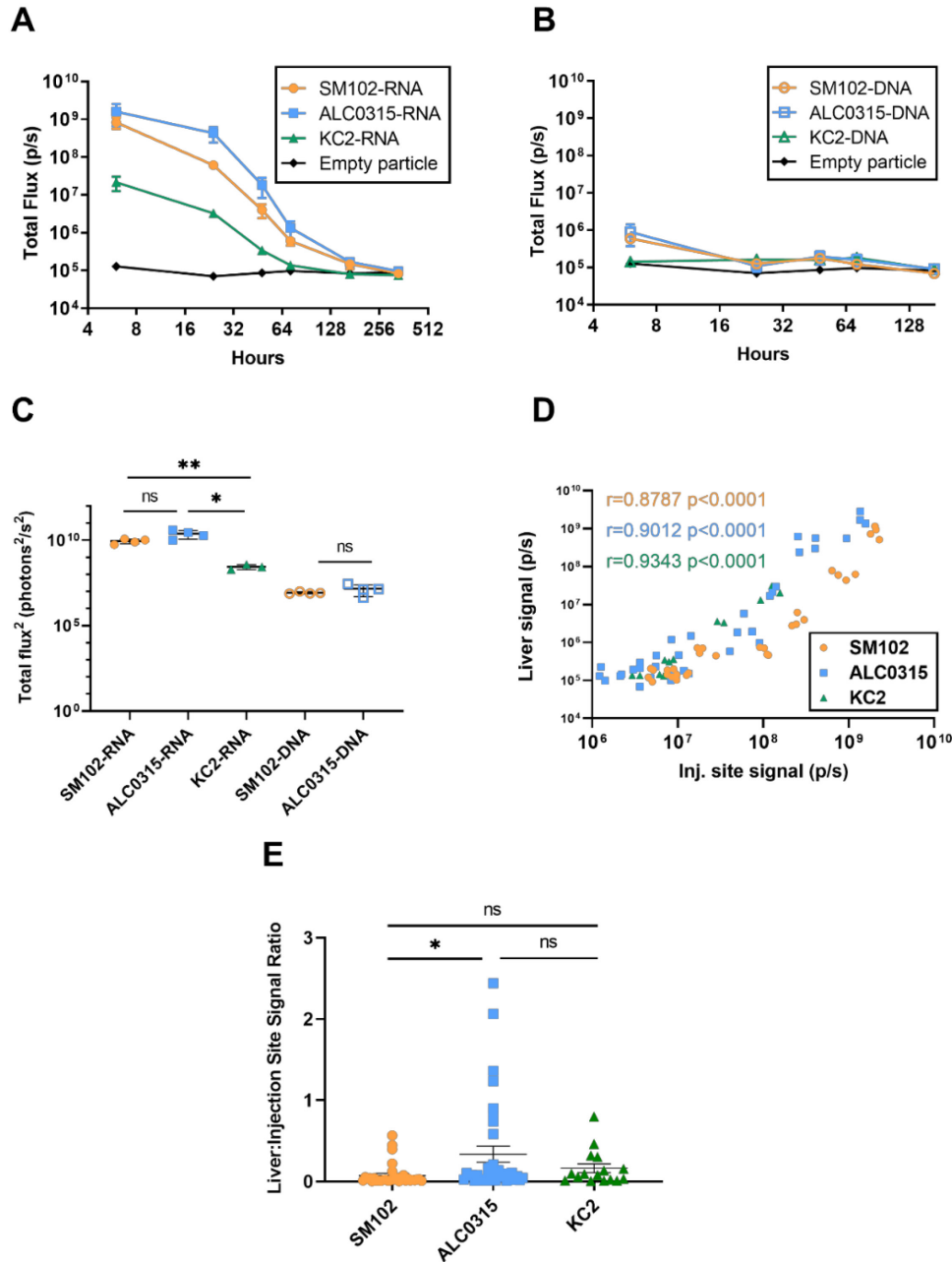
**Table 5.2.** Luciferase signal duration at the injection site and liver. Signal duration was determined as the last time point where the luminescence was higher than the cut-off value. The cut-off value was defined as the mean of the signal from the empty particle control group plus three times the standard deviation. Duration listed as not applicable (N/A) if all time points had signal below the cut-off value.

#### 5.3.4. Biodistribution: LNPs Result in Protein Expression in the Liver Proportional to the Expression at the Injection Site

Significant luciferase expression was observed in the livers of all mice injected with each of the LNP formulations, with the exception of the KC2-DNA group. The cut-off value for significant luciferase expression was defined as the mean of the signal from the empty particle group plus three times the standard deviation. This finding is in line with previous reports that have also demonstrated antigen expression in the liver following vaccination [329,485]. Here, we found that luciferase expression in the liver of both SM102-RNA and ALC0315-RNA groups lasted for the longest duration of seven days. In comparison, the liver signal in KC2-RNA and ALC0315-DNA mice was present for 72 h, while SM102-DNA mice had liver signal for 48 h (Figure 5.4A,B, Table 5.2). Similar to the expression kinetics at the injection site, all groups had the highest liver signal at six hours. ALC0315-RNA and SM102-RNA resulted in the greatest peak liver signal,



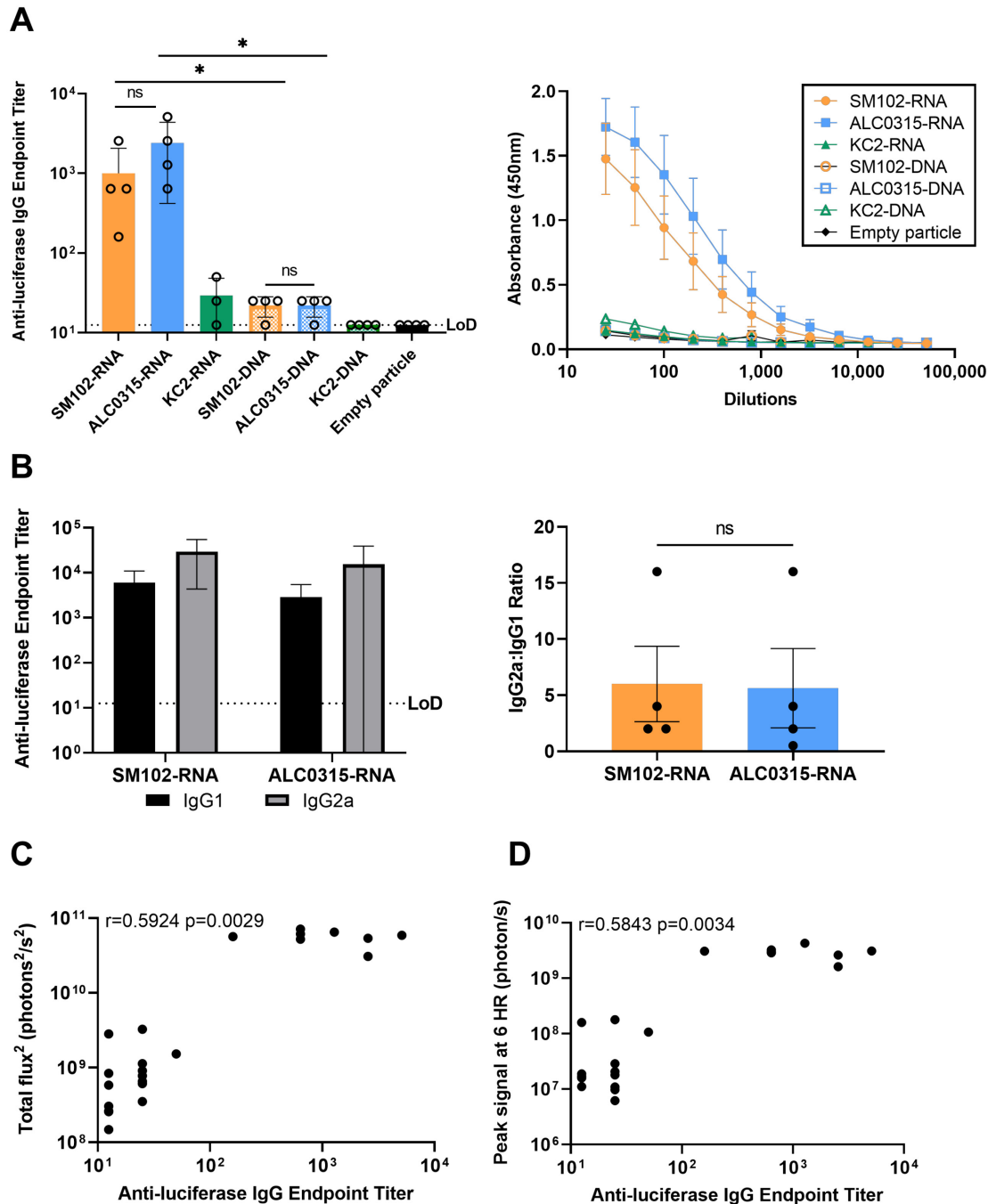
while the KC2-RNA group was over 50-fold lower than ALC0315-RNA ( $p = 0.0267$ ) (Figure 5.4A). In addition, both ALC0315-DNA and SM102-DNA resulted in much lower liver signal than their mRNA counterparts at six hours (ALC0315:  $p = 0.0428$ , SM102:  $p = 0.0093$ ). Similar trends were observed for the total luciferase signal in the liver over the 28-day time course. The total signal was not significantly different between ALC0315-LNPs and SM102-LNPs when both encapsulated the same nucleic acid. Furthermore, both SM102-RNA and ALC0315-RNA particles induced significantly greater total signal than the KC2-RNA group (Figure 5.4C). Consequently, we sought to determine if the protein expression in the liver was correlated to expression levels at the injection site. When considering all time points with detectable liver signal, the expression at the two sites was strongly correlated for all LNP formulations (SM102:  $r = 0.8787$ ,  $p < 0.0001$ ; ALC0315:  $r = 0.9012$ ,  $p < 0.0001$ ; KC2:  $r = 0.9343$ ,  $p < 0.0001$ ) (Figure 5.4D). Finally, we determined the ratio of liver signal to injection site signal. The ALC0315 formulation was found to have a significantly higher liver-to-injection site ratio than the SM102 formulation (Figure 5.4E). Taken together, these results suggest that LNPs lead to significant protein expression in the liver, which is correlated with the expression levels at the injection site and may vary depending on the LNP formulation.



**Figure 5.4.** LNPs result in protein expression in the liver proportional to the expression at the injection site. Quantification of liver bioluminescent signal in BALB/c mice injected with (A) RNA- or (B) DNA-encoding luciferase encapsulated in various particle formulations. (C) Total liver signal (total flux<sup>2</sup>) produced for each formulation over the complete time-course. Total liver signal as calculated as area under the curve (AUC) of the fitted liver signal curves minus the average AUC of the empty control group. (D) The correlation between liver and injection site signal and (E) ratio of liver and injection site signal at time points with detectable liver signal. Error bars represent standard deviation (SD). ns: not significant, \* *p*-value < 0.05, \*\* *p*-value < 0.01.

### 5.3.5. Immunogenicity: SM102-RNA and ALC0315-RNA Particles Result in the Highest Antibody Response with Th1 Bias

Finally, we sought to investigate the luciferase-specific antibody response in the serum 28 days after the initial injection. Despite generating significant luciferase expression, all DNA-LNP groups and the KC2-RNA group did not induce a significant antibody response when compared to mice injected with the negative control LNP (empty particle) (Figure 5.5A). In comparison, SM102-RNA and ALC0315-RNA both induced significant anti-luciferase antibody titres, with no significant difference in titres between the two groups. Further analysis of the IgG subclass antibody titres revealed that both formulations induced more IgG2a than IgG1, indicating a Th1-biased immune response (Figure 5.5B). In addition, the antibody titres from all groups were found to moderately correlate with both the 6 h peak luciferase expression ( $r = 0.5843$ ,  $p = 0.0034$ ) and the total luciferase expression over the 28-day time course (AUC,  $r = 0.5924$ ,  $p = 0.0029$ ) (Figure 5.5C,D). In conclusion, the SM102-RNA and ALC0315-RNA groups were found to be the most effective at inducing a robust antibody response after one dose.



**Figure 5.5.** SM102-RNA and ALC0315-RNA particles result in the highest antibody response with Th1 bias. **(A)** ELISA determination of luciferase-specific IgG antibody titres in serum of mice 28 days post-injection. **(B)** IgG2a and IgG1 luciferase-specific serum antibody titres and ratios of IgG2a and IgG1 endpoint titre. Dashed line represents limit of detection (LoD). Error bars represent standard deviation (SD). \*  $p$ -value < 0.05. Correlation between luciferase-specific IgG antibody titres and **(C)** total in vivo signal or **(D)** peak signal at 6 h post-injection.

## 5.4. Discussion

In this study, we compared three different LNP formulations delivering an mRNA or pDNA surrogate vaccine based on four key parameters: (1) potency (defined as reporter gene expression), (2) expression kinetics, (3) biodistribution, and (4) immunogenicity. Two of the lipid formulations evaluated in this study are identical to those used in COVID-19 mRNA vaccines, here referred to as SM102- and ALC0315-LNPs. We also included a formulation previously shown to effectively deliver pDNA, referred to as KC2-LNPs [484]. Each of the LNP formulations was used to encapsulate either pDNA- or mRNA-encoding firefly luciferase. Luciferase was used as a representative vaccine antigen as it has been shown previously to be immunogenic and its expression is easily visualized via bioluminescent imaging [486].

First, we evaluated the potency of the LNP formulations in vitro using HEK293T cells. We found that all LNP formulations were effective at delivering DNA, with SM102-DNA > KC2-DNA > ALC0315-DNA. To our knowledge, this is the first demonstration that SM-102- and ALC-0315-based LNPs are effective for in vitro transfection of pDNA. Previous studies have suggested that ionizable lipids with higher pKa values result in a greater quantity of charged ionizable lipids in the endosome leading to improved endosomal release of DNA into the cytoplasm and higher levels of protein expression in vitro [487]. Our results support this conclusion since ionizable lipids with higher pKa values (SM-102 = 6.75 and KC2 = 6.7) were found to have greater reporter gene expression than ALC-0315 (pKa = 6.09). The difference in reporter gene expression between SM102-DNA and KC2-DNA LNPs is likely attributed to SM-102 disrupting the endosomal membrane to a greater extent due to its more cone-like shape [484]. Compared to ALC-0315 and KC2, SM-102 has a more asymmetric tail, which has been shown to have higher transfection efficiency [328]. Interestingly, for RNA-LNPs a different pattern was observed with SM102-RNA > ALC0315-RNA > KC2-RNA suggesting that factors other than pKa, such as LNP morphology,

inner structure, and RNA microenvironment, may account for the observed differences [488]. In another recent publication, the authors observed several lipid structure activity relationships that correlated with improved protein expression, including number of carbons on the lipid tails on the ester side and the effect of carbon spacing on the disulfide arm of the lipids [489]. The results of our study are consistent with a recent publication by Escalona-Rayó et al. that demonstrated that SM102-RNA particles resulted in greater in vitro protein expression than ALC0315-RNA particles following transfection into mouse primary bone marrow dendritic cells [490].

Next, we assessed the potency of each LNP formulation in vivo following injection in BALB/c mice. SM102-RNA and ALC0315-RNA resulted in higher total luciferase signal compared to KC2-RNA. This result is most likely due to the branched ester chains of the SM-102 and ALC-0315 lipid tails, which have been shown to increase functional delivery of mRNA, and not the apparent pKa of the ionizable lipid [491,492]. These results are in accordance with a previous study comparing ALC0315-RNA, SM102-RNA, and MC3-RNA particles administered intravenously into zebrafish embryos, in which ALC0315-RNA and SM102-RNA demonstrated elevated EGFP protein expression compared to MC3-RNA (pKa = 6.4) [490]. In addition, we observed some similarities between the in vitro and in vivo potency, with SM102-RNA and ALC0315-RNA resulting in higher luciferase expression than all other groups in both cases. In comparison, both DNA and RNA formulations of SM-102 and ALC-0315 LNPs demonstrated similar levels of potency and were significantly greater than KC2 LNPs when evaluated in vivo, but not in vitro. Ultimately, the predictive capacity of in vitro screening may be greatly influenced by the cell line and the use of primary cells should be considered to improve in vitro–in vivo translation [404,493,494]. Moreover, this is also the first demonstration that LNPs formulated with SM-102 or ALC-0315 are effective for the delivery of pDNA in vivo.

We then investigated the biodistribution of the luciferase signal following intramuscular injection. In addition to the expected signal surrounding the tibialis anterior, we observed signals in the livers of all mice, except those injected with KC2-DNA, which peaked at six hours. These findings are in line with previous reports that observed localization of LNPs to the liver following intramuscular injection of LNPs formulated with ALC-0315 or SM-102 [329,485]. The uptake of ionizable LNPs by hepatocytes is thought to occur through low-density lipoprotein LDL receptor-mediated endocytosis facilitated by binding to apolipoprotein E [495–497]. In the present study, we also found that the injection site signal was strongly correlated with the liver signal for all three LNP formulations. This result indicates that the liver signal is likely proportional to the magnitude of the injection site signal. This is further supported by the fact that KC2-DNA, which resulted in the lowest total luciferase expression of all groups, had no detectable liver signal. To further characterize the biodistribution, we compared the liver-to-injection site ratio among the three formulations, which revealed a significantly higher ratio for the ALC0315-LNPs. This result suggests that the ALC-0315-based particle may have increased hepatic tropism compared to the SM-102-based formulation. This is the first head-to-head comparison of the biodistribution of ALC-0315- and SM-102-based LNPs, and future studies should continue to explore how differences in biodistribution may be beneficial for the treatment or prevention of different diseases.

Finally, we analyzed the immunogenicity of each nanoparticle formulation by quantifying the antibody response to luciferase 28 days after injection. While it is valuable to understand the expression kinetics of these delivery systems, the usefulness of these LNP formulations for mRNA and DNA vaccine delivery is largely determined by their ability to induce a robust antibody response against the encoded antigen [498,499]. Only the SM102-RNA and ALC0315-RNA

groups, which had the highest total luciferase expression, induced significant anti-luciferase serum antibody titres when compared to the empty particle control. Antibody responses to both particles were predominated by IgG2a, suggesting a proinflammatory Th1 bias that may be preferential for vaccination against some pathogens [500–502]. We found that the antibody titre moderately correlated with both the peak and total luciferase expression suggesting that in vivo bioluminescence imaging may be a viable method for screening novel LNP formulations. In addition to humoral immunity, effective vaccines may also induce a cellular immune response against the desired pathogen. Although T cell responses were not investigated in the present study, a previous comparison of SM102-RNA, AL0315-RNA, and MC3-RNA found that subcutaneous injection of all three particles induced similar levels of intracellular cytokine production by antigen-specific T cells [490]. We suggest that further analyses of the immune mechanisms stimulated by leading LNP formulations are required to better inform the development of effective DNA vaccines.

To our knowledge, our study is the first to demonstrate that LNPs formulated with SM-102 and ALC-0315 are effective for in vivo delivery of pDNA and result in significant and prolonged protein expression. Since both the SM-102 and ALC-0315 LNP formulations have been approved for clinical use, this finding could accelerate the development and approval of DNA vaccines and therapeutics for a variety of diseases. Despite the many advantages of the DNA vaccines, there are inherent obstacles associated with this platform. First, DNA vaccines are typically considered weakly immunogenic and are thought to require an adjuvant for sufficient immune activation [503,504]. However, the use of LNPs for DNA delivery has the potential to overcome this barrier due to their immunostimulatory properties [311,505]. Second, DNA vaccines have been historically associated with possible safety concerns, including integration into the host genome



and the development of anti-DNA antibodies [506,507]. More recent investigations into these claims have largely negated these concerns; however, future studies should continue to evaluate the safety of the DNA vaccine platform [479,508,509].

One limitation of our study is the use of the luciferase protein as a surrogate antigen. Luciferase is a relatively weak immunogen, and its use may not accurately reflect the efficacy of other potential vaccine antigens [510]. Therefore, LNP formulations in this study that did not induce a significant antibody response, including all DNA groups, may be immunogenic when encoding a more immunogenic antigen. Furthermore, in this study, only a single dose of each vaccine was administered. Considering that most vaccines require a multi-dose regimen for optimal efficacy, subsequent injections of the surrogate vaccine used in this study, or the inclusion of an adjuvant, may induce a significant antibody response. In fact, a recent study by our group demonstrated that a DNA-KC2 LNP, the poorest performer in our study in terms of *in vivo* potency and immunogenicity, encoding the outer surface protein C antigen of *B. burgdorferi*, elicited a significant immune response after two 12 ug doses of pDNA, a lower dose than what was administered here [387]. Based on the results of the present study, future studies should explore the immunogenicity and protection conferred by DNA-LNP vaccines formulated with SM-102 or ALC-0315 delivering varying doses of pDNA encoding a relevant antigen.

In conclusion, this study provides important insights into the comparison of three different LNP formulations for RNA and DNA vaccine delivery. We elucidated the potency, expression kinetics, biodistribution, and immunogenicity of some of the most widely used LNP formulations. Specifically, we found that formulations containing the ionizable lipids SM-102 and ALC-0315 delivering an RNA payload were the most potent and immunogenic, while the same lipid formulations delivering DNA resulted in the longest duration of luciferase signal. In addition, the

ALC0315-RNA group was also found to have increased hepatic tropism compared to other LNP groups. Our study is the first to demonstrate the utility of LNP formulations identical to those approved for COVID-19 mRNA vaccines for DNA vaccine delivery. Overall, this research could inform the development and optimization of nucleic acid vaccines used for the prevention of a variety of infectious diseases.

**Supplementary Materials:** Appendix - Figure S5.1: DNA-LNPs are more thermostable than RNA-LNPs.

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# Chapter 6: Polyvalent mRNA vaccine targeting outer surface protein C affords multi-strain protection against Lyme disease

## Preface

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## Abstract

There is currently no Lyme disease (LD) vaccine available for use in humans. Outer surface protein C (OspC) of the causative agent, *Borrelia burgdorferi*, is a promising LD vaccine target. However, the extensive genetic variation of OspC poses a challenge in affording broad protection. Here, we developed a monovalent mRNA vaccine encoding OspC type A and a polyvalent vaccine encoding OspC types A, C, I, K, and N. The monovalent vaccine conferred complete protection against homologous challenge in mice, inducing functional OspC-specific antibodies and CD4<sup>+</sup> T cell responses. The polyvalent formulation elicited antibodies to all encoded OspC types and protected against strains expressing OspC types A, I, and K, but not C or N. Increasing the dose enhanced protection against the OspC type C strain. This study is the first demonstration of an effective OspC-targeted mRNA vaccine and supports the development of OspC-based vaccines for broad LD prevention.

## 6.1. Introduction

Lyme disease (LD) is a significant public health concern in temperate regions of North America and Europe. It remains the most common vector-borne disease across both continents, with an estimated 480,000 cases occurring in North America and over 120,000 reported cases in Europe annually [1–3,511,512]. These case numbers represent a substantial increase in incidence since monitoring began, driven largely by the expansion of associated tick habitats and greater human encroachment into endemic regions [4,6,8].

LD is caused by spirochetes of the *Borrelia burgdorferi* sensu lato (sl) genospecies complex. The genospecies that cause most cases of LD are *B. burgdorferi* sensu stricto (ss) in

North America and *B. afzelii* and *B. garinii* in Europe [336,337]. These spirochetes are maintained in nature via enzootic cycles involving wildlife hosts and ticks in the genus *Ixodes*, which transmit the spirochetes into the host skin. The bacteria then disseminate to various organs including the joints, heart, and nervous system. In humans, *B. burgdorferi* ss infection initially presents with flu-like symptoms and the characteristic bull's-eye rash, known as *erythema migrans*, in 70-80% of cases [28,151,152]. Severe manifestations of disseminated LD can include arthritis, carditis, and neurocognitive dysfunction [145,161,162,429]. Although antibiotics are available and effective for the treatment of LD, 10-20% of patients experience continued symptoms after appropriate treatment, termed post-treatment Lyme disease syndrome (PTLDS) [513–515]. Furthermore, many LD cases suffer from delayed diagnoses or misdiagnoses, emphasizing the need for improved preventative measures [447,516].

LYMERix™, a recombinant subunit vaccine for LD, was previously approved by Health Canada and the United States Food and Drug Administration (FDA) in 1998 [19]. The LYMERix™ vaccine targeted outer surface protein A (OspA), an immunogenic lipoprotein found on the surface of *B. burgdorferi*. Four years after approval, LYMERix™ was voluntarily withdrawn from the market by the manufacturer due to poor sales amidst reports of vaccine-induced arthritis, which were later unsubstantiated by an FDA review [19,268]. Since OspA is expressed by the spirochete during infection of the tick but is downregulated upon tick feeding and throughout mammalian infection, vaccines that target OspA must generate and maintain high levels of circulating antibodies that can enter the tick midgut during the tick blood meal to kill the spirochetes inside the tick before transmission to the host can occur [50,58,331]. As a result, annual booster injections were recommended for the LYMERix™ vaccine, introducing an additional burden on patients that may reduce vaccine compliance. A potentially more promising strategy for LD vaccine

development is to target an alternative *B. burgdorferi* lipoprotein, such as outer surface protein C (OspC). In contrast to OspA, OspC is highly expressed during infection of the mammalian host and is required for early dissemination [87,331]. Vaccines targeting OspC may therefore require less frequent booster vaccinations [517].

A major challenge in developing OspC-targeted vaccines is the high genetic diversity of the *ospC* gene within each *B. burgdorferi* s.l. genospecies. In North American strains of *B. burgdorferi* ss, the *ospC* gene is highly polymorphic with over 20 major allele groups that are referred to as OspC types or major groups [95,97,107]. Cross-protection between OspC types is limited as previous infection or vaccination with recombinant OspC only affords protection against subsequent infection with homologous OspC types [70,94,98,101]. In areas where LD is endemic, human patients can be repeatedly infected with *B. burgdorferi* strains carrying different OspC types [518]. Additionally, studies examining *B. burgdorferi* strain diversity in populations of *Ixodes* ticks have repeatedly shown that a high percentage of infected ticks contain more than one OspC type [97,102,519,520]. An effective LD vaccine must therefore include a majority of the predominant OspC types in a given region.

The global deployment of the BNT162b2 and mRNA-1273 vaccines during the COVID-19 pandemic unequivocally demonstrated the immunogenicity, safety, and real-world efficacy of mRNA vaccines [521–523]. mRNA vaccines are highly adaptable and can be rapidly synthesized at large scales using cell-free systems. Recent research also suggests the utility of this platform to target bacterial infections, including two *B. burgdorferi* OspA-targeting vaccines undergoing preclinical evaluation [301,302,524,525]. Furthermore, the mRNA vaccine platform is particularly suited to the development of polyvalent vaccines, as mRNA encoding multiple antigens or antigen variants can be encapsulated within lipid nanoparticles (LNPs) and delivered together.

One clinically available veterinary vaccine consisting of a chimeric recombinant OspC (crOspC) protein in combination with OspA, Vanguard® crLyme, has demonstrated considerable efficacy and safety for the prevention of LD in canines [125]. Additionally, crOspC vaccines consisting of the immunodominant epitopes from a variety of different OspC types are capable of generating antibodies that recognize over 20 different OspC proteins spanning multiple *B. burgdorferi* genospecies and demonstrate borreliacidal activity *in vitro* [116,118,139,219]. Nevertheless, the breadth of protection conferred by OspC vaccines in the absence of OspA has not been thoroughly investigated.

Here, we developed both a monovalent and polyvalent nucleoside-modified mRNA-LNP vaccine encoding either OspC type A alone (OspC-A mRNA) or in conjunction with four other OspC types that are among the most prevalent in human and tick infections in North America (OspC-ACIKN mRNA) [97,105,108,526]. We first investigated the protection conferred by the monovalent vaccine against homologous *B. burgdorferi* needle challenge in a C3H/HeN mouse model. We then characterized the antibody and T cell immune responses induced by the monovalent OspC-A mRNA vaccine. Finally, we assessed the breadth of protection afforded by the polyvalent OspC-ACIKN mRNA against the five corresponding infection strains. Ultimately, our results demonstrate that OspC-targeting mRNA vaccines elicit both antigen-specific humoral and cellular mediated immunity and can confer simultaneous protection against multiple *B. burgdorferi* strains.

## 6.2. Materials and methods

### 6.2.1. Bacterial cell culture

The *B. burgdorferi* strains used in this paper were characterized in detail in a previous publication, including B31-A3 (OspC type A), JD1 Clone SK143, (OspC type C), Bb16-54 (OspC type I), 297

Ah130 (OspC type K), and Bb16-126 (OspC type N) [527]. Strain B31-A3, and strain B31-A3 engineered to constitutively express OspC (B31-A3-OspC), were kindly provided by Dr. Patricia Rosa (National Institute of Allergy and Infectious Disease, MD, USA) [383,528]. Strain JD1 and strain 297 Ah130 were kindly provided by Dr. George Chaconas (University of Calgary, AB, Canada). Strain Bb16-54 and strain Bb16-126 were obtained from the *B. burgdorferi* isolate collection of the Public Health Agency of Canada. Maintenance of the *B. burgdorferi* strains was performed as previously described [381,387,527]. In brief, *B. burgdorferi* was cultured at 35°C and 1.5% CO<sub>2</sub> in BSK-H media containing 6% rabbit serum and supplemented with 5 µg/mL Amphotericin B, 100 µg/mL Phosphomycin, and 50 µg/mL Rifampicin. Cultures were grown to mid-log phase and were not subcultured to prevent plasmid loss. Cell counts were determined using a Petroff-Hausser counting chamber under dark-field microscopy.

### 6.2.2. Animal care

Female C3H/HeN mice were purchased from Charles River (Senneville, Quebec, Canada). Mice arrived at 6 weeks old and were allowed to acclimatize for one week before experimental procedures began. All animal procedures in this study were reviewed and approved by the Health Canada Animal Care Committee and were conducted under protocols #2020-011, #2023-003, and #2024-003 according to institutional guidelines.

### 6.2.3. mRNA and lipid nanoparticle synthesis

mRNA encoding firefly luciferase, ospC type A of *B. burgdorferi* strain B31 (GenBank accession #AAC66329.1), ospC type C of strain JD1 (GenBank accession #ADQ31294.1), ospC type I of strain Bb16-54 (GenBank accession #PHOQ01000005.1), OspC type K of strain 297 (GenBank accession #NC\_018983.1), or ospC type N of strain Bb16-126 (GenBank accession



#PHQD00000000.1) were commercially synthesized by TriLink Biotechnologies (San Diego, CA, USA) with a CleanCap®AG Cap 1 structure and a 120A polyadenylated tail. mRNA was DNase and phosphatase treated and purified by a silica membrane. OspC mRNA contained a human tyrosinase signal peptide (MLLAVLYCLLWSFQTSAGHFPRA; GenBank accession #AH003020) at the N-terminus. All mRNA was fully substituted with N1-methyl-pseudo-uridine and was codon optimized for expression in mice.

Lipid nanoparticles (LNPs) were produced using a NanoAssemblr BT™ or Ignite™ instrument (Precision Nanosystems, Inc., Vancouver, BC) as described previously, encapsulating mRNA instead of plasmid DNA [387]. The percent molar ratio was 50 : 10 : 38.5 : 1.5, respectively, of lipids heptadecan-9-yl-8-((2-hydroxyethyl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate (SM-102) (MedKoo Biosciences, Morrisville, NC, USA), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC, Avanti Polar Lipids, Inc., Alabaster, AL, USA), ovine cholesterol (Millipore Sigma, Burlington, ON, CA), and 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG2000, Millipore Sigma). Lipids in ethanol were mixed at a ratio of 3:1 to aqueous 50mM sodium citrate, pH4, containing the mRNA at an N/P ratio of 6. For empty lipid nanoparticle controls, the aqueous phase contained 50mM sodium citrate only.

#### 6.2.4. LNP characterization

LNP size was characterized by nanoparticle tracking analysis (NTA) using a NanoSight 300 instrument (Malvern Panalytical, Westborough, MA, USA) as described previously [387]. Average particle size ranged from 68 to 83 nm. The efficiency of nucleic acid encapsulation was determined using Triton X-100 (Millipore Sigma) and SYBR™ Gold dye (ThermoFisher, Ottawa, ON, CA) as described previously [387]. In brief, LNPs were disrupted with 1% Triton X-100 in a 96-well plate and nucleic acids were quantified by addition of 1X SYBR™ Gold dye and measured

using a Synergy MX plate reader (Ex/Em: 495/537 nm) (BioTek, Winooski, Vermont, USA). Disrupted LNPs were measured alongside undisrupted LNPs and a nucleic acid standard curve. The encapsulation efficiency was determined by subtracting the unencapsulated mRNA from the total amount of mRNA in a sample and then dividing by the total amount of mRNA. The encapsulation efficiencies ranged from 88% to 98%.

#### 6.2.5. Mass spectrometry confirmation of OspC expression

Expression of the OspC proteins was confirmed by mass spectrometry following transfection of HEK293T cells *in vitro* with OspC-ACIKN mRNA encapsulated in LNPs (Figure S6.1). HEK293T cells were incubated with 500 ng of OspC-ACIKN mRNA encapsulated in LNPs at 37 °C and 5% CO<sub>2</sub>. After 24 hours, the cells were lysed in Radio-Immunoprecipitation Assay buffer and then pulse sonicated. The samples were processed with a single pot solid phase-enhanced sample preparation (SP3) strategy, consisting of reduction with 10 mM DTT followed by alkylation with 20 mM IAA. Protein was bound to SeraMag Magnetic carboxylate beads in the presence of 80% ACN, then impurities were removed with 3 washes of 80% ACN. Trypsin was added at a ratio of 1:25 enzyme: protein and incubated overnight at 37°C.

Tryptic peptides were analyzed using an Orbitrap Astral Mass Spectrometer coupled with a Vanquish Neo UHPLC System (ThermoFisher Scientific Inc.). For each injection 1 ul (500 ng peptides) were analyzed by loading onto a PepMap Neo C18 trap and desalting with 0.1% formic acid in water (solvent A) before separating on 75 µm I.D. × 150 mm fused silica analytical column packed in-house with 3µm ReproSil-Pur C18 beads (100 Å; Dr. Maisch GmbH, Ammerbuch, Germany). Chromatographic separation was achieved at a flow rate of 500 nl/min. The gradient was set as 5–45% buffer B in 28.5 min, the spray voltage was set to 2.6 kV, and the temperature

of the heated capillary was 300 °C. Scan range was from 380 to 980 m/z for both MS and DIA. The DIA window was set to 2 Dalton, with HCD energy of 25%. The mass resolution was 240000 for ms1. A real-time internal calibration by the lock mass was used. All data were recorded with Xcalibur software (ThermoFisher Scientific, San Jose, CA).

The software package DiaNN (v2.1.0) was used to analyze the data with a library-free search against the reviewed Uniprot Human database (downloaded 20241120, entries) containing the sequences of the five OspC types added. The resulting protein and peptide results were filtered to view only OspC. Relative abundance was manually calculated by the Top3 method by averaging the three most abundant precursors, ignoring shared peptides. The relationship between MS signal response and protein concentration is constant within a CV of less than +/-10%.

#### 6.2.6. Immunizations and bacterial challenge

For the monovalent protection study, eight-week-old female C3H/HeN mice were randomly assigned to groups and injected intramuscularly in the hind leg with LNPs containing 1 µg of mRNA encoding OspC-A (OspC-A mRNA, n = 5) or an empty LNP negative control containing a matched amount of lipid (Control, n = 5) (Figure 6.1A). For the polyvalent protection study, eight-week-old female C3H/HeN mice were randomly assigned to groups and injected intramuscularly with LNPs containing 3 µg of each mRNA encoding the five OspC types (OspC-ACIKN, n = 10) or 3 µg of mRNA encoding firefly luciferase (Control, n = 10) (Figure 6.6A). The total injection volume was 50 µL divided between the two hind legs. Mice received an identical boost injection four weeks later and were bled three weeks after the boost injection for collection of serum. Bacterial challenge was performed four weeks after the boost injection by subcutaneous needle injection between the shoulder blades.

For the challenge, spirochetes were grown to mid-log phase, centrifuged at 5000 x g for 20 min, and resuspended in 100 µL of incomplete BSK-H media without rabbit serum. For the monovalent challenge, mice were injected with 10<sup>6</sup> spirochetes of strain B31-A3. For the OspC-ACIKN vaccine study challenge, mice were injected with 10<sup>6</sup> spirochetes of strain B31-A3 (OspC type A), Bb16-54 (OspC type I), or 297 Ah130 (OspC type K), or 10<sup>4</sup> spirochetes of strain JD1 (OspC type C) or Bb16-126 (OspC type N). Challenge doses were determined based on our previous study characterizing strain infectivity [527]. An additional group of five mice per study were injected with phosphate-buffered saline (PBS) instead of LNPs at day 0 and 28 and incomplete BSK-H media without bacteria instead of challenge at day 56 to serve as naive controls for histopathological analysis. Fourteen days after the challenge (d70), mice were euthanized by cardiac puncture exsanguination and cervical dislocation under isoflurane anesthesia for sample collection.

For the immunogenicity study, eight-week-old female C3H/HeN mice were randomly assigned to groups and injected intramuscularly with either 1 µg of mRNA encoding OspC-A encapsulated in a lipid nanoparticle (OspC-A mRNA, n = 10) or 1 µg of mRNA encoding firefly luciferase encapsulated in a lipid nanoparticle (Control, n = 10). Mice received an identical boost injection four weeks later and were euthanized four weeks after the boost for sample collection. Euthanasia was performed by cardiac puncture exsanguination and cervical dislocation under isoflurane anesthesia.

#### 6.2.7. Tissue culture for bacterial identification

Live spirochetes can be cultured from mouse tissues to determine mouse infection status and spirochete viability. The skin surrounding the bacterial injection site and the right ear were

collected two weeks after challenge and cultured in complete BSK-H media for 28 days at 35°C and 1.5% CO<sub>2</sub>. Cultures were examined under darkfield microscopy across five fields of view and were considered positive if at least one motile spirochete was observed.

#### 6.2.8. Quantitative PCR (qPCR) for bacterial burden

Two weeks after the bacterial challenge, the mice were euthanized and 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 extraction from mouse tissues was performed using a DNAeasy kit (Qiagen, Toronto, ON, CA) according to the manufacturer's instructions and qPCR analysis was performed as described previously, targeting the *flaB* gene of *B. burgdorferi* and standardized per 10<sup>6</sup> mouse  $\beta$ -actin gene copies [387].

#### 6.2.9. Determination of lymphadenopathy

Quantification of lymphadenopathy was performed as described previously [381]. Briefly, the left axillary lymph nodes were collected two weeks after bacterial challenge, pressed between two microscope slides, and filtered through a 70  $\mu$ m cell strainer to create a single-cell suspension. White blood cell counts were determined using a Sysmex XT-2000iV haematology analyzer.

#### 6.2.10. Histopathology

Histopathology analysis of the heart and axillary lymph nodes was performed as described previously [381,387]. In brief, the superior half of the heart and the right axillary lymph nodes were collected two weeks after bacterial challenge into 10% neutral buffered formalin for routine histopathological processing and hematoxylin and eosin staining. Inflammation in the heart tissue and lymph nodes was graded on a scale from 0-5 based on International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice (INHAND) standards, where

0 was normal, 1 was minimal, 2 was mild, 3 was moderate, 4 was marked, and 5 was severe [441,442]. Lymph nodes were graded individually 0-5 for follicular hyperplasia, paracortical hyperplasia, medullary cord plasma cell hyperplasia and the combined pathology score represents the sum of these three grades.

#### 6.2.11. Enzyme-linked immunosorbent assay (ELISA)

OspC-specific antibody titres were quantified according to the method described previously [381,387]. Briefly, recombinant OspC type A, C, I, K or N were used to coat 96-well Nunc Maxisorp™ flat bottom plates (ThermoFisher) at a concentration of 1 µg/mL in 100 µL overnight at 4°C. Plates were blocked with 1% (w/v) bovine serum albumin (IgG-Free, Protease-Free, Jackson ImmunoResearch, West Grove, PA) and incubated with serum collected four weeks after boost-vaccination serially diluted in PBS containing 0.05% Tween-20 (PBS-T) for 1 h at 37°C. For total IgG ELISAs, the serum dilutions were two-fold from 1:50 to 1:102,400. For IgG1 and IgG2a ELISAs, the serum dilutions were three-fold from 1:50 to 1:8,857,350. Following serum incubations and washing, HRP-conjugated goat anti-mouse IgG (Cytiva, Marlborough, MA), HRP-conjugated goat anti-mouse IgG1 (Jackson ImmunoResearch), or HRP-conjugated goat anti-mouse IgG2a (Jackson ImmunoResearch) was added to each plate at 1:5,000 dilution in PBS-T. Tetramethyl-benzidine (TMB) substrate (Cell Signaling Technology, Danvers, MA, USA) was added to each well, followed by 0.16 M sulphuric acid, and then the absorbance was read at 450 nm using a spectrophotometer. 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 control mice plus three times the standard deviation.

### 6.2.12. Antibody-dependent phagocytosis (ADCP) assay

ADCP activity was measured as previously described [381]. In brief, mouse serum samples were collected four weeks after boost vaccination, diluted 1:10 in Dulbecco's Modified Eagle Medium (DMEM) containing no serum or antibiotics, and heat-inactivated at 56°C for 30 min. In a 96-well plate, 20 µL of diluted inactivated serum was incubated with *B. burgdorferi*-OspC stained with a FITC-conjugated anti-Borrelia polyclonal antibody (PA1-73005, ThermoFisher) at a concentration of  $3.75 \times 10^7$  cells/mL for 15 min at 37°C and 300 rpm to enable opsonization. Then, 10 µL of RAW 264.7 cells (ATCC TIB-71), a mouse macrophage cell line, at a concentration of  $7.5 \times 10^6$  cells/mL were added to each well before shaking for 15 min at 37°C and 300 rpm and then incubating for 1 h at 37°C without shaking. Phagocytosis was arrested by addition of 100 µL of cold PBS and ADCP activity was represented by the percentage of FITC+ RAW 264.7 cells using a BD FACSymphony™ A1 flow cytometer.

### 6.2.13 Serum borreliacidal assay

Serum borreliacidal activity was measured as previously described [387]. In brief, 4 µL of mouse serum samples collected four weeks after boost vaccination were incubated with 4 µL of mid-log phase *B. burgdorferi*-OspC culture diluted to  $5 \times 10^7$  cells/mL and 4 µL of guinea pig serum (GPS, Complement Technologies, Typer, Texas, USA) for 24 hours at 35°C and 1.5% CO<sub>2</sub>. Motile cells were then quantified using a Petroff-Hausser counting chamber under dark-field microscopy and the percentage of live spirochetes was determined by dividing the cell count by the average cell count of control samples without serum.

#### 6.2.14. Generation of single-cell suspensions from the draining lymph nodes

Four weeks after OspC-A mRNA or Control boost vaccination, the draining popliteal and inguinal lymph nodes were collected in RPMI media. The nodes were gently squished between two microscope slides and passed through a 70  $\mu$ m cell strainer before centrifugation at 400 x g for 5 min. Cells were resuspended in RPMI and white blood cells were counted using a Sysmex XT-2000iV haematology analyzer.

#### 6.2.15. Multiplex cytokine ELISA

The multiplex cytokine ELISA was performed using a ProcartaPlex 17-plex Immunoassay kit (EPX170-26087-901, ThermoFisher) as described previously [381]. In brief, 1 million lymph node cells in single-cell suspension from OspC-A vaccinated or control mice (n = 5 per group) were stimulated with a custom overlapping peptide pool of OspC type A (GenScript, Piscataway, NJ, USA) for 24 h at 37°C before centrifugation at 400 x g for 5 min. The supernatant was collected and frozen at -80°C until the assay was performed according to the manufacturer's instructions. A Luminex 200 system (Millipore Sigma) was used to read the plate and MILLIPLEX Analyst software version 5.1 was used to determine the concentration of each cytokine.

#### 6.2.16. Flow cytometry of activation-induced markers

In a 96-well plate, 1 million mouse lymph node cells (n = 5 per group) in single-cell suspension from OspC-A vaccinated or control mice were stimulated individually with a custom 15-mer overlapping peptide pool of OspC type A containing 1  $\mu$ g of each peptide (GenScript) in RPMI media for 18 h at 37°C, 5% CO<sub>2</sub>. Unstimulated lymph node cells were included as a negative control and lymph node cells stimulated with phorbol 12-myristate-13-acetate (Sigma-Aldrich, St. Louis, MO, USA) at 20 ng/mL and ionomycin (Sigma-Aldrich) at 1  $\mu$ g/mL (PMA+ionomycin)



were included as a positive control. Following the incubation, lymph node cells were centrifuged at 500 x g for 5 min, washed twice with FACS buffer, and resuspended in FACS buffer containing rat anti-mouse CD16/CD32 (Mouse Fc Block™, BD Biosciences, Mississauga, ON, CA). The plate was incubated for 15 min at 4°C and then centrifuged at 500 x g for 5 min. The lymph node cells were resuspended in antibody cocktail containing anti-CD3 Alexa Fluor® 700 (Clone 17A2, BD Biosciences), anti-CD4 BB700 (Clone RM4-5, BD Biosciences), anti-OX40 BV786 (Clone OX-86, BD Biosciences), anti-CD25 PE (Clone OX-39, BD Biosciences), and LIVE/DEAD™ Fixable Blue Dead Cell Stain (ThermoFisher) diluted in Brilliant Stain Buffer (BD Biosciences) and incubated for 30 min at 4°C in the dark. Lymph node cells were washed twice with FACS buffer, fixed in Cytofix™ fixation buffer (BD Biosciences) for 15 min at room temperature, washed twice again and then resuspended in FACS buffer. Acquisition was performed using a BD FACS Aria™ Fusion flow cytometer and data analysis was performed using FlowJo v10 software (Treestar) according to the gating strategy depicted in Figure S6.2.

#### 6.2.17. Enzyme-linked immunosorbent spot (ELISpot) assay

The ELISpot assay was performed using a Mouse IFN- $\gamma$ /IL-4 Double-Color ELISPOT kit according to the manufacturer's instructions (ImmunoSpot by Cellular Technology Limited, Shaker Heights, Ohio, USA). For stimulation, 1 million lymph node cells in single-cell suspension from OspC-A vaccinated or control mice (n = 5 per group) were stimulated individually with a custom 15-mer overlapping peptide pool of OspC type A containing 1  $\mu$ g of each peptide (GenScript). The plates were read using an ImmunoSpot® S6 Analyzer and data analysis was performed using ImmunoSpot® v7.0 software.

### 6.2.18. Intracellular cytokine staining

Intracellular cytokine staining and flow cytometry analysis of antigen-specific T cells was performed as described previously using mouse lymph node cells instead of splenocytes [381]. In brief, 1 million lymph node cells in single-cell suspension from OspC-A vaccinated or control mice ( $n = 5$  per group) were stimulated individually with a custom 15-mer overlapping peptide pool of OspC type A containing 1  $\mu\text{g}$  of each peptide (GenScript) for 5 h. Unstimulated and PMA+ionomycin-stimulated samples were included as negative and positive controls, respectively. GolgiPlug™ (Brefeldin A, BD Biosciences) was added to each well after the first hour. After the 5 h incubation, lymph node cells were washed with PBS, blocked with rat anti-mouse CD16/CD32 (Mouse Fc Block™, BD Biosciences), and surface stained for 30 min at 4°C with anti-CD3 Alexa Fluor® 700 (Clone 17A2, BD Biosciences), anti-CD4 BB700 (Clone RM4-5, BD Biosciences), anti-CD8a FITC (Clone 53-6.7, BD Biosciences) and LIVE/DEAD™ Fixable Blue Dead Cell Stain (ThermoFisher) diluted in Brilliant Stain Buffer (BD Biosciences). Lymph node cells were fixed and permeabilized before intracellular staining with anti-IFN- $\gamma$  BV786 (Clone XMG1.2, BD Biosciences), anti-TNF BV650 (Clone MP6-XT22, BD Biosciences), and anti-IL-2 (Clone JES6-5H4, BD Biosciences) diluted in Brilliant Stain Buffer (BD Biosciences) for 30 min at 4°C. Cells were resuspended in PBS and acquired immediately using a BD FACS Aria™ Fusion flow cytometer. Data analysis was performed using FlowJo v10 software (Treestar) according to the gating strategy described previously [381].

### 6.2.19. Statistical analysis

Statistical significance of data or log10-transformed data was calculated using unpaired two-tailed t-tests or Fisher's exact test at a significance value of  $\alpha = 0.05$ . All statistical analyses were

performed using GraphPad Prism 9 (Dotmatics Inc, Boston, MA, USA). ns, not significant, \* p-value < 0.05, \*\* p-value < 0.01, \*\*\* p-value < 0.001, \*\*\*\* p-value < 0.0001.

## 6.3. Results

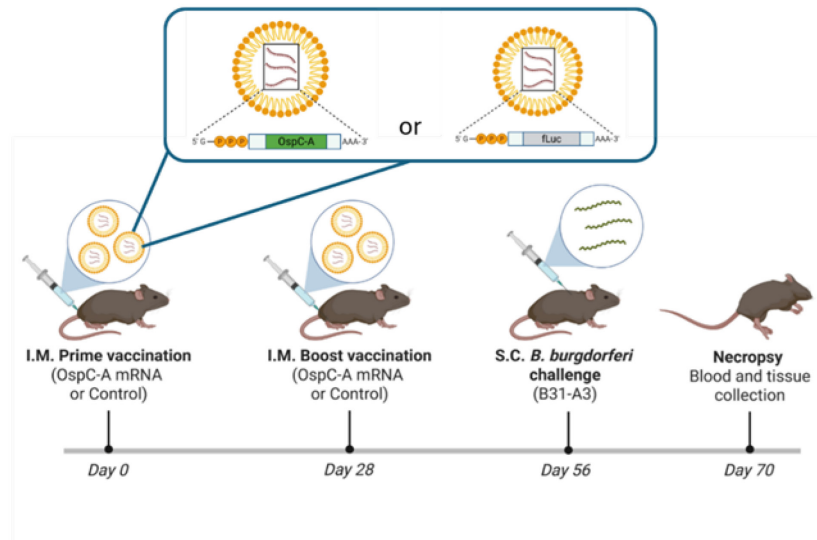
### 6.3.1. OspC-A mRNA affords complete protection against *B. burgdorferi* infection and dissemination

To evaluate the ability of the candidate mRNA vaccine to protect against *B. burgdorferi* infection, female C3H/HeN mice were vaccinated with a prime-boost regimen and then challenged with a homologous strain of *B. burgdorferi*. On day 0, mice were injected intramuscularly with 1 µg of OspC-A mRNA or an empty LNP control containing an equivalent amount of lipid (Control). An identical boost injection was administered on day 28 and then four weeks later mice were challenged subcutaneously with 10<sup>6</sup> spirochetes expressing the matching OspC type A. Fourteen days later, mice were sacrificed for tissue collection (Figure 6.1A).

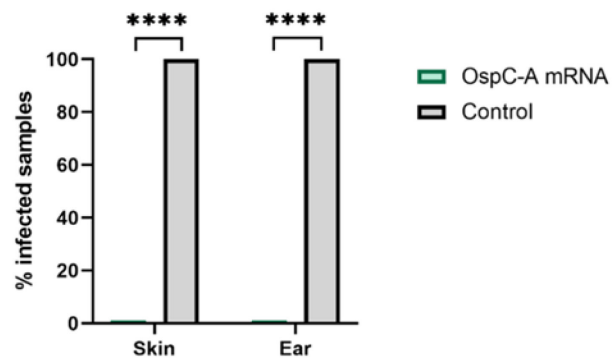
Infection status of the mice was determined by culturing spirochetes from explants of the mouse skin and ears. The presence of motile spirochetes was detected in the samples using dark field microscopy. All mice injected with the empty LNP control were positive for *B. burgdorferi* infection in both the skin and ears (Figure 6.1B), demonstrating robust infection via the needle challenge. In comparison, all skin and ear samples collected from mice vaccinated with OspC-A mRNA were negative for the presence of spirochetes. Furthermore, qPCR was performed to quantify the bacterial burden across a wider array of tissues including the ear, bladder, lymph nodes, tibiotarsal joint, and heart. Significant *B. burgdorferi* DNA loads were detected in all of these tissues in the control mice, whereas the bacterial DNA was below the limit of detection in all tissues of the OspC-A mRNA vaccinated mice (Figure 6.1C). Taken together, these results suggest that the candidate OspC-A mRNA vaccine completely protects against infection and

dissemination of *B. burgdorferi* strains carrying OspC type A in mice one month after boost vaccination.

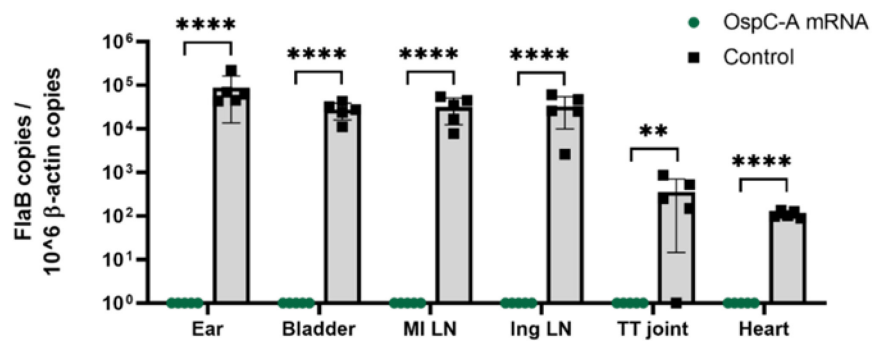
**A**



**B**



**C**



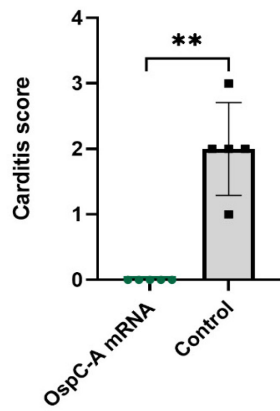
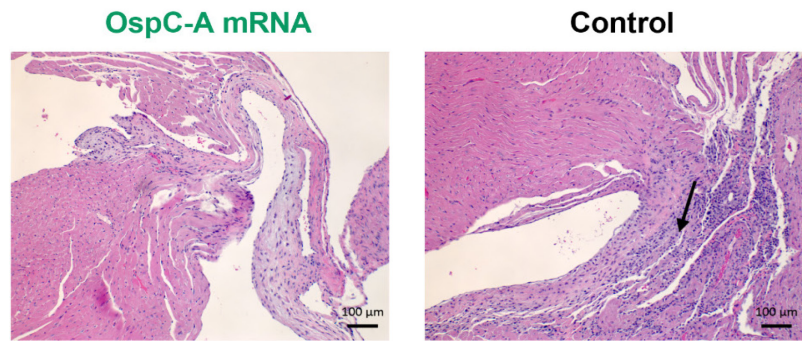
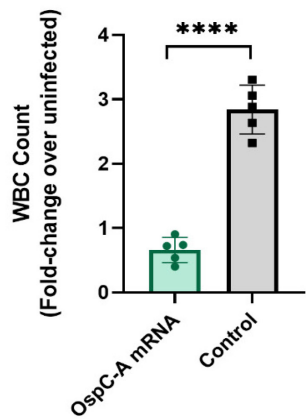
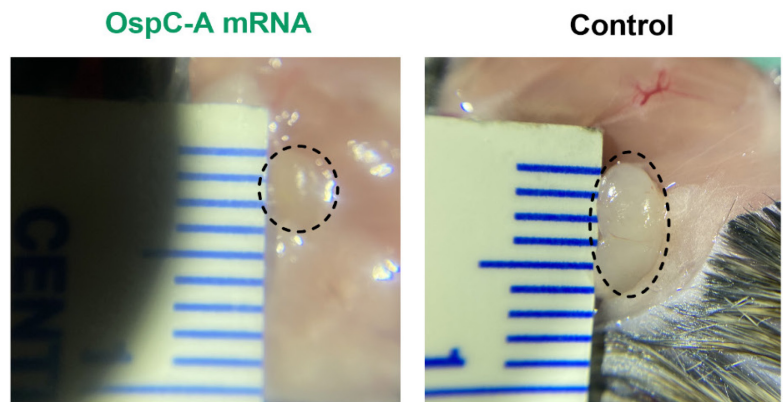
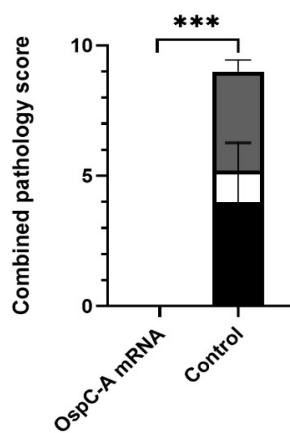
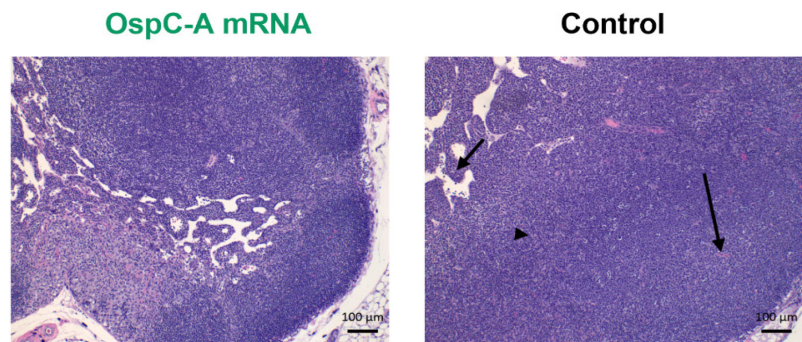
**Figure 6.1.** OspC-A mRNA affords protection against infection and dissemination with homologous *B. burgdorferi*. **A)** Schematic depicting the procedure schedule for C3H/HeN mice. Mice were injected with the OspC-A mRNA vaccine or an empty LNP control (Control) on day 0 and 28 (n = 5 per group), challenged with homologous *B. burgdorferi* B31-A3 on day 56, and euthanized on day 70. Created using BioRender.com. **B)** Percentage of infected mice following challenge. The skin surrounding the spirochete injection site and right ear were collected at necropsy and placed in BSK-H culture media for 28 days at 35°C, 1.5% CO<sub>2</sub> before examination by dark-field microscopy. Samples were considered positive for infection if at least one spirochete was identified across five fields of view. Statistical significance was calculated using Fisher's exact test. **C)** qPCR analysis of the bacterial DNA burden represented by *B. burgdorferi* FlaB gene copies per 10<sup>6</sup> mouse  $\beta$ -actin copies. Samples below the threshold of amplification are represented by a value of 1. TT joint, tibiotarsal joint; MI LN, medial iliac lymph node; Ing LN, inguinal lymph node. Statistical significance was calculated using independent two-tailed t-tests. Error bars represent standard deviation. \*\* p-value < 0.01, \*\*\*\* p-value < 0.0001.

### 6.3.2. OspC-A mRNA protects against Lyme carditis and lymphadenopathy

Disseminated infection with *B. burgdorferi* causes multisystem inflammation in both humans and mice. In human cases, Lyme carditis can cause life-threatening dysfunction of the conduction system, such as atrioventricular blocks [529]. To evaluate whether the candidate vaccine could prevent the development of carditis in mice, histopathological analysis of the heart tissue was conducted at 14 days post-infection. Control mice developed mild carditis, characterized by mixed mononuclear-neutrophilic infiltration of the heart base with variable involvement of adjoining structures (Figure 6.2A, B). In comparison, mice vaccinated with the OspC-A mRNA vaccine did not exhibit any detectable carditis.

Dissemination of *B. burgdorferi* to the lymph nodes causes extensive extrafollicular proliferation of B cells [158]. This accumulation of B cells leads to swelling of the lymph nodes systemically and especially close to the site of infection [30,390]. Accordingly, we observed severe enlargement of the axillary lymph nodes in all control mice, whereas all OspC-A mRNA vaccinated mice had lymph nodes of a similar size to uninfected mice. In addition, white blood

cell (WBC) counts from axillary lymph nodes of control mice were approximately four-fold higher than the WBC counts from the OspC-A mRNA group (Figure 6.2C, D). Inflammation of the axillary lymph nodes in the control mice was characterized by marked follicular and medullary cord plasma cell hyperplasia and mild paracortical hyperplasia (Figure 6.2E, F). Conversely, hyperplasia was not detected in any OspC-A mRNA vaccinated mice. In summary, these data demonstrate that the candidate OspC-A mRNA vaccine completely protects mice against carditis and lymphadenopathy associated with *B. burgdorferi* infection.

**A****B****C****D****E****F**

Follicular Hyperplasia
  Paracortical Hyperplasia
  Medullary Cord Plasma Cell Hyperplasia



**Figure 6.2.** Vaccination with OspC-A mRNA protects mice against the development of *B. burgdorferi*-induced carditis and lymphadenopathy. **A)** Summary of carditis pathology scores from mice vaccinated with OspC-A mRNA or the empty LNP control (Control). **B)** Representative images of H&E-stained heart tissue from mice vaccinated with OspC-A mRNA or the empty LNP control (Control). Arrow indicates moderate mononuclear-neutrophilic inflammation in the heart base. **C)** White blood cell (WBC) counts of the left axillary lymph node extracted from OspC-A mRNA vaccinated and empty LNP control mice two weeks after bacterial challenge normalized to the WBC counts from unchallenged and uninfected mice. **D)** In situ images of the left axillary lymph node from mice injected with OspC-A mRNA or the empty LNP control at the time of necropsy (day 70). **E)** Summary of lymph node pathology scores. **F)** Representative images of H&E-stained axillary lymph nodes from mice vaccinated with OspC-A mRNA or the empty LNP control. Arrows indicate marked follicular hyperplasia (long arrow), severe medullary cord hyperplasia (short arrow), and mild paracortical hyperplasia (arrowhead).  $n = 5$  per group, error bars represent standard deviation. Statistical significance was calculated using independent two-tailed t-tests. \*\*  $p$ -value  $< 0.01$ , \*\*\*  $p$ -value  $< 0.001$ , \*\*\*\*  $p$ -value  $< 0.0001$ .

### 6.3.3. OspC-A mRNA generates a functional antibody response

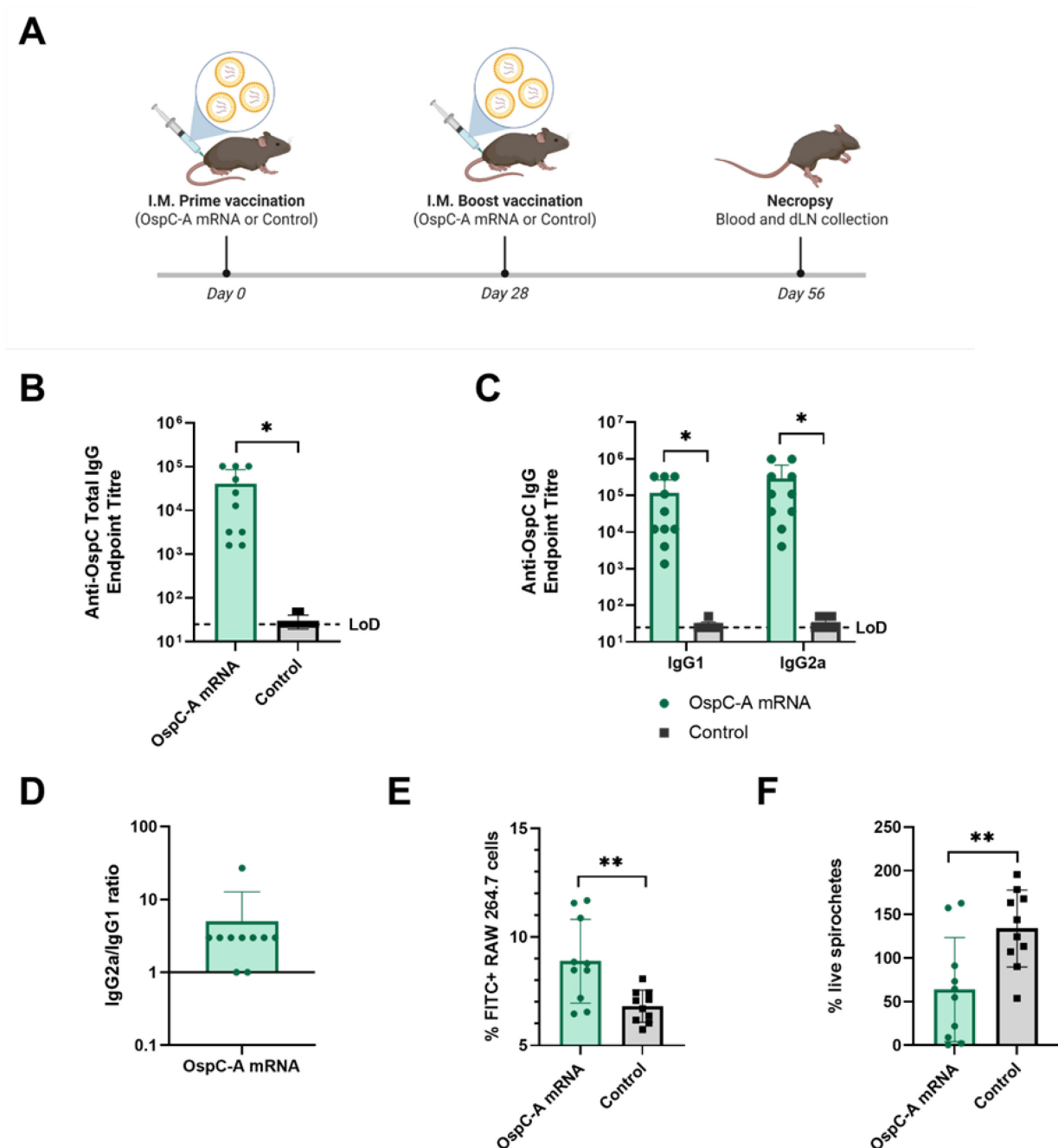
To investigate the immunological mechanisms conferring the observed protection against *B. burgdorferi* infection and disease, mice were vaccinated with either the OspC-A mRNA vaccine or a negative control mRNA-LNP vaccine encoding firefly luciferase (Control). Mice were vaccinated with 1  $\mu$ g of mRNA on day 0 and day 28. Four weeks post-boost, the mice were euthanized without bacterial challenge (Figure 6.3A).

Enzyme-linked immunosorbent assay (ELISA) was used to measure anti-OspC antibody titres in the serum of mice collected at four weeks post-boost. Vaccination with OspC-A mRNA produced significant anti-OspC antibody titres relative to the control (Figure 6.3B). OspC-A mRNA vaccination induced both IgG1 and IgG2a subtypes, with an IgG2a:IgG1 ratio greater than 1 (average =  $5 \pm 7.8$ ), indicating a Th1-biased immune response (Figure 6.3C, D).

Complement-mediated killing and phagocytosis are two of the principal mechanisms of *B. burgdorferi* clearance by the immune system [91,418,461,462]. Antibody binding to *B. burgdorferi* surface proteins can mark the spirochetes for phagocytosis in a process referred to as



opsonization or antibody-dependent cellular phagocytosis (ADCP). To evaluate whether the antibodies generated by vaccination with OspC-A mRNA were capable of inducing ADCP, fluorescently labelled *B. burgdorferi* were incubated with a RAW 267.4 mouse macrophage cell line in the presence of vaccinated serum. Since OspC is not highly expressed by *B. burgdorferi* *in vitro*, the B31-A3 strain used in this assay was engineered to constitutively express OspC (*B. burgdorferi*-OspC) [383,408]. Serum from mice vaccinated with OspC-A mRNA induced significantly higher levels of phagocytosis than serum from the control group, demonstrated by a greater percentage of fluorescent macrophages and a higher median fluorescence intensity (Figure 6.3E). Additionally, incubation of *B. burgdorferi*-OspC with serum from mice vaccinated with OspC-A mRNA in the presence of guinea pig serum, resulted in a two-fold decrease in live spirochetes after 24 hours, compared to the control vaccine (Figure 6.3F). Overall, these data suggest that the antibody response elicited by the candidate vaccine is functional and capable of inducing pertinent effector functions.

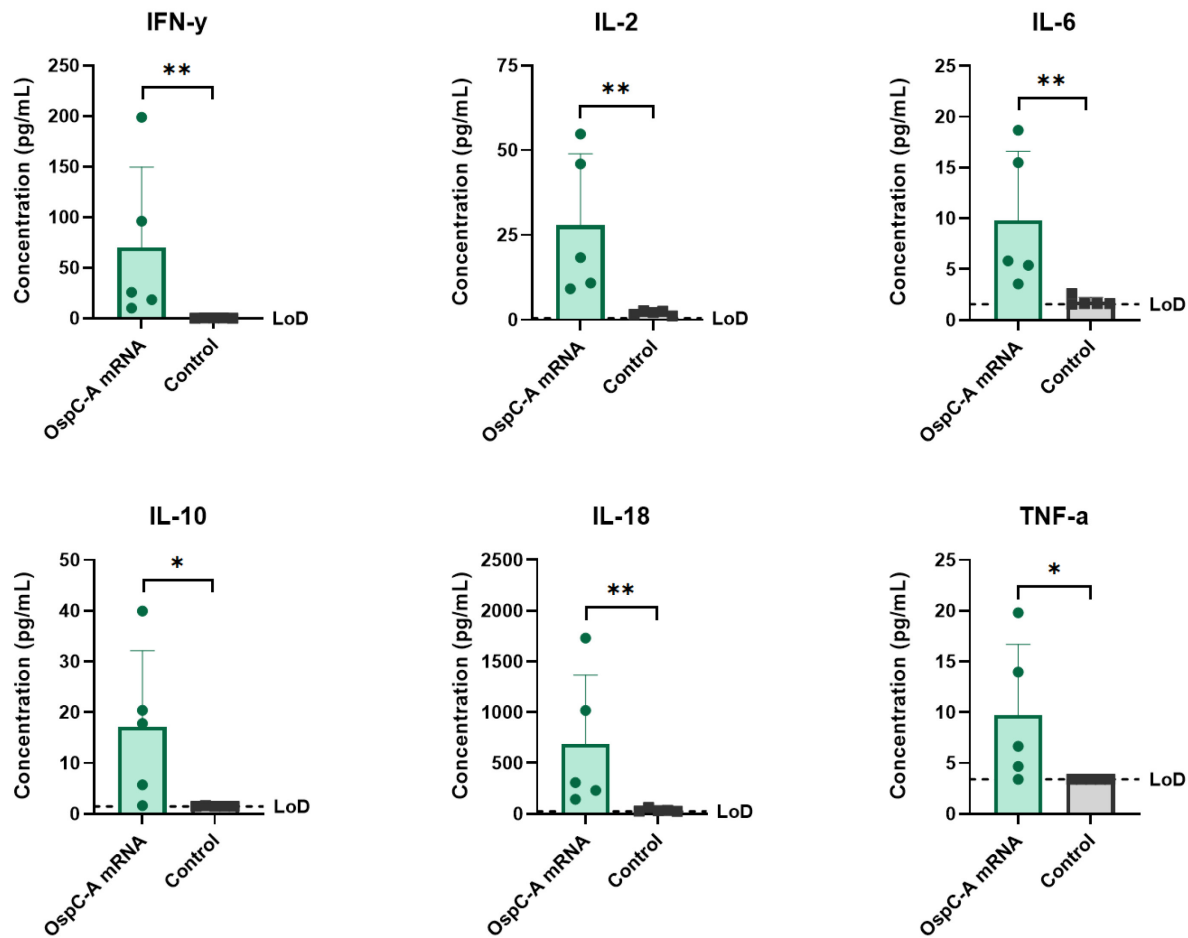


**Figure 6.3.** Vaccination with OspC-A mRNA induces a functional Th1-skewed anti-OspC antibody response in mice. **A)** Schematic depicting the procedure schedule for C3H/HeN mice. Mice were injected with the OspC-A mRNA vaccine or luciferase mRNA control (Control) on day 0 and 28 ( $n = 10$  per group) and euthanized on day 56 for collection of blood and draining lymph nodes (dLNs). Created using BioRender.com. ELISA quantification of **B)** anti-OspC total IgG and **C)** anti-OspC IgG1 and IgG2a antibody titres in serum of mice at four weeks after boost

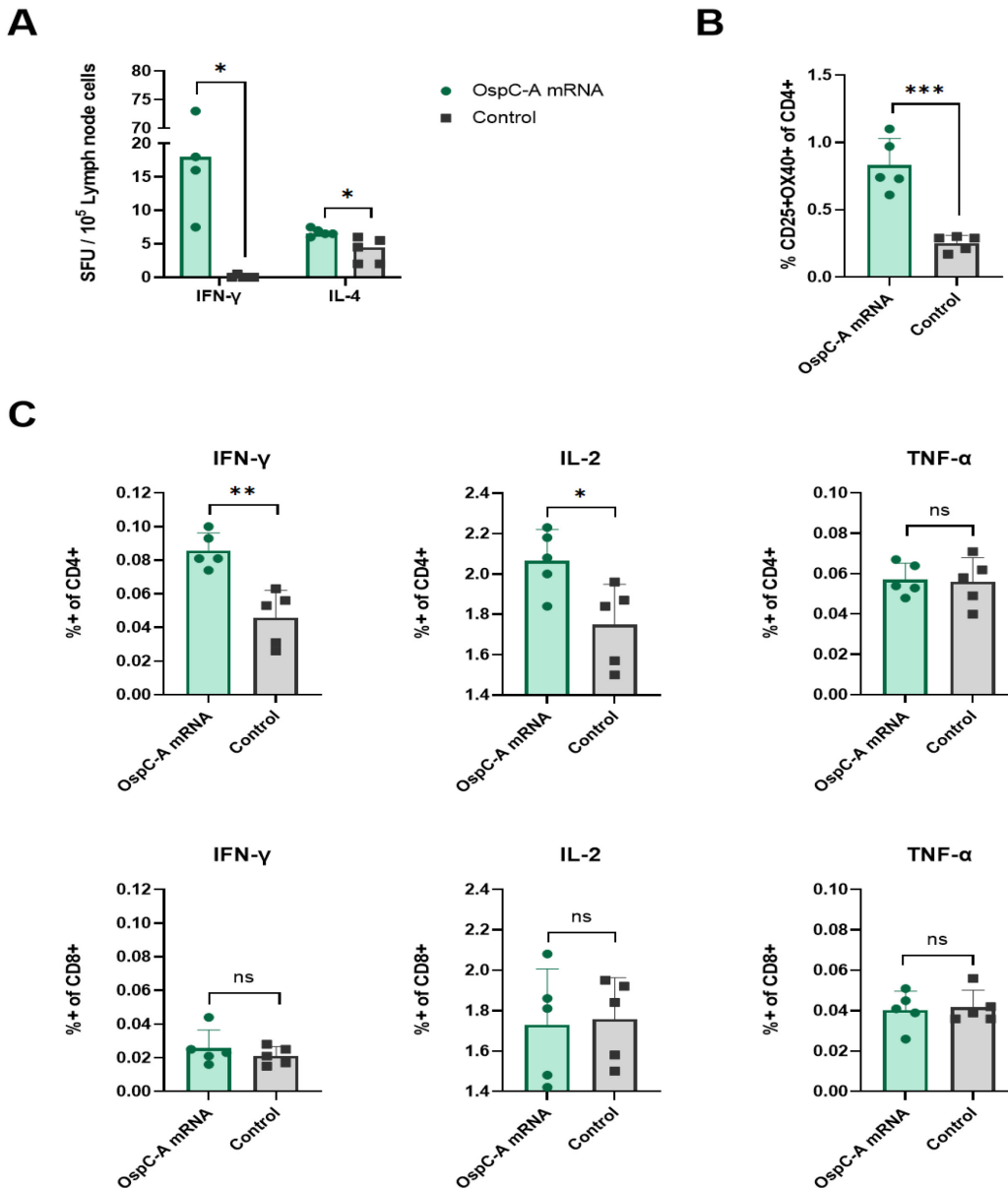
vaccination. Dashed line indicates the limit of detection (LoD). Samples pertaining to data points on the dashed line have antibody titres below the limit of detection **D**) Ratio of IgG2a to IgG1 OspC-specific serum antibodies four weeks after boost vaccination. **E**) Flow cytometry determination of the percentage of FITC+ RAW 264.7 cells resulting from ADCP assay. **F**) Percentage of live cells compared to no serum control following serum borreliacidal assay. Error bars represent standard deviation. Statistical significance was calculated using independent two-tailed t-tests. \* p-value < 0.05, \*\* p-value < 0.01.

#### 6.3.4. OspC-A mRNA elicits antigen-specific CD4+ T cell responses

Next, we assessed the cell-mediated immune response elicited by the OspC-A mRNA vaccine. Lymphocytes collected from the draining lymph nodes four weeks after the boost injection were stimulated with an overlapping peptide pool of OspC type A. Following stimulation, lymph node cells from OspC-A mRNA vaccinated mice secreted significantly higher levels of IFN- $\gamma$ , IL-2, IL-6, IL-10, IL-18, and TNF- $\alpha$  than the control mice (Figure 6.4). Secretion of Th2 cytokines, including IL-4, IL-5, and IL-13, was not elevated in the OspC-A mRNA group, nor was secretion of GM-CSF, IL-1 $\beta$ , IL-9, IL-12p70, IL-17A, IL-22, IL-23, and IL-24 (Figure S6.3). ELISpot analysis of the stimulated lymph node cells detected considerable IFN- $\gamma$  production and minimal IL-4 production in the OspC-A mRNA group compared to the control (Figure 6.5A). In response to stimulation, OspC-A mRNA vaccinated mice had significantly higher populations of activated CD4 T cells (CD25+OX40+) than control mice (Figure 6.5B). Furthermore, intracellular cytokine staining demonstrated that CD4+ T cells but not CD8+ T cells produced IFN- $\gamma$  and IL-2 following OspC-A mRNA vaccination (Figure 6.5C). In conclusion, these findings provide robust evidence that the candidate OspC-A mRNA vaccine induces a strong antigen-specific Th1-biased CD4+ T cell response.



**Figure 6.4.** Vaccination of mice with OspC-A mRNA induces antigen-specific immune responses in the draining lymph nodes. dLNs were harvested from mice four weeks after boost vaccination and were stimulated with an overlapping peptide library of OspC type A for 24 h. The concentration of each cytokine in the supernatant was determined by ProcartaPlex 17-plex Immunoassay kit. LoD, limit of detection.  $n = 5$  per group, error bars represent standard deviation. Statistical significance was calculated using independent two-tailed t-tests. \*  $p$ -value  $< 0.05$ , \*\*  $p$ -value  $< 0.01$ .



**Figure 6.5.** OspC-A induces OspC-specific T cell responses in the draining lymph nodes of OspC-A-vaccinated mice. **A)** ELISpot determination of IFN- $\gamma$  and IL-4 production by draining lymph node cells following 24 h stimulation with an overlapping OspC peptide pool represented by spot forming units (SFU) per  $10^5$  lymph node cells. **B)** Flow cytometry analysis of activation induced markers (AIM) expression (CD25+ OX40+) as a percentage of CD4+ T cells in the dLNs collected four weeks after boost vaccination following 18 h stimulation with an overlapping OspC peptide pool. **C)** Flow cytometry analysis of the percentage of CD4+ (top) and CD8+ (bottom) T cells producing IFN- $\gamma$ , IL-2, or TNF- $\alpha$ .  $n = 5$  per group, error bars represent standard deviation. Statistical significance was calculated using independent two-tailed t-tests. ns, not significant, \*  $p$ -value  $< 0.05$ , \*\*  $p$ -value  $< 0.01$ , \*\*\*  $p$ -value  $< 0.001$ .

### 6.3.5. Polyvalent OspC mRNA affords protection against multiple *B. burgdorferi* strains

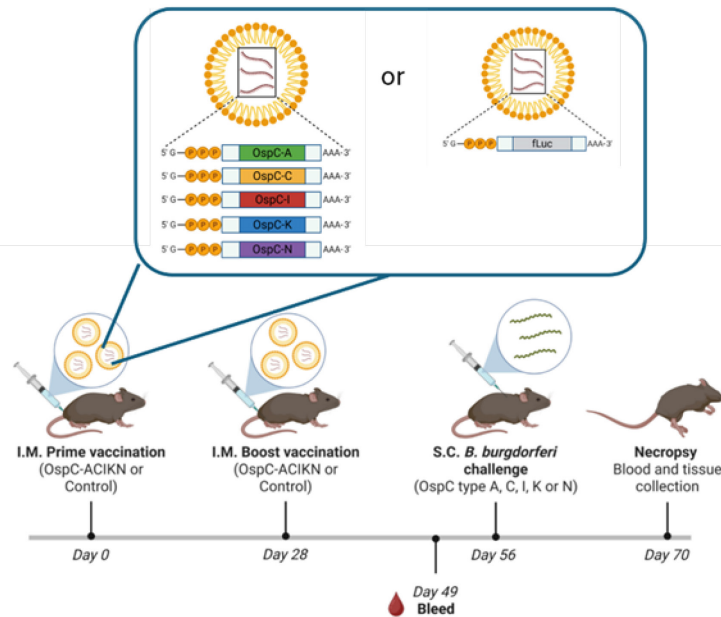
To address the challenge presented by the genetic diversity of OspC, we generated a polyvalent mRNA vaccine encoding five of the most prevalent OspC types in North America, namely type A, C, I, K, and N (OspC-ACIKN) [94,97,526]. Mice were vaccinated on day 0 and day 28 with 1 µg of each OspC type or a negative control mRNA-LNP vaccine encoding firefly luciferase (Control). Three weeks after the boost vaccination, mice were bled for serum collection. One week later, mice were challenged subcutaneously with one of the five *B. burgdorferi* strains carrying OspC types A, C, I, K, or N. Two weeks after the challenge, mice were euthanized for tissue collection (Figure 6.6A).

OspC-ACIKN vaccination induced significant serum IgG titres at three weeks after the boost against all included OspC types relative to the control (Figure 6.6B). Following bacterial challenge, infection status was determined by culture of the skin surrounding the bacterial injection site for 28 days and live spirochetes were identified by darkfield microscopy. 90-100% of control mice were positive for motile spirochetes in the skin across all *B. burgdorferi* strains (Figure 6.6C). In comparison, only 10% of the mice vaccinated with OspC-ACIKN mRNA were positive for motile spirochetes with the type A strain, 20% were positive for the type K strain, and no mice vaccinated with OspC-ACIKN were positive for the type I strain. Interestingly, OspC-ACIKN vaccination only reduced infection with type C and type N strains by 10% compared to the control. These results were further confirmed by quantification of the *B. burgdorferi* DNA burden in the ear, bladder, tibio-tarsal joint, and heart, which found that vaccination with OspC-ACIKN significantly reduced the tissue spirochete loads of the type A, I, and K strains but not the type C and N strains (Figure 6.6D, Figure S6.4).

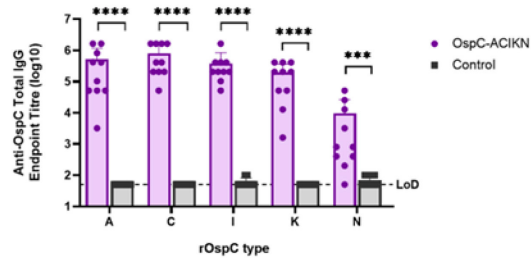
We previously demonstrated that infection with each of the five *B. burgdorferi* strains causes inflammation of the heart base and hyperplasia of the axillary lymph nodes in mice at two weeks post-infection [527]. Here, we observed that the infection status, as determined by qPCR and explant culture, was associated with the development of carditis and lymphadenopathy. All control mice developed mild-to-marked Lyme carditis following infection with each strain (Figure 6.6E). Additionally, OspC-ACIKN vaccinated mice with breakthrough infection of any strain developed minimal-to-marked carditis, with the exception of one mouse in the group challenged with the type C strain, which was previously shown to cause the lowest levels of carditis [527]. Overall, vaccination with OspC-ACIKN significantly reduced the development of Lyme carditis from infection with the type A and type I strains, but not types C, K, or N.

Similarly, control mice had severe lymph node enlargement and consistently developed moderate-to-severe lymph node hyperplasia following infection with all strains (Figure 6.6F, G). Additionally, OspC-ACIKN vaccinated mice with breakthrough infection developed mild-to-severe lymph node hyperplasia. In comparison, OspC-ACIKN vaccinated mice that were protected from infection had significantly reduced lymph node cellularity and hyperplasia. Vaccination with OspC-ACIKN therefore reduced lymph node pathology resulting from infection with *B. burgdorferi* strains type A, I, and K, but not C and N. Overall, the OspC-ACIKN vaccine afforded significant protection against pathologies associated with infection of three of the five *B. burgdorferi* strains.

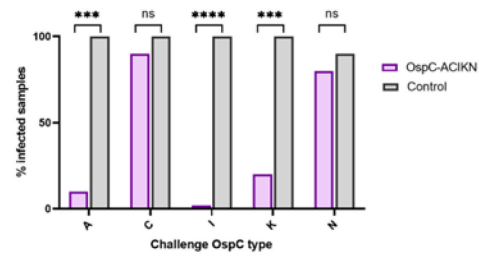
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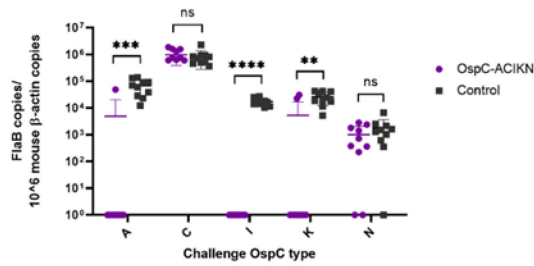
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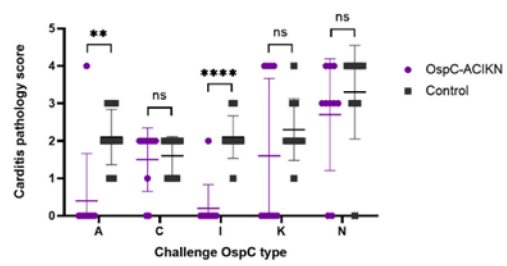
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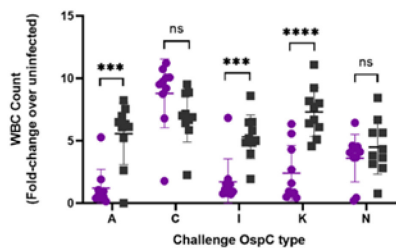
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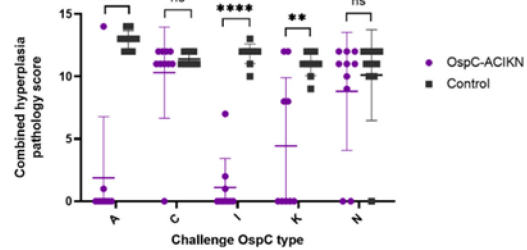
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**F**



**G**





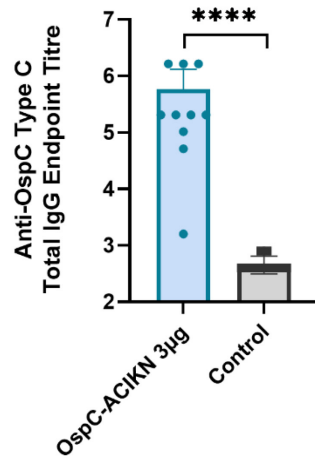
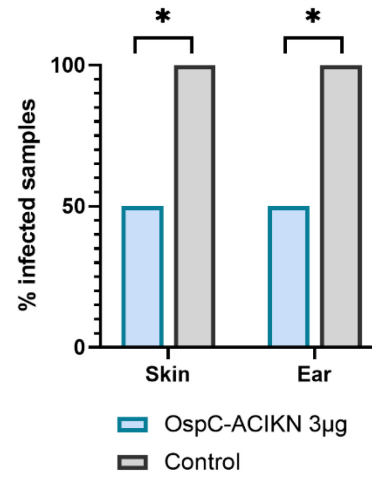
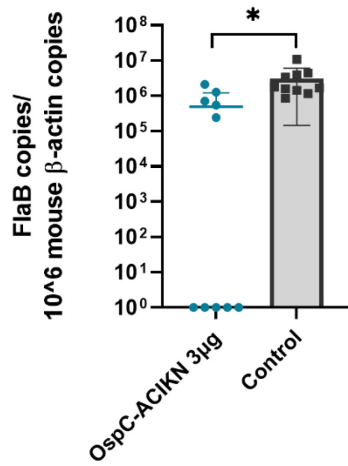
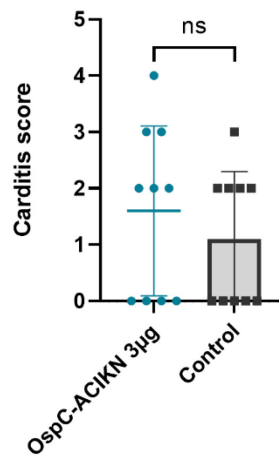
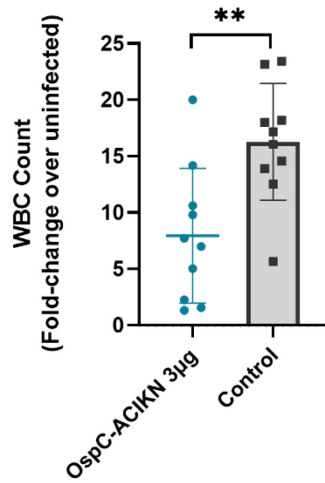
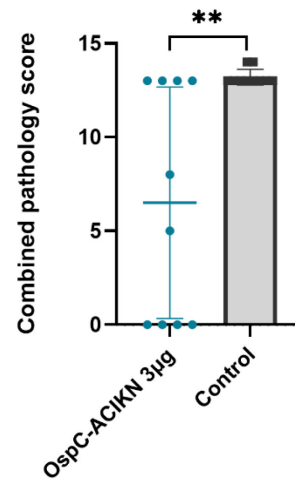
**Figure 6.6.** Vaccination with OspC-ACIKN mRNA protects mice against infection with multiple *B. burgdorferi* strains. **A)** Schematic depicting the procedure schedule for C3H/HeN mice. Mice were injected with the OspC-ACIKN or luciferase mRNA vaccine on day 0 and 28, were bled on day 49, and challenged with either *B. burgdorferi* strain B31-A3 (OspC type A), JD1 (OspC type C), Bb16-54 (OspC type I), 297 Ah130 (OspC type K), or Bb16-126 (OspC type N) on day 56 (n = 10 per strain per vaccine group), and euthanized on day 70. Created using BioRender.com. **B)** ELISA quantification of anti-OspC IgG antibody titres by recombinant OspC type in serum of mice three weeks after boost vaccination and prior to challenge with the matching strain. **C)** Percentage of infected mice following challenge. The skin surrounding the injection site was collected at necropsy and placed in BSK-H culture media for 28 days at 35°C, 1.5% CO<sub>2</sub> before examination by dark-field microscopy. Samples were considered positive for infection if at least one spirochete was identified across five fields of view. **D)** qPCR analysis of the bacterial DNA burden in the left ear represented by *B. burgdorferi* FlaB gene copies per 10<sup>6</sup> mouse  $\beta$ -actin copies. Samples below the threshold of amplification are represented by a value of 1. **E)** Summary of carditis pathology scores two weeks after challenge. **F)** White blood cell (WBC) counts of the left axillary lymph node extracted from vaccinated and control mice two weeks after bacterial challenge normalized to the WBC counts from unchallenged and uninfected mice. **G)** Summary of lymph node pathology scores two weeks after challenge. Statistical significance was calculated using independent two-tailed t-tests for **B**, **D-G** and Fisher's exact test for **C**. Error bars represent standard deviation. ns, not significant, \*\* p-value < 0.01, \*\*\* p-value < 0.001, \*\*\*\* p-value < 0.0001.

### 6.3.6. Increasing vaccine dose improves protection against *B. burgdorferi* expressing OspC type C

Given that OspC-ACIKN vaccination of the mice provided minimal protection against *B. burgdorferi* strains with OspC types C and N, we performed a follow-up study to evaluate whether increasing the vaccine dose from 1  $\mu$ g to 3  $\mu$ g of mRNA would better prevent infection. In this study, mice were vaccinated with 3  $\mu$ g of mRNA of each of the five OspC types or the luciferase mRNA as a control. Mice were bled for serum collection and then challenged four weeks post-boost with *B. burgdorferi* strain OspC type C, as this strain resulted in the highest rates of infection in mice vaccinated with 1  $\mu$ g of OspC-ACIKN (Figure 6.6C). Finally, the mice were euthanized on day 70 for tissue collection.

The 3 µg dose of OspC-ACIKN mRNA induced significant IgG antibody titres in the serum three weeks post-boost when compared to the control (Figure 6.7A). Moreover, bacterial culture from the right ear and skin surrounding the spirochete injection site demonstrated that vaccination with OspC-ACIKN protected 50% of the mice from infection with the type C strain (Figure 6.7B), an increase relative to the 10% protection observed at the 1 µg dose (Figure 6.6C). This finding was further confirmed by qPCR, which demonstrated a significant reduction in spirochete DNA of the type C strain in OspC-ACIKN vaccinated mice compared to control mice (Figure 6.7C).

Infection with the OspC type C strain did not cause detectable carditis in half of the control mice (Figure 6.7D). We have previously reported that the OspC type C strain causes the least carditis of the five strains evaluated in this study [527]. Consequently, vaccination with OspC-ACIKN did not significantly reduce inflammation in the heart compared to the control mice. In contrast, following the type C challenge, control mice developed significant hyperplasia of the axillary lymph nodes, as demonstrated by the substantially elevated white blood cell counts (Figure 6.7E) and histopathological analysis (Figure 6.7F). Mice vaccinated with 3 µg of OspC-ACIKN had significantly lower white blood cell counts and less hyperplasia of the axillary lymph nodes compared to the control group. Taken together, these results demonstrate that increasing the vaccination dose of mRNA can lead to improved protection against infection and pathology with the type C strain.

**A****B****C****D****E****F**

**Figure 6.7.** Vaccination of mice with a higher dose of OspC-ACIKN mRNA affords greater protection against infection with *B. burgdorferi* expressing OspC type C. **A)** ELISA quantification of anti-OspC type C IgG antibody titres in serum of mice three weeks after boost vaccination with a 3 µg dose of OspC-ACIKN or luciferase mRNA. **B)** Percentage of infected mice following challenge. Two weeks after challenge with the *B. burgdorferi* OspC type C strain, the right ear and skin surrounding the injection site were collected and placed in BSK-H culture media for 28 days at 35°C, 1.5% CO<sub>2</sub> before examination by dark-field microscopy. Samples were considered positive for infection if at least one spirochete was identified across five fields of view. **C)** qPCR of the bacterial DNA burden in the left ear represented by *B. burgdorferi* FlaB gene copies per 10<sup>6</sup> mouse β-actin copies. Samples below the threshold of amplification are represented by a value of 1. **D)** Summary of carditis pathology scores two weeks after challenge with *B. burgdorferi* OspC type C strain. **E)** White blood cell (WBC) counts of the left axillary lymph node extracted from vaccinated mice two weeks after type C challenge, normalized to the WBC counts from uninfected mice. **F)** Summary of lymph node pathology scores two weeks after type C challenge. n = 10 per group, error bars represent standard deviation. Statistical significance was calculated using independent two-tailed t-tests for **A**, **C-F** and Fisher's exact test for **B**. ns, not significant, \* p-value < 0.05, \*\* p-value < 0.01, \*\*\* p-value < 0.001, \*\*\*\* p-value < 0.0001.

## 6.4. Discussion

The causative agent of LD, *B. burgdorferi*, is estimated to infect nearly half a million individuals in North America each year [2,3,512]. Climate change models project that by 2050, Canada will have 120,000 to 500,000 cases of LD per year [530]. Despite the availability and efficacy of oral and intravenous antibiotic treatments, 10-20% of these patients will develop PTLDS or Lyme IACI, significantly reducing their quality of life [513,531–533]. In the United States, LD, PTLDS, and Lyme IACI cases are a substantial economic burden, costing the healthcare system between 700 million and 1.3 billion USD annually [11]. For Canada, climate change models suggest that the economic costs of LD in 2050 could range between 0.5 and 2.0 billion Canadian dollars per year [530]. Vaccines are therefore an essential component of a comprehensive prevention plan for Lyme disease.

Since the removal of LYMERix™ in 2002, there is no option to vaccinate against LD in humans. LYMERix™, as well as the current clinical trial candidate, VLA15, are both recombinant

protein vaccines targeting OspA [19,72]. Most preclinical and veterinary vaccine development efforts have focused on recombinant protein or subunit vaccine platforms. Logistically, mRNA vaccine production is more rapid and scalable than protein purification and mRNA vaccines facilitated the rapid response to the COVID-19 pandemic [534]. The ionizable lipid component of LNPs has been shown to have an adjuvanting effect on T follicular helper cells, thereby increasing antibody production [311]. Moreover, nucleoside-modified mRNA vaccines have demonstrated greater induction of germinal center and T cell responses leading to higher levels of antigen-specific memory B cells, long-lived plasma cells, and antibody titres than recombinant protein vaccines, including in the context of LD vaccination [301,302,535,536].

crOspC vaccines composed of immunodominant epitopes from diverse OspC types can elicit antibodies that recognize more than 20 distinct OspC types and exhibit borreliacidal activity *in vitro* [116,118,139,219]. Here, we focused on an mRNA vaccine platform encoding OspC along with in-depth mechanistic investigations. We first developed a monovalent OspC mRNA vaccine encoding only OspC type A (OspC-A mRNA) to determine the ability of an mRNA vaccine to protect against homologous infection. The candidate vaccine afforded complete protection against infection and spirochete dissemination to the ear, bladder, heart, ankle joint, and lymph nodes. The vaccine also prevented the development of Lyme carditis and lymph node hyperplasia, as shown through *in situ* visualization, enumeration of white blood cells, and histopathology [108].

The OspC-A mRNA vaccine induced significant serum antibody titres with a Th1 bias, indicated by high levels of IgG2a antibodies compared to IgG1 antibodies. The optimal balance between Th1- and Th2-biased responses for *B. burgdorferi* clearance is not clearly established, however, IgG2a antibodies act as opsonins to facilitate ADCP of extracellular bacteria, such as *B. burgdorferi* [537,538]. Accordingly, the antibodies elicited by OspC-A mRNA vaccination

induced ADCP *in vitro*, demonstrating their functionality. Vaccination with an mRNA vaccine targeting OspA previously demonstrated robust IgG titres that persisted for up to 6 months [301]. In this study, we did not investigate the kinetics of the antibody response. As protection against LD has been correlated with antibody titres, further studies will need to be performed to assess the longevity of the protection conferred by OspC mRNA vaccines, especially in comparison to well-established OspA-based vaccines [539,540].

Next, we examined the antigen-specific cellular immune response to vaccination. In the draining lymph nodes, OspC-A mRNA vaccination induced broad antigen-specific Th1-skewed cytokine responses with high levels of IFN- $\gamma$  production. CD4<sup>+</sup> T cells in the lymph nodes of vaccinated mice also displayed high levels of activation induced markers (OX40 and CD25) and IFN- $\gamma$  and IL-2 production in response to stimulation with OspC peptides. Following vaccination, CD4<sup>+</sup> T cells may contribute to clearance of *B. burgdorferi* infection through the induction of *B. burgdorferi*-specific class-switched antibody responses, long-lived plasma cells, and memory B cells [232,301].

One limitation of OspC-targeting vaccines that must be addressed is the genetic variability of the OspC antigen. Therefore, we developed a prototype polyvalent OspC vaccine containing five of the most common major OspC types (A, C, I, K and N) in North America. OspC types A, I, K, and N are among the most prevalent OspC alleles detected in human LD infection [526]. In North America, OspC types A, K, C, and N are among the most common OspC types detected in populations of *I. scapularis* ticks [97,105]. We demonstrated that two 1  $\mu$ g doses of OspC-ACIKN mRNA vaccine elicited significant antibody titres against all five included OspC types and afforded 80-100% protection against infection with *B. burgdorferi* strains expressing OspC types A, I, and K. At the 1  $\mu$ g dose, significant protection was not observed for type C and N strains,

which we have previously shown to be highly infectious [527]. It should be noted that throughout this study our candidate vaccines displayed an “all-or-nothing” response, in which mice that were protected against infection did not develop carditis and lymphadenopathy, but those that were infected had similar levels of pathology to the control mice. This may be due to the transient nature of OspC expression during infection, potentially suggesting a limited window of vaccine efficacy. In a proof-of-concept follow-up study, we demonstrated that a 3 µg dose of the polyvalent vaccine increased protection against the type C strain from 10% to 50%. Together, these findings suggest that multi-strain protection can be afforded by polyvalent OspC vaccines and that protection against highly infectious strains, such as JD1 (type C) and Bb16-126 (type N) may require dose optimization to achieve full protection. Nevertheless, since approximately 60% of LD infections are caused by OspC type A or K, the protection demonstrated by the OspC-ACIKN vaccine in this study has the potential to prevent a substantial portion of human LD infections.

One limitation of this study is that the vaccinated mice were challenged by needle injection of spirochetes rather than via tick bite, which is the natural mode of infection. However, previous studies have demonstrated that vaccination with recombinant OspC proteins protects mice against infection via both needles and ticks [70,101,115,125,517,541]. Thus, we expect that our mRNA-based OspC vaccines will protect mice and humans against the bites of *B. burgdorferi*-infected ticks. Anti-OspC antibodies are thought to neutralize the spirochetes in the host at the tick bite site after they had been transmitted by a feeding tick [98]. However, a recent study demonstrated that the infectivity of tick-borne spirochetes (as measured via needle inoculation into naïve mice) was greatly reduced if these ticks had fed on mice that had previously developed a strong antibody response against the homologous strain [98]. This study suggests that anti-OspC IgG antibodies might play an additional role in preventing tick-to-host transmission of *B. burgdorferi* that cannot

be captured by needle inoculation studies. Thus, future studies should investigate whether mRNA vaccines protect mice against an infectious tick bite challenge.

In conclusion, our study is the first to demonstrate the efficacy of OspC-targeting mRNA vaccines for LD. Compared to historical LD vaccine designs, our candidate vaccines offer the advantages of targeting OspC with the benefits of the mRNA platform, including an excellent safety profile and robust immunogenicity. To our knowledge, it is also the first study to conduct a systematic challenge of multiple *B. burgdorferi* strains expressing different OspC types for the assessment of a polyvalent OspC vaccine. Future studies should evaluate the efficacy of polyvalent mRNA OspC vaccines in short- and long-term tick challenge models. Ultimately, these results support the advancement of OspC-targeting mRNA vaccines toward clinical development to combat the rising prevalence and health care burden of Lyme disease.

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## Chapter 7: Discussion and conclusion

Lyme disease persists as a pressing public health concern across North America and Europe. Since the removal of LYMERix™ in 2002, there has been no vaccine available for the prevention of LD in humans. Although previous vaccine development efforts have largely focused on targeting OspA due to its relatively conserved genetic sequence and consistent expression across strains, OspC presents several advantages as an alternative vaccine antigen and remains relatively understudied in the context of modern vaccine technology. The main objective of this thesis was to evaluate the suitability of OspC as a vaccine target for LD using next-generation vaccine platforms through the interrogation of both humoral and cell-mediated immune responses. This thesis investigates the protective efficacy and immune mechanisms induced by monovalent DNA and mRNA vaccines delivered by LNPs, as well as a vaccinia virus-vectored vaccine. Given the genetic diversity of OspC, the findings from these studies were applied to the development of a polyvalent mRNA vaccine encoding multiple OspC types to evaluate the breadth of protection against multiple challenge strains. Ultimately, this thesis informs the development of safe and effective next-generation vaccines for LD capable of affording broad and long-lasting immunity.

### 7.1. OspC is a protective vaccine antigen across next-generation platforms

The first objective of this thesis was to evaluate the protective efficacy and underlying humoral and cell-mediated immune responses of monovalent next-generation vaccines targeting OspC. *B. burgdorferi*, like other bacterial pathogens, is known to exhibit strain-specific differences in infectivity and host-responses. Therefore, I first characterized the infection profile of a variety of Bbss strains (297 Ah130, Bb16-54, B31-A3, Bb16-126 and JD1) in **Chapter 2**, prior to performing the subsequent vaccine challenge studies. Using a C3H/HeN mouse model, I assessed

the infectivity, bacterial burden in the heart tissue and ankle joint, resulting pathologies – including carditis and lymphadenopathy – and antibody responses following subcutaneous injection with each strain at doses ranging from  $10^4$  to  $10^6$  spirochetes. Strains Bb16-126 and JD1 were found to be the most infectious and resulted in the greatest heart burden and carditis levels, respectively. Meanwhile, B31-A3 infection resulted in the greatest burden in the ankle joint and the greatest induction of IgM antibodies two weeks after infection. All strains caused substantial lymphadenopathy and induction of IgM antibodies.

To address the first objective of this thesis, I then evaluated vaccines encoding OspC type A against homologous *B. burgdorferi* B31-A3 needle challenge in **Chapters 3, 4, and 6** (Figure 7.1). The vaccine platforms tested in these chapters have either previously demonstrated conflicting results with regards to protection or have never been tested for OspC delivery. First, in **Chapter 3**, I demonstrated that a pDNA vaccine delivered by LNP (pVAX1-OspC:LNP) afforded complete protection against infection and dissemination to a variety of tissues. Vaccination with pVAX1-OspC:LNP also prevented the development of carditis and lymphadenopathy associated with LD. These findings add to previous literature which report mixed findings on the efficacy of DNA vaccines encoding OspC [83,290,297–299]. While one study by Wallich et al. reported that a “naked” pDNA vaccine using a pUC plasmid backbone encoding OspC failed to induce antibodies in mice in the absence of OspA, more recent studies employing the pCMV or pVAX1 vector demonstrated significant protection against homologous infection [83,297–299]. However, these vaccines require intradermal administration or the use of gene guns, which are not ideal for mass immunization. A major factor in the success of the candidate vaccine in **Chapter 3** is likely the lipid nanoparticle delivery system, which facilitates efficient nucleic acid delivery while providing immune stimulation, unlike the unaided delivery employed by Wallich et al. [297]. The

results shown in **Chapter 3** add to the body of evidence that pDNA vaccines encoding OspC are effective for the prevention of LD in preclinical models. Furthermore, advancements in DNA vaccine design, such as the pVAX1 vector and LNP-based intramuscular delivery, overcome previous challenges to generate easy-to-produce and clinically translatable LD vaccines. To my knowledge, this is the first study demonstrating an effective intramuscular DNA vaccine targeting OspC for LD.

|                                                     | pVAX1-OspC                                          | VV-OspC                                             | OspC mRNA                                           |
|-----------------------------------------------------|-----------------------------------------------------|-----------------------------------------------------|-----------------------------------------------------|
| Vaccine type                                        | pDNA, LNP                                           | Viral-vector<br>(Vaccinia Virus)                    | mRNA, LNP                                           |
| Protection                                          | 100%,<br>reduced carditis<br>and<br>lymphadenopathy | 100%,<br>reduced carditis<br>and<br>lymphadenopathy | 100%,<br>reduced carditis<br>and<br>lymphadenopathy |
| Antibody response<br>Skew<br>Borrelia-cidal<br>ADCP | Th1<br>Yes<br>Not determined                        | Th1<br>Not determined<br>Yes                        | Th1<br>Yes<br>Yes                                   |
| Cellular immune<br>response                         | Not determined                                      | Th1<br>CD4+ T cells                                 | Th1<br>CD4+ T cells                                 |

**Figure 7.1.** Summary of the protection and immune responses elicited by the next-generation monovalent OspC vaccines evaluated in Chapters 3, 4, and 6.

In **Chapter 4**, I demonstrate that a modified vaccinia virus-vectored vaccine expressing OspC (VV-OspC) affords complete protection against homologous *B. burgdorferi* infection, dissemination, and associated pathologies. This finding is in contrast to a previous study using an NDV-based viral vector vaccine targeting OspC, which failed to induce an antibody response in C3H mice. While NDV-OspC did elicit an antibody response in hamsters, it failed to afford protection against needle challenge. Compared to NDV, vaccinia virus may be a superior vector for the delivery of OspC, however, this should be confirmed by a direct comparison controlling for the use of secretion signals and the immunization schedule. Additionally, the NDV-based vaccine was administered intranasally, which can preferentially induce local mucosal immune responses with potentially reduced systemic antibody effector functions [542]. Furthermore, Xiao et al. performed their challenge with a relatively high dose of  $10^8$  *B. burgdorferi* only one week after the boost vaccination, in comparison to the  $10^6$  dose four weeks post-boost used in **Chapter 4**, which may have obfuscated any protection they would have observed. Nevertheless, the study in **Chapter 4** provides a foundation for further research efforts to pursue the use of viral vector OspC vaccines for LD.

Finally, the efficacy of an mRNA vaccine targeting OspC had never been tested. Prior to the study in **Chapter 6**, several LNP formulations were evaluated in **Chapter 5** and one of the most potent and immunogenic formulations, SM102-mRNA, was chosen to progress into the **Chapter 6** study. In **Chapter 6**, I demonstrate for the first time that an mRNA vaccine encoding OspC vaccine affords complete protection against homologous *B. burgdorferi* infection, dissemination, and associated pathologies. Given the ease of production and excellent safety profile of the mRNA vaccine platform, these findings show substantial promise for the development of a safe and effective LD vaccine.

In **Chapters 3, 4, and 6**, immune responses associated with the observed protection were also investigated. The pVAX1-OspC:LNP, VV-OspC, and OspC-A mRNA vaccines all elicited significant functional antibody responses with Th1-bias three weeks after boost injection. Although not directly compared in the same study, when administered according to the same schedule, the antibody titres elicited by OspC-A mRNA were the highest, followed by VV-OspC, and then pVAX1-OspC:LNP. The pVAX1-OspC and OspC-A mRNA vaccines elicited borreliacidal antibodies, while the OspC-A mRNA and VV-OspC vaccines elicited opsonic antibodies capable of inducing ADCP *in vitro*. Furthermore, OspC-specific CD4<sup>+</sup> T cell responses were observed after both VV-OspC and mRNA-OspC vaccination and studies are currently in progress to characterize the T cell response to pVAX1-OspC vaccination. Notably, when compared to the licensed veterinary rOspC vaccine, Vanguard® CrLyne, VV-OspC elicited greater OspC-specific T cell responses, highlighting the potential advantages of these next-generation vaccine platforms.

In sum, this thesis demonstrates that the OspC antigen is protective against homologous infection using a variety of next-generation vaccine platforms that are each associated with certain advantages and limitations. For example, the pDNA-based vaccine, while fully protective against infection, induced the lowest antibody titres of the three candidate vaccines. However, DNA-based vaccines are generally easier and cheaper to produce than both mRNA and viral vector vaccines [543]. While both mRNA and DNA vaccines are non-infectious, DNA is more thermostable than mRNA and does not require ultra-cold storage, which is costly and can hinder vaccine deployment to remote regions. Therefore, enhancing DNA vaccine immunogenicity with the use of adjuvants may be the optimal strategy to capitalize on DNA vaccine benefits while minimizing the disadvantages associated with the platform. Still, DNA vaccines are nascent in their clinical use,

whereas mRNA vaccines have demonstrated robust effectiveness and an excellent safety profile following global deployment. Ultimately, all three of these platforms have been shown to afford protection against LD and induce both cellular and humoral immunity. Therefore, each of the aforementioned factors should be weighed as LD vaccine development progresses to choose the most appropriate platform for large-scale production and administration.

One limitation of these studies is the administration of the spirochete challenge by subcutaneous needle injection, rather than by a more ecologically relevant tick vector challenge model. Nevertheless, previous studies have shown that OspC vaccine-induced protection against needle challenge translated to protection in tick challenge models [70,101,115,125,541,544]. Another limitation of these studies is that each study tested OspC type A using a homologous challenge with strain B31-A3. Some evidence suggests that whether homologous protection will occur is dependent on the BbsI strain, therefore these findings may not be generalizable to all strains or OspC types [99]. Finally, these studies examined the efficacy of monovalent vaccines against a single strain. An effective LD vaccine should afford broad protection against multiple strains. Therefore, the highly immunogenic mRNA vaccine platform was further investigated for multi-strain protection in **Chapter 6**.

## 7.2. LNPs effectively deliver pDNA vaccines

Effective and practical delivery of pDNA vaccines has been a long-standing barrier to the widespread clinical use of this vaccine technology. pDNA vaccine constructs are relatively large (2-20 kB) and highly negatively charged, limiting passage across the cell membrane. Additionally, unprotected DNA is highly susceptible to degradation by nucleases. Intramuscular and intradermal needle injection of naked DNA results in relatively low transfection efficiency [545,546]. Advanced delivery methods, including electroporation, gene gun delivery, and needle-free jet

injectors have been explored to improve cellular uptake and immunogenicity of DNA vaccines [253]. However, these technologies require invasive and painful procedures or specialized equipment that are not easily deployed in the clinic. Similarly, various polymers and cationic lipids have been investigated to improve delivery, but have been shown to induce cytotoxicity or unfavourable inflammatory responses [321,322]. LNPs, which efficiently deliver mRNA vaccines *in vivo*, can be adapted for the encapsulation of pDNA, yet the most successful modern mRNA-LNP formulations have not been explored in the context of pDNA delivery.

The second objective of this thesis was to characterize the potency, expression kinetics, biodistribution, and immunogenicity of pDNA vaccines delivered by modern LNP formulations. In **Chapter 3**, I demonstrated that a pDNA vaccine can be effectively delivered by an LNP formulated with the second-generation KC2 ionizable lipid, resulting in potent antibody responses and complete protection against *B. burgdorferi* infection. Third-generation LNPs have facilitated efficient mRNA transfection *in vivo* and robust immunogenicity with an excellent safety profile. Yet, these approved LNP formulations had never been tested for pDNA vaccine delivery, which has the potential to alleviate the ultra-cold storage requirements of mRNA vaccines. Therefore, in **Chapter 5**, I sought to compare the SM-102 and ALC-0315 ionizable lipid formulations to the previously used KC2 formulation used in **Chapter 3** for pDNA delivery. Both the SM102-DNA and ALC0315-DNA formulations were more potent and immunogenic than the KC2-DNA formulation, suggesting that previously approved formulations that have been shown to be highly tolerable can be easily adapted to pDNA vaccination. Future pDNA vaccine studies should employ these third-generation LNPs and further optimize them for pDNA delivery. For example, higher N/P ratios (the molar ratio of amine groups on the ionizable lipids (N) to phosphate groups on the nucleic acid (P)) have been shown to improve immunogenicity of DNA-LNP vaccines [547].



Additional immune stimulation can also be achieved through the incorporation of adjuvants such as CpG or co-expression of cytokines or co-stimulatory ligands, which may alleviate concerns of the relatively low immunogenicity associated with DNA vaccination [548–550].

### 7.3. Polyvalent OspC vaccines can afford multi-strain protection

An effective vaccine for LD must afford broad protection against the vast genetic diversity of *B. burgdorferi*. With over 20 different major allele groups and limited cross-protection between OspC types, LD vaccines targeting OspC face particular challenges in conferring multi-strain protection [70,94–96,98–101]. Targeting conserved regions of OspC has not proven to be a practical vaccine strategy thus far, since conserved regions are typically not surface-exposed or immunogenic [94,551]. One possible strategy is to develop polyvalent vaccines encoding multiple OspC types, particularly the most prevalent types in a given area. This idea has been thoroughly explored by Marconi and colleagues using recombinant protein vaccines, however, no polyvalent OspC vaccines have been tested using next-generation vaccine platforms, such as nucleic acid vaccines [116,118,125,219].

The final objective of this thesis was to determine the breadth of protection conferred by a polyvalent mRNA vaccine targeting OspC against challenge with characterized *B. burgdorferi* strains. In **Chapter 5**, I compared the expression profiles and immunogenicity of three different leading LNP formulations delivering pDNA and mRNA encoding firefly luciferase as a surrogate vaccine antigen. The SM102-RNA and ALC0315-RNA formulations demonstrated superior antigen expression and immunogenicity than the corresponding pDNA formulations and KC2-RNA formulation. No significant differences in potency and antibody titres were identified between the SM102-RNA and ALC0315-RNA formulations. Therefore, one of these two formulations, SM102-RNA, was chosen for the development of a polyvalent OspC vaccine

encoding five of the most common OspC types found in tick and human infection (A, C, I, K, N) [97,105,106,108].

In **Chapter 6**, the breadth of protection of this pentavalent OspC vaccine (OspC-ACIKN) was evaluated in mice against challenge with the five strains characterized in **Chapter 2**. Two 1 µg doses of the vaccine afforded 80-100% protection against *B. burgdorferi* expressing OspC type A, I, or K, but failed to protect against *B. burgdorferi* expressing OspC type C or N. However, increasing the vaccine dose to 3 µg improved the efficacy of the vaccine to 50% against type C challenge. Much remains to be understood about the differences in efficacy between the OspC types. It is possible that certain strains express lower levels of OspC *in vivo*, however, it is likely that decreasing OspC expression would reduce strain fitness since OspC is required for early mammalian infection [87,88]. Certain strains may also better mask or shield immunogenic regions of the OspC protein. Additionally, some strains may have better immune evasion mechanisms or the antibodies elicited against certain OspC types may better induce antibody effector functions, such as ADCC or complement-mediated killing. To test some of these hypotheses, future studies may assess the protection conferred by monovalent vaccines for OspC type C and type N. If OspC-C or OspC-N monovalent vaccines are more protective against homologous infection than their polyvalent counterpart, this may suggest that certain OspC types, such as A, I, or K are more immunodominant. The results of **Chapter 6** also suggest that further dose optimization or the use of adjuvants may be required to overcome strain-specific differences that could hinder the use of OspC polyvalent vaccines. In sum, this study represents the first OspC-based mRNA vaccine for LD and the first systematic challenge of a polyvalent OspC vaccine using individual strains of *B. burgdorferi* expressing different OspC types rather than ticks collected from the wild harbouring undetermined *B. burgdorferi* strains.

## 7.4. Future directions

This thesis examines the protective adaptive immune mechanisms induced following OspC-based vaccination against *B. burgdorferi* infection. Further investigation into the complex nature of these immune responses may enhance the strategic development of future LD vaccines. To determine whether OspC-specific antibodies are sufficient to confer protection, sera from vaccinated mice could be passively transferred to naive mice before *B. burgdorferi* challenge. Furthermore, the complement pathway or Fc receptors of the naive recipient mice could be first depleted to examine whether antibody-mediated clearance *in vivo* is complement-dependent or reliant on ADCP and other antibody effector functions. Adoptive transfer of CD4<sup>+</sup> or CD8<sup>+</sup> T cells from vaccinated mice could also be performed to examine whether OspC-specific T cell responses are sufficient to confer protection against infection in naive mice. Together, these studies would help elucidate the aspects of the adaptive immune response that are critical for conferring protection to guide rational OspC vaccine design.

As LNP technology continues to be applied to pDNA vaccination, differences in the immune responses initiated by DNA-LNPs and mRNA-LNPs should continue to be investigated. In a recent study, DNA- and mRNA-LNPs were shown to differentially modulate innate immune responses [547]. For example, DNA-LNPs more strongly activate mDCs and recruit substantially more neutrophils to the dLN than mRNA-LNPs. DNA-LNPs were also reported to induce stronger CD8<sup>+</sup> T cell responses than mRNA-LNP vaccines in response to an influenza hemagglutinin antigen. Given the ease of manufacturing and storing DNA-LNP vaccines compared to mRNA vaccines, further studies should continue to compare the advantages and limitations of these platforms with respect to the elicited immune responses, including duration and breadth of protection.

The majority of LD vaccines have been shown to afford protection for 1-1.5 years following injection, necessitating frequent booster vaccinations [19,73,552,553]. As these vaccines have predominantly targeted OspA, which requires the maintenance of high circulating antibody titres to afford protection, the question of whether OspC-based vaccines can afford longer-lasting protection with fewer rounds of immunization should be evaluated [268,332,333]. In conjunction, the germinal center and memory responses to OspC-based vaccination should be further elucidated. Technological advancements, such as the rise of single-cell RNA sequencing, has enabled TCR and BCR lineage tracing to track clonal expansion and affinity maturation [554,555]. These methods can be used to investigate the development of broadly reactive immune responses for highly diverse antigens, such as OspC, and to examine the progression of the immune response. Comparisons of the duration of protection afforded by different next-generation vaccine platforms should also be investigated. Furthermore, the study in **Chapter 5** and others have shown that DNA-LNPs have longer protein production kinetics than their mRNA counterparts, perhaps stimulating a more durable memory response.

While this thesis is focused on the efficacy of OspC as a vaccine antigen for LD, combination vaccines targeting multiple *B. burgdorferi* antigens are a promising strategy for LD vaccination. For example, the recombinant protein canine Vanguard® CrLyme vaccine includes both the broadly conserved OspA antigen and the highly expressed OspC antigen [125]. Similarly, O'Bier et al. have recently published a protein vaccine including an OspC chimeritope, OspA, OspB, FltA and FltB, which afforded 80-90% protection against infection with *B. burgdorferi* B31 challenge [139]. These strategies could be adapted to the next-generation vaccine platforms discussed in this thesis, including vaccinia virus-vectored, pDNA-LNP, and mRNA vaccines.

Additionally, other antigens, such as CspZ, could also be investigated as targets for a *B. burgdorferi* combination vaccine.

Finally, the development of LD vaccines should be considered from a broader public health lens. Transmission of *B. burgdorferi* occurs by tick vectors, which can harbour multiple bacterial and viral pathogens. Co-infections of *B. burgdorferi* with *Anaplasma phagocytophilum* or *Babesia microti* occur in 4% and 22% of cases, respectively [556]. In accord with LD cases, rates of other tick-borne infections, such as anaplasmosis, babesiosis, and Powassan virus, are on the rise [8,557–559]. Ideally, *B. burgdorferi* antigens should be included in a combination vaccine that affords protection against multiple common tick-borne pathogens.

## 7.5. Final remarks

Lyme disease is the most common vector-borne illness in North America and Europe. Since the removal of the OspA-targeting LYMERix™ vaccine from the market, the development of a novel LD vaccine remains a pressing public health matter. This thesis provides considerable evidence for the use of an alternative *B. burgdorferi* surface protein, OspC, as a vaccine antigen delivered by next-generation vaccine platforms, including a vaccinia virus-vectored, a pDNA-LNP, and an mRNA-LNP vaccine. Ultimately, the work presented in this thesis provides a foundation for the rational design of broadly protective OspC vaccines, with the goal of mitigating the growing global burden of LD.

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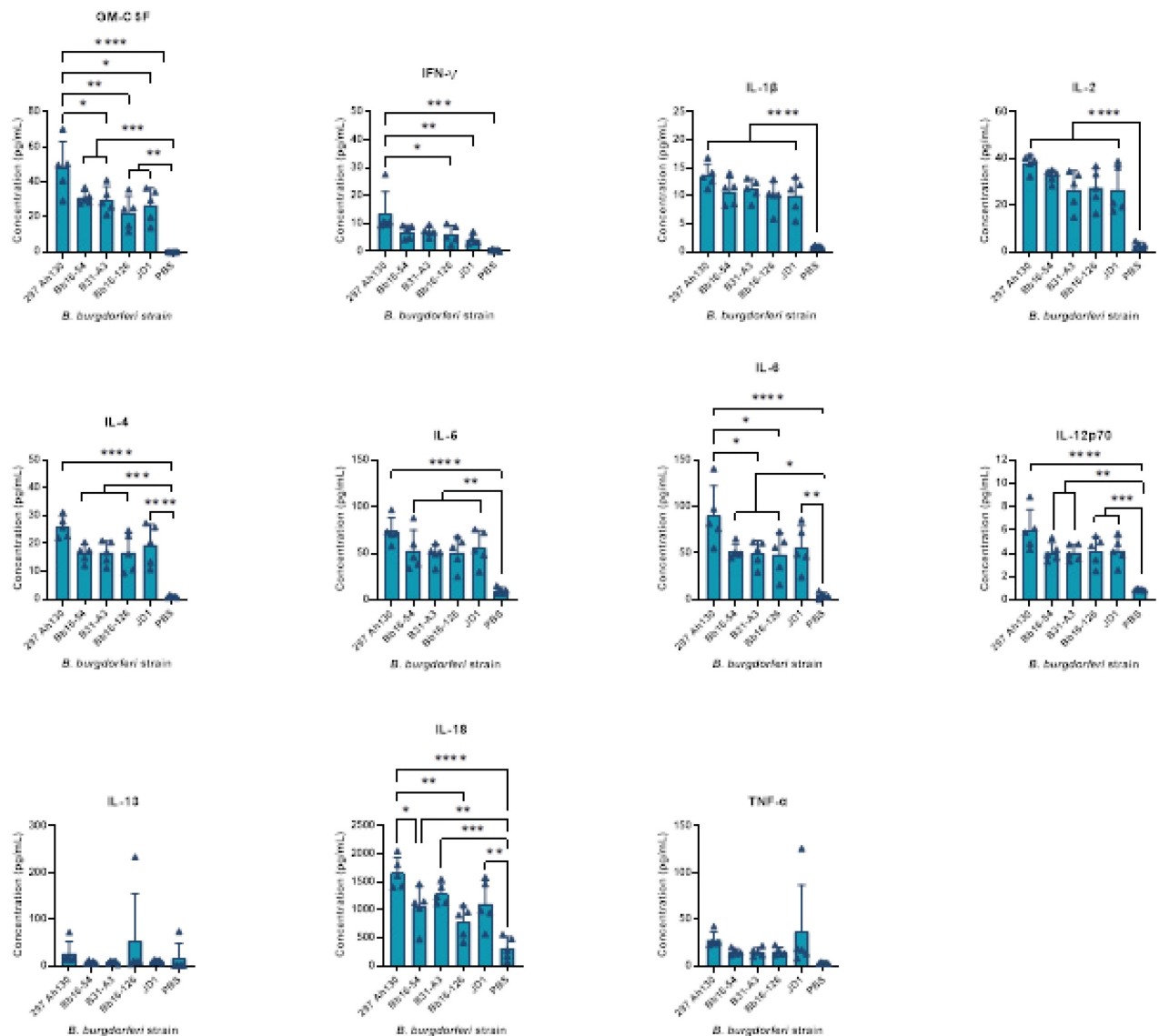
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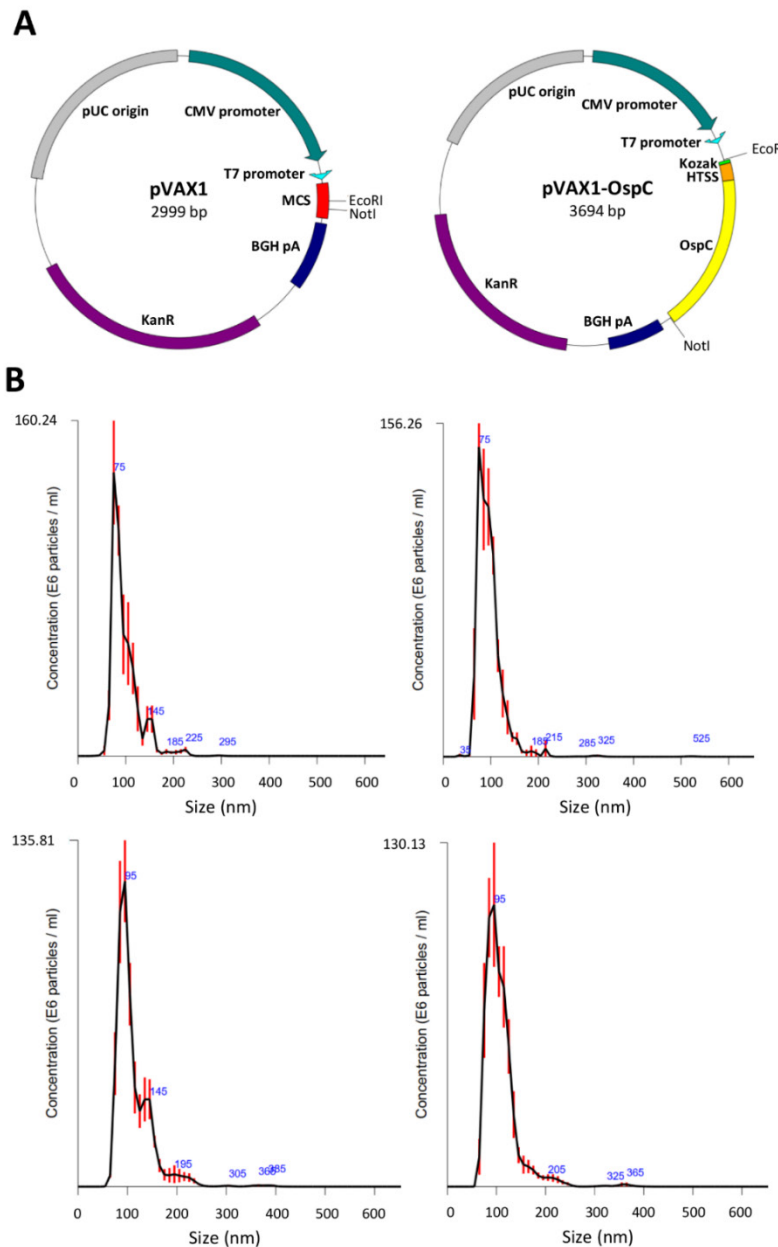
# Appendix: Supplementary Figures

## Chapter 2



**Figure S2.1.** Serum cytokine levels following infection with *B. burgdorferi* strains. Serum was collected from mice 14 days after subcutaneous infection with  $10^6$  spirochetes of Bbss strain 297 Ah130, Bb16-54, B31-A3, Bb16-126, or JD1. The concentration of each cytokine was determined by a ProcartaPlex 11-plex cytokine Immunoassay kit. Error bars represent standard deviation.\* p-value < 0.05, \*\* p-value < 0.01, \*\*\* p-value < 0.001, \*\*\*\* p-value < 0.0001.

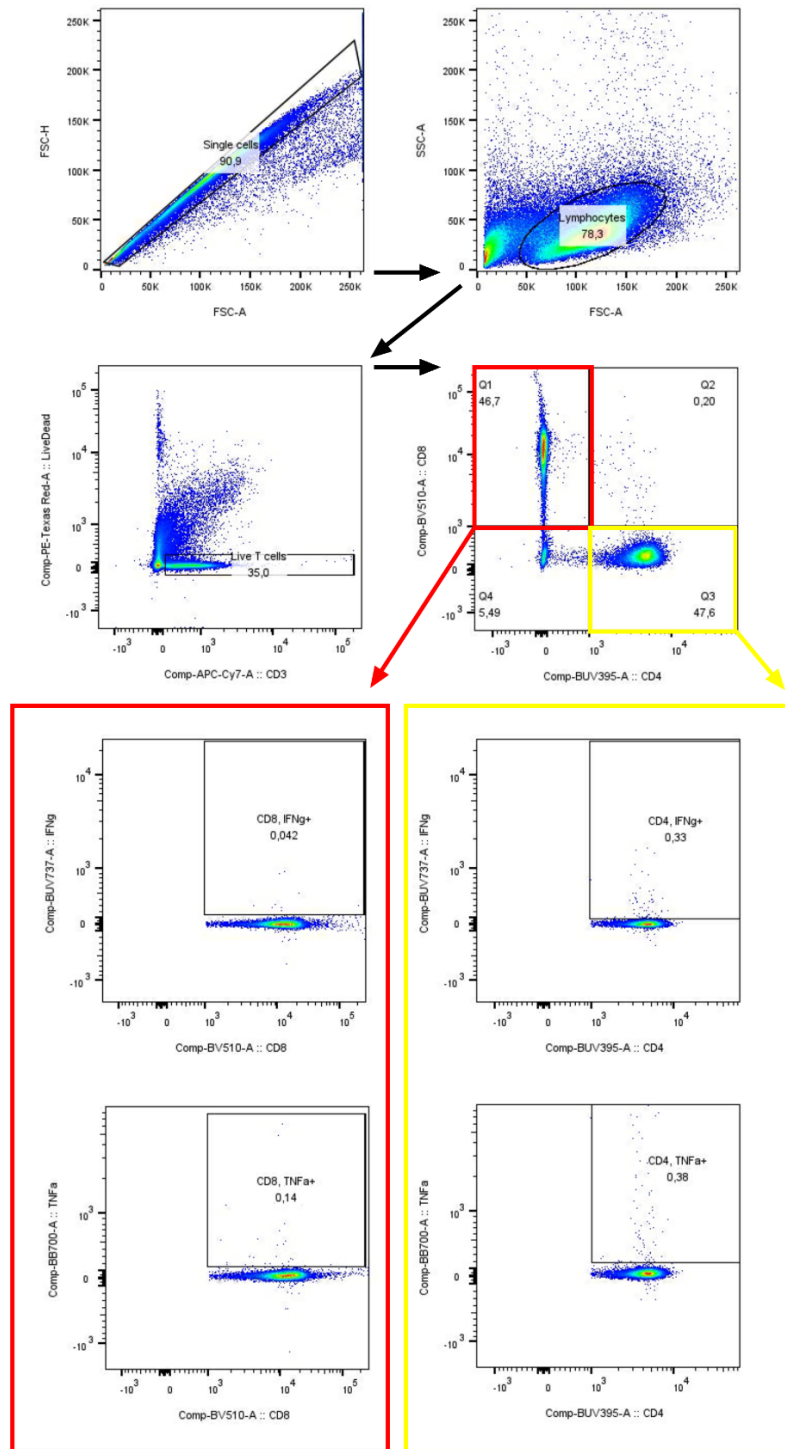
## Chapter 3



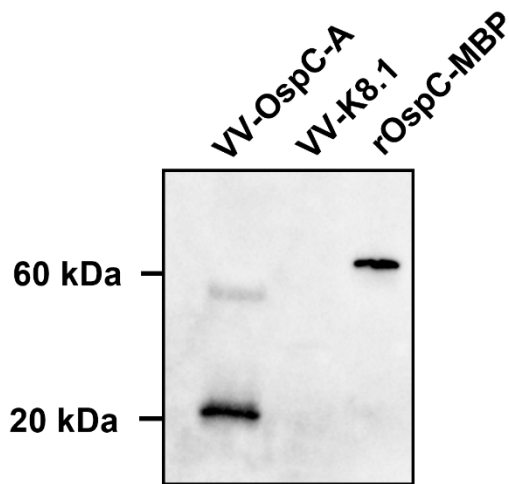
**Figure S3.1.** DNA lipid nanoparticle vaccine design and characterization. **(A)** Schematics depicting the parental pVAX1 plasmid (pVAX1) and pVAX1 plasmid engineered to express outer surface protein C (pVAX1-OspC). **(B)** Size distributions of pVAX1:LNP (left) and pVAX1-OspC:LNP (right) formulations determined by nanoparticle tracking analysis. Top row: preparations for prime injections, bottom row: preparations for boost injections. Red error bars indicate standard error of the mean. CMV: cytomegalovirus, MCS: multiple cloning site, BGH pA: bovine growth hormone polyadenylation signal, KanR: kanamycin resistance gene, hTSS: human tyrosinase signal sequence.



## Chapter 4

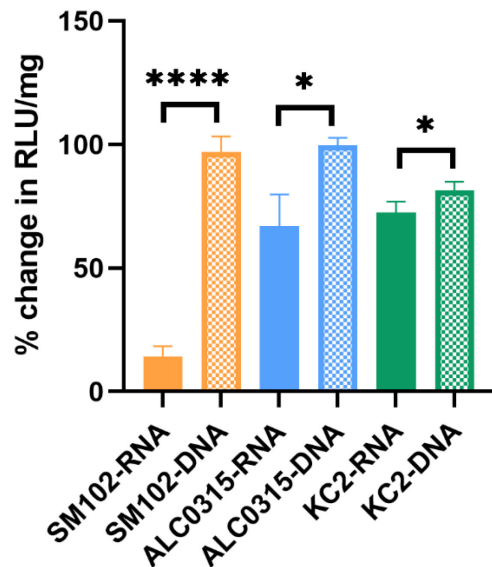


**Figure S4.1.** Gating strategy for flow cytometry analysis of antigen-specific T cells. Representative plots for splenocytes harvested from mice 28 days after vaccination with two doses of VV-OspC-A.



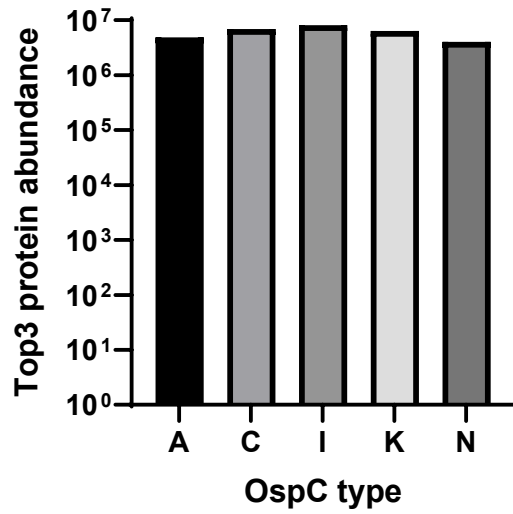
**Figure S4.2.** Verification of OspC expression *in vitro*. BHK21 cells were infected with VV-OspC-A or the negative control VV-K8.1. OspC protein in whole cell lysates were detected by Western Blot. Recombinant OspC with a maltose-binding protein tag (rOspC-MBP) was included as a positive control. The expected band size for OspC and OspC-MBP are 21 kDa and 63 kDa, respectively.

## Chapter 5

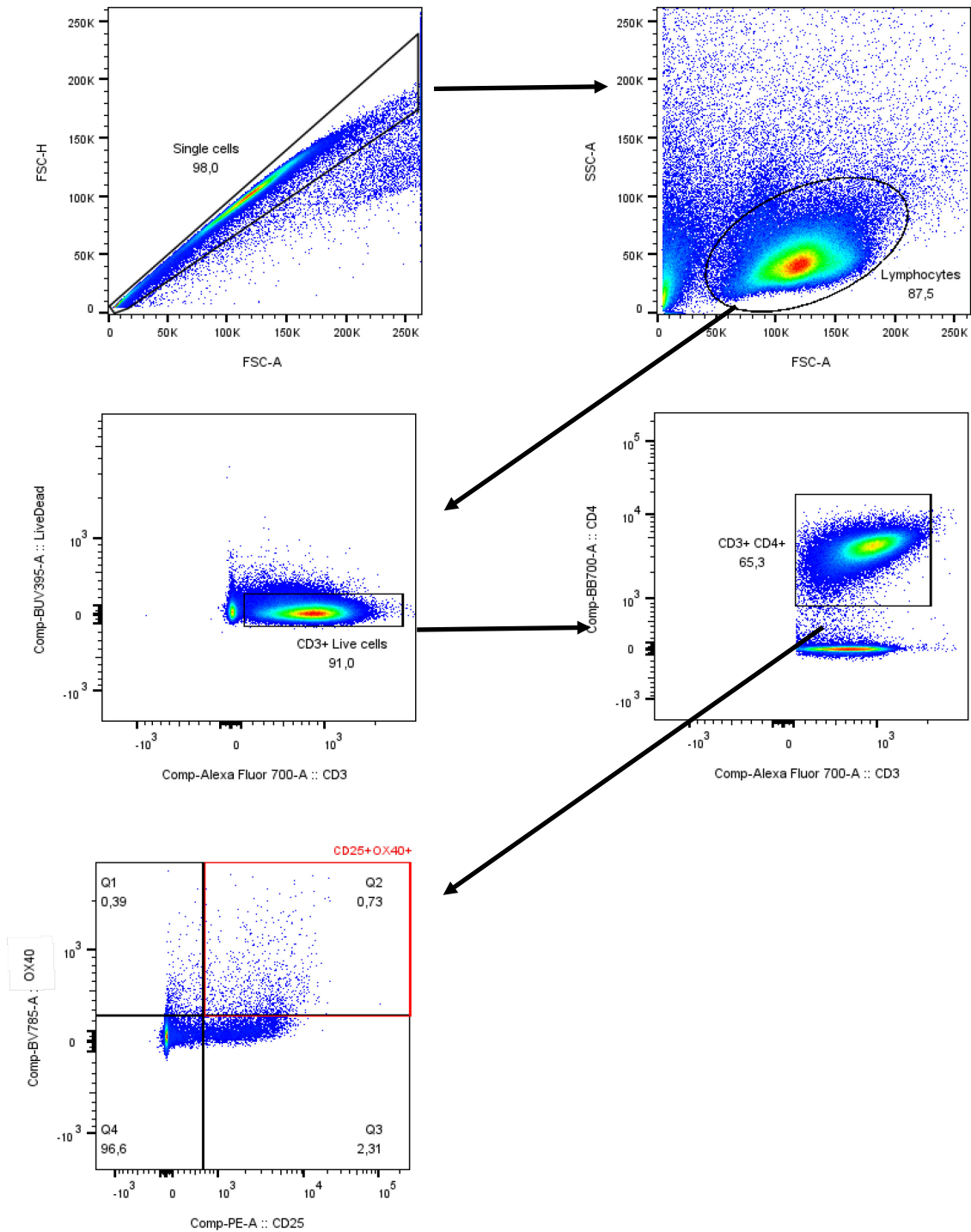


**Figure S5.1.** DNA-LNPs are more thermostable than RNA-LNPs. LNPs encapsulating mRNA encoding firefly luciferase were stored at 37°C for seven days and then used to transfect HEK293T cells according to the *in vitro* transfection assay protocol described in the methods section. % change in RLU/mg represents the change in RLU/mg when compared to that of the freshly prepared LNP formulation on day 0. RLU: relative luminescence units. Error bars represent standard deviation (SD). \* p-value < 0.05, \*\*\*\* < 0.0001.

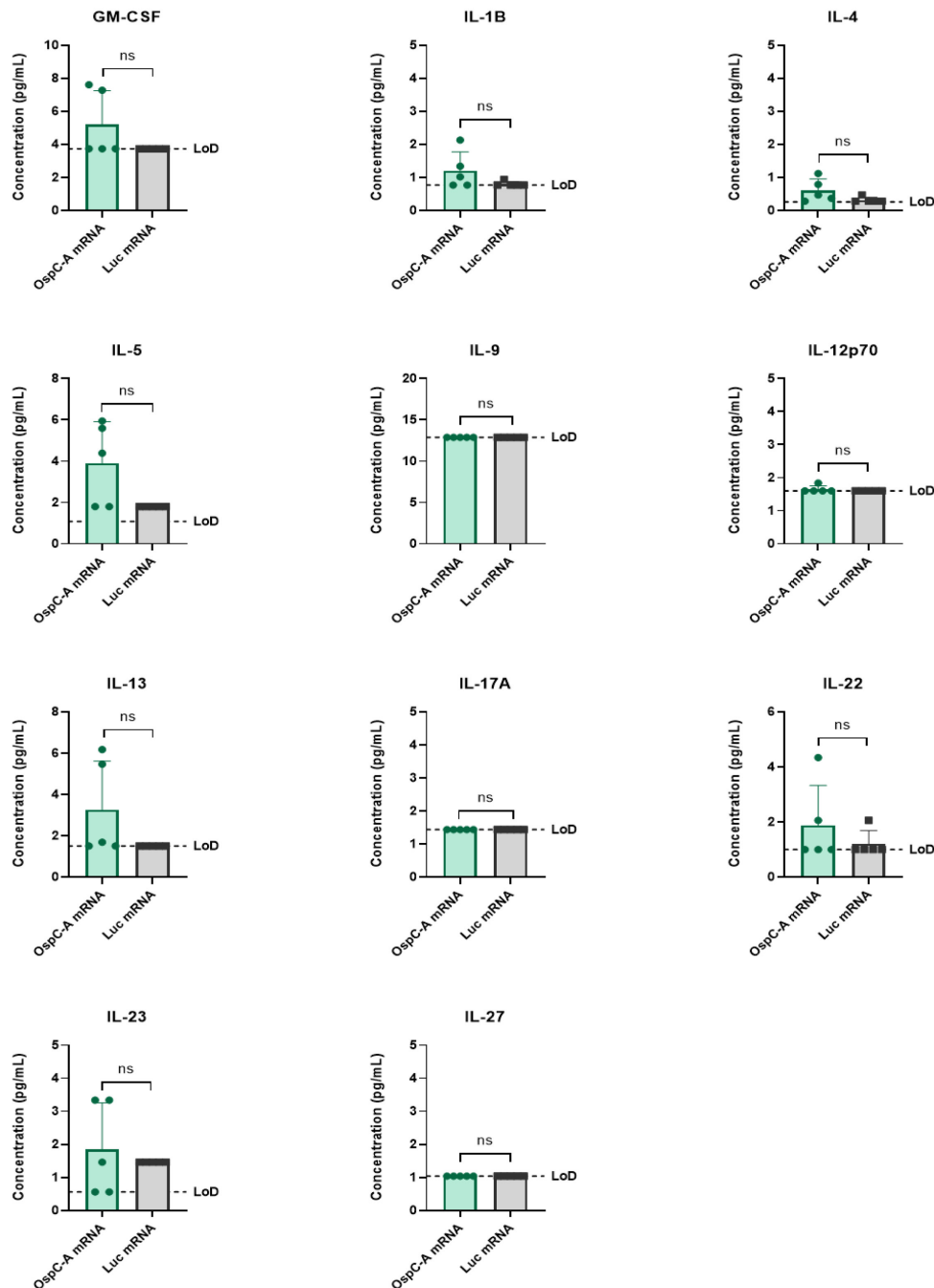
## Chapter 6



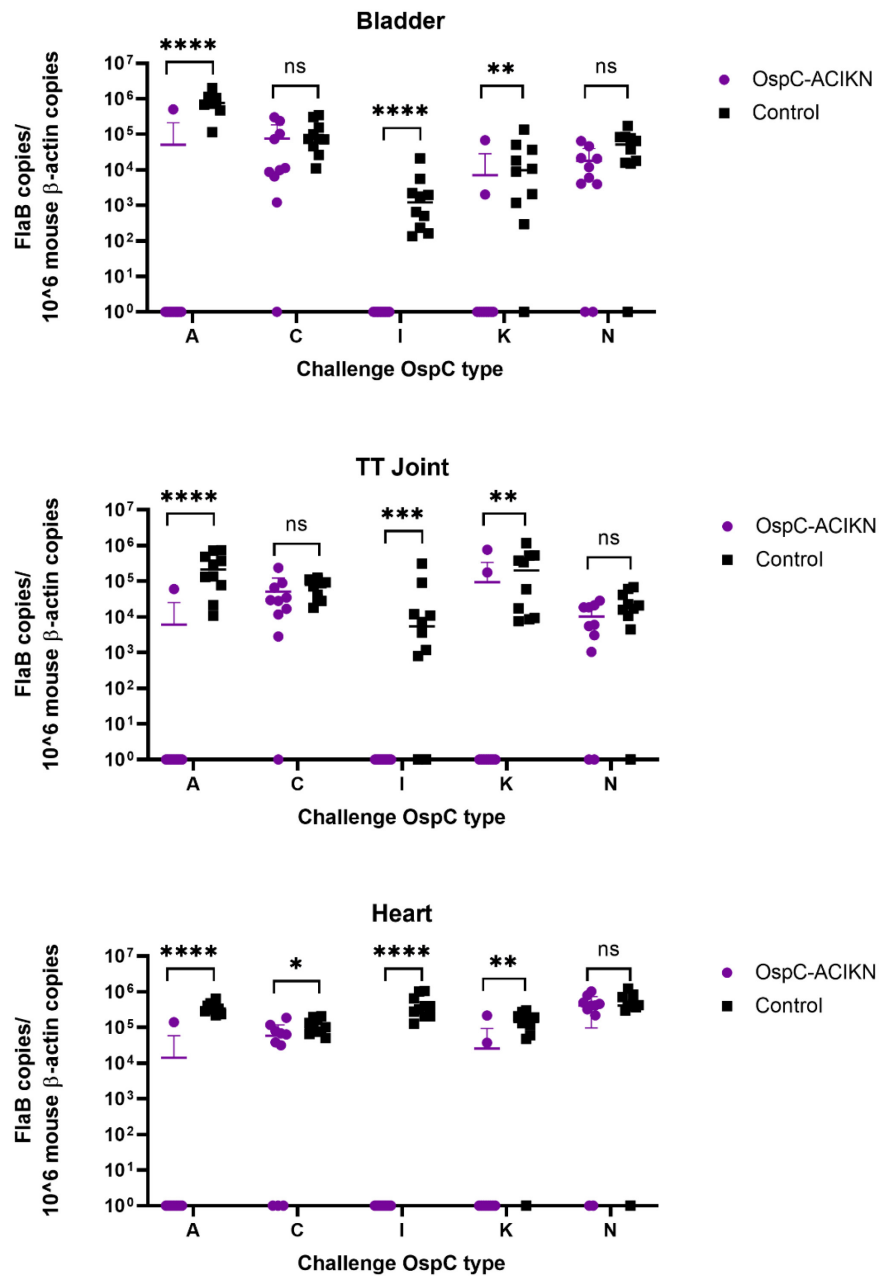
**Figure S6.1.** Protein abundance by OspC type following *in vitro* transfection of HEK 293T cells with LNPs encapsulating OspC-ACIKN.



**Figure S6.2.** Representative gating strategy for the activation induced marker (AIM) flow cytometry assay. Activated T cells were CD4<sup>+</sup> CD25<sup>+</sup> OX40<sup>+</sup>.



**Figure S6.3.** Cytokines that were not significantly elevated in the OspC-A mRNA group following stimulation of the draining lymph nodes with an OspC overlapping peptide pool. dLNs were harvested from mice four weeks after boost vaccination with the OspC-A mRNA vaccine or Control. dLNs were stimulated with an overlapping peptide library of OspC for 24 h. The concentration of each cytokine in the supernatant was determined by a ProcartaPlex 17-plex Immunoassay kit. LoD, limit of detection. Error bars represent standard deviation. Statistical significance was calculated using unpaired two-tailed t-tests. ns, not significant.



**Figure S6.4.** Vaccination with OspC-ACIKN mRNA protects mice against disseminated infection with multiple *B. burgdorferi* strains. qPCR analysis of the bacterial DNA burden in the bladder (top), tibiotarsal joint (TT joint) (middle) and heart (bottom) represented by *B. burgdorferi* FlaB gene copies per 10<sup>6</sup> mouse β-actin copies. Samples below the threshold of amplification are represented by a value of 1. Statistical significance was calculated using independent two-tailed t-tests. Error bars represent standard deviation. \* p-value < 0.05, \*\* p-value < 0.01, \*\*\* p-value < 0.001, \*\*\*\* p-value < 0.0001.