OspA Antibodies Inhibit the In vitro Transmigration of *Borreliellia burgdorferi*

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running title: Transmigration of *B. burgdorferi*

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Keywords:

ABSTRACT

Antibodies against Outer surface protein A (OspA) block tick-to-mammal transmission of the Lyme disease spirochete, *Borreliella burgdorferi*. Evidence suggests that antibodies ingested by a tick during a blood meal entrap OspA-expressing *B. burgdorferi* within the tick midgut and prevent spirochete migration to the salivary gland and host dermis. In this study, we employed a recently described Transwell system to quantitate the impact of OspA antibodies on *B. burgdorferi* transmigration. We demonstrate that spirochete movement from the lower Transwell chamber to the upper chamber over a 20-hour period was reduced by $\geq$99% in the presence the transmission-blocking mouse monoclonal IgG antibody (mAb) LA-2. In the case of LA-2, transmigration inhibition coincided with the formation of large antibody-induced bacterial aggregates in the lower Transwell chamber, as determined by dark field microscopy and flow cytometry. Further examination of a panel of human OspA mAbs of varying affinities and epitope specificities revealed that virtually all could block *B. burgdorferi* transmigration, although most did so in the absence of measurable spirochete agglutination. We propose that transmigration arrest may represent a possible mechanism by which OspA antibodies entrap *B. burgdorferi* in the tick midgut and limit transmission to mammalian hosts.

INTRODUCTION

The spirochete *Borreliella burgdorferi* sensu latu is the etiologic agent of Lyme disease, the most prevalent tick-borne disease in the United States [1]. *B. burgdorferi* is 10-20 microns in length (0.3 microns wide) and swims with a planar (flatwave) morphology driven by 7-10 periplasmic flagella [2,3]. Motility enables the spirochete to disseminate in a variety of viscous environments and tissues [4-6]. Motility is paramount in the context of tick-mediated transmission in that the spirochete migrates during the course of a blood meal from the tick midgut to the hemocoel and salivary glands before being deposited into the dermis of the mammalian host [7]. Breaching the tick midgut epithelium is a particularly dynamic process that occurs over a 24-72 h period, as revealed through in situ confocal imaging of fluorescent spirochetes [8]. The importance of motility to *B. burgdorferi*'s infectious life cycle is underscored by the fact that flagella mutants are not only attenuated for mammalian infection but inept for tick to mouse transmission [9,10].

During egress from the tick midgut, *B. burgdorferi* is also vulnerable to host factors, including antibodies. In fact, Lyme disease vaccines and monoclonal antibodies directed against Outer surface protein A (OspA) afford protection by entrapping spirochetes within the tick midgut during a feeding event [11-16]. OspA is ~30 kDa lipoprotein expressed at high levels on the surface of *B. burgdorferi* that reside within the midgut of unfed ticks. OspA is downregulated as the spirochete migrates to the salivary gland and eventually extinguished when the bacteria are within mammalian hosts [16]. It was recognized almost three decades ago in mouse (and other rodent) models that circulating OspA polyclonal and monoclonal antibodies (mAbs) are highly effective at blocking tick-mediated transmission of *B. burgdorferi* [11,17]. These and other observations eventually led to the development of a first and second generation human and veterinary OspA-based Lyme disease vaccines [18-20].

The molecular mechanisms by which OspA antibodies interfere with *B. burgdorferi* transmission are not fully understood, but evidence suggests that the interactions likely occur within the midgut and are not dependent on human complement [13-15,21,22]. Since those early reports very little has been done in this area, probably

because of the technical challenges associated with sorting out antibody-spirochete interactions within the context of tick tissues [8]. However, Van Gundy and colleagues recently developed a Transwell method to evaluate *B. burgdorferi* migration in response to chemotactic gradients, including salivary proteins [23]. Transwells units consist of an insert with a microporous membrane (3.0 micron) that sits within 96-well microtiter plates. Spirochetes suspended in a low-nutrient medium and seeded in the lower chamber of 96 well Transwell units migrate against gravity into the upper chamber in response to chemoattractant, including BSK-II complete medium [23]. In this report, we employed the Transwell assay as a model system to investigate the impact of OspA antibodies on *B. burgdorferi* migration across a physical barrier.

MATERIALS AND METHODS

Strains and culture conditions

B. burgdorferi strain B31-A, a high-passage, non-infectious clone of wild-type B31 carrying the plasmid pBSV2G_P_{flaB}-msfGFP (Addgene 118231) that constitutively expresses GFP expression, was used for most of the assays [24,25]. For the assays with different OspA serotypes, *B. burgdorferi* strain HB19-R1, a high-passage derivative of human blood isolate HB19 lacking linear plasmid 54 (Δ*ospAB*) was transformed with the IPTG-inducible mScarlet-I reporter plasmid expressing each of the seven different OspA variants (ST 1-7), regulated by the P_{ospAB} promoter from *B. burgdorferi* B31 (Willsey et al, *manuscript submitted*) [26]. All the strains were maintained as frozen glycerol stocks at -80°C and cultured routinely in gelatin-free BSK-II complete media, supplemented with 50 µg/mL of gentamicin, in microaerophilic and static growth conditions at 33°C with 2% CO_2, unless otherwise stated.

Monoclonal antibodies (mAbs)

The OspA-specific mAbs used in this study were expressed and purified by Dr. Lisa Cavacini (UMass Chan Medical School, Worcester, MA), as described [27]. Chimeric LA-2 IgG1 was custom synthesized by ZabBio, Inc (San Diego, CA).

Transmigration assays

The Transwell® migration assays were performed essentially as described by Van Gundy [23], with several modifications. HTS Transwell® 96 well permeable supports (Corning, Corning, NY) with tray inserts containing 3.0 µm pore size polycarbonate membrane in each well were used for the assays. Frozen glycerol stocks of respective *B. burgdorferi* strains were thawed and cultured for ~3 days, then diluted in fresh medium at a starting cell concentration of 1.5×10^6 spirochetes/mL and grown till mid-exponential phase (2-5$\times10^7$ spirochetes/mL; ~40-42 hours). Spirochetes were collected by centrifugation at 1800×*g* at room temperature. The cell pellet was washed once with phosphate buffered saline (PBS), pH 7.4, and then resuspended in the low-nutrient, methylcellulose-free *Borrelia* motility buffer [28], supplemented with 50 µg/mL of gentamicin. For the HB19-R1 strains, the buffer was also supplemented with 1 mM IPTG for inducing mScarlet production.

Cell suspensions were added to the lower chambers (LC) of the Transwell units along with appropriate dilutions of mAbs (**Table 1**) or polyclonal serum that had been heat-inactivated at 56°C for 30 min. In the end, the lower chamber contained 2×10^6 HB19-R1 spirochetes or 2×10^7 B31-A spirochetes in 200 µL. The upper chambers (UC) of the Transwell unit were filled with 150 µL of BSK-II complete medium, free of gelatin and Phenol Red, and supplemented with 50 µg/mL of gentamicin (and 1 mM IPTG for the HB19-R1 strains). The Transwell plate was sealed with Parafilm® and incubated at 33°C with 5% CO_2 for 18-20 h to enable spirochete migration across the polycarbonate membrane from the lower to the upper chamber. After the incubation, 75 µL volume was collected, without touching the membrane, from the upper chamber of each well, and diluted in 425 µL of PBS (total volume 0.5 mL) in a Falcon® 5 mL polystyrene test tube (Corning) for flow cytometry. The transmigration assays were done with 3-5 technical replicates (wells/plate) per assay and the assays were independently repeated three times. Technical replicates were averaged for each independent assay and average migration of spirochetes in antibody treated conditions were normalized to that of untreated controls for statistical analyses [29].

Flow cytometry

To quantitate migration, B31-A spirochetes were enumerated using a FACSCalibur (BD Biosciences) for 1 min at the 'Lo' flow rate setting (12 µL/min), with an excitation wavelength of 488 nm and a 530/30 emission filter (FL1). Events were gated to exclude debris, based on their size (forward scatter, FSC), granularity (side scatter, SSC), and GFP fluorescence intensity, using the BD CellQuest™ Pro software. HB19-R1 spirochetes, on the other hand, were counted on a BD FACSymphony A3 for 1 min at the 'Hi' flow rate setting (60 µL/min) with an excitation wavelength of 561 nm and a 610/20 emission filter (PE-CF594). Events were gated based on FSC, SSC, and mScarlet fluorescence intensity, using the BD FACSDiva™ software.

To quantitate antibody-mediated spirochete agglutination, aliquots (200 µL) from the lower chamber were added to 300 µL of PBS (total volume 0.5 mL) in a 5 mL polystyrene test tube. A total of 20,000 events were collected by gating to exclude debris, and size and granularity of events were measured using FSC-SSC dot plots with appropriate quadrants, as described [30]. Agglutination was represented as the percentage of events having increased FSC and SSC due to aggregation (sum of events in the lower-right, upper-left, and upper right quadrants) relative to free cells (events in lower-left quadrant). Percent agglutination of all treatments was normalized relative to untreated controls.

Analysis of normalized data was done using GraphPad Prism version 10.6.0 for Windows (GraphPad Software, Boston, MA). Statistically significant differences between treatments and their respective controls were determined using Ordinary one-way ANOVA followed by Dunnett's or Tukey's multiple comparisons *post hoc* test.

Dark-field Microscopy

For examination of the spirochetes in the lower chamber of the Transwell units, 7 µL aliquots of were spotted onto glass microscope slides and covered with cover glass (22×22, No. 1, 0.13-0.16 mm thickness). Spirochetes were imaged using an Olympus BH-2 RFCA upright microscope (Evident Scientific; Waltham, MA) paired with a 20X objective (N.A. 0.70) in a dark-field setting. Images and videos were acquired using an Excelis™ MPX-20RC microscopy camera (Accu-Scope, Commack, NY) paired with CaptaVision+ software version 2.4.9.0 for image analysis.

RESULTS

Van Gundy and colleagues developed a Transwell® assay to quantify the transmigration of *B. burgdorferi* in response to chemotactic factors, like tick salivary gland proteins [23]. Transwells consist of an insert with a microporous membrane (3.0 micron) that sits within 96-well microtiter plates. Spirochetes suspended in a low-nutrient medium and seeded in the lower chamber of 96 well Transwell units migrate against gravity into the upper chamber in response to chemoattractant, including BSK-II complete medium [23]. We sought to adopt this system to assess the impact of OspA antibodies on spirochete movement across physical barrier such that might be encountered during transmission (**Figure 1A**).

In our model, *B. burgdorferi* B31-A cells expressing GFP or HB19-R1 cells expressing mScarlet were suspended in low-nutrient motility buffer and seeded in the lower chamber of 96 well Transwell units at a range of concentrations (10^{4-7} total) for 2, 4 or 20 h (**Figure 1B, C**). We found that maximal migration was observed when 2×10^{7} spirochetes were seeded in the chamber (**Figure 1B**) and allowed to migrate for 20 h (**Figure 1C**). Migration was dependent on a chemoattractant, as spirochetes were detected in the upper chamber when it was filled with BSK-II medium but not the *Borrelia* motility buffer (consisting of Dulbecco's PBS, bovine serum albumin, and EDTA). No measurable migration of spirochetes from the lower to the upper chamber was observed when BSK-II or motility buffer were on both sides of the membrane, demonstrating that a chemoattractant gradient is necessary for *B. burgdorferi* migration (**Figure 1D**).

Van Gundy layered the polycarbonate Transwell membrane with 6% gelatin as a diffusion barrier [4,31]. We similarly investigated the impact of a range of gelatin (2-6%) on spirochete migration. Consistent with the literature, *B. burgdorferi* migration was significantly enhanced in the presence of 2% gelatin, compared to absence of gelatin, but reduced at higher concentrations (**Figure 1E**) [4]. However, the addition of 2% gelatin in our migration assays proved to be a confounding factor because the gelatin liquefied

at 33°C and affected both pipetting and background values in the flow cytometer. For that reason, we eventually conducted our assays without the addition of gelatin.

LA-2 blocks Bb migration in a dose-dependent manner

LA-2 is a well-characterized transmission-blocking OspA mAb [17,32]. It targets an epitope on OspA's C-terminus encompassing *β*-strands 16-21 and has potent complement-dependent borreliacidal activity in vitro [33]. To evaluate the impact of LA-2 IgG on *B. burgdorferi* B31-A transmigration, spirochetes were mixed with 100 μg mL^{-1} LA-2 or an isotype control just prior to being seeded in the lower chamber of the Transwell unit. The number of spirochetes in the upper chamber was enumerated 20 h later. We found that LA-2 treatment resulted in >99% reduction in spirochete migration relative to the isotype control (**Figure 2A**). We therefore conducted a dose response analysis in which a fixed number of *B. burgdorferi* B31-A cells were mixed with ten-fold serial dilutions of LA-2 IgG (10 - 0.001 μg mL^{-1}). Within this series, 10 μg mL^{-1} of LA-2 (roughly equivalent to 2×10^6 molecules per spirochete) resulted in significant inhibition of spirochete migration (~98%; $p<0.05$), while inhibitory activity diminished $\leq$1 μg mL^{-1} (**Figure 3A**). Monovalent LA-2 Fab' fragments (10 μg mL^{-1}) evaluated under identical conditions in reduced migration of B31-A by ~43% relative to the isotype control, although statistical significance was not achieved (**Figure 4A**). From these studies, we conclude that LA-2 is a potent inhibitor of *B. burgdorferi* migration and that maximal effects occur in bivalent form.

While the transmigration assays with LA-2 were done in the absence of exogenous complement in the BSK-II (upper chamber) or low motility media (lower chamber), we were concerned that trace amounts of complement may have carried over from the *B. burgdorferi* B31-A culture. The presence of even trace amounts of complement might have borreliacidal (or bacteriostatic) effects on spirochetes and thereby impact migration. To address this concern, we repeated the transmigration assays with an "Fc silent" derivative of LA-2 known as LA-2 LALAPG. LA-2 LALAPG carries three point mutations in the Fc region of IgG1 that render the molecule unable to fix complement (Palmer et al., *manuscript in preparation*). We found that LA-2 LALAPG functioned as effectively as LA-2 IgG in transmigration inhibition (**Figure S2**), thereby demonstrating

the effects are independent of any Fc activity and likely restricted to Fv region of the antibody.

LA-2-induced agglutination of *B. burgdorferi* inversely correlates with loss of transmigration activity.

A hallmark of certain OspA antibodies, including LA-2, is their capacity to induce agglutination of *B. burgdorferi* cells in culture [30,34-38]. We reasoned that agglutination might account, at least in part, for the lack of spirochete transmigration observed in the Transwell assays in the presence of LA-2. To examine this possibility, we collected the volumes in the lower chambers of the Transwell units in which *B. burgdorferi* B31-A had been treated with 100 µg mL^{-1} LA-2 IgG or an equivalent concentration of an isotype control. By dark-field microscopy, LA-2 IgG treated cells formed large, dense, multicellular aggregates even as cells on the periphery of the aggregates retained wriggling behavior (**Figure 2C**). In contrast, spirochetes treated with 100 µg mL^{-1} of an IgG1 isotype control appeared as single, free-swimming cells that were indistinguishable from cells in the untreated volume (**Figure 2C**). These observations suggest that agglutination may indeed be a factor in influencing spirochete transmigration. In a separate experiment, we treated spirochetes with 100 µg mL^{-1} LA-2, and observed their behavior immediately after, instead of incubating them according to the Transwell assay conditions. We found one end of the spirochetes looping back on itself, while the other end remained free, consistent with what Frye et al. described as “lariats”.

We employed a microvolume flow cytometry protocol developed in our laboratory to quantitate spirochete agglutination as a function of LA-2 IgG concertation [30,39]. Using this assay, we noted a sizeable shift in the forward scatter (FSC) and side scatter (SSC) values upon the addition of LA-2 consistent with agglutination (**Figure 2B**). We observed that LA-2 at 10 µg/mL of resulted in ~40% spirochetes agglutination, whereas agglutination was negligible (<5%) at lower LA-2 concentrations (**Figure 3B**). Under the same conditions, LA-2 Fab’ (10 µg mL^{-1}) induced ~2% of the spirochetes to agglutinate (**Figure 4B**).

Impact of OspA human MAbs *B. burgdorferi* transmigration

We next tested whether other OspA mAbs besides LA-2 effect transmigration of *B. burgdorferi* in the Transwell assay. We previously characterized a panel of human OspA mAbs with varying epitope specificities and binding affinities, including three (319-44, 221-7, 857-2) shown to passively protect mice from tick-mediated *B. burgdorferi* challenge (**Table 1**) [27,30,32,40]. When tested at 10 μg mL^{-1} in the Transwell assay, 10 of the 12 OspA mAbs significantly reduced *B. burgdorferi* migration, albeit to varying degrees (30-90%). The three mAbs (LA-2, 319-44 and 3-24) that target epitopes in Bin 3, which is located at the C-terminus of OspA, had the most pronounced impact on spirochete migration with inhibition values ranging from 89-93% (**Figure 5A**). The two mAbs in Bin 2, 212-55 and 319-28, reduced spirochete transmigration by ~65% and ~35%, respectively. Finally, the Bin 1 MAbs, which target OspA's central *β*-sheet (*β*-strands 8-11) had the most varied impacts: 227-2 reduced migration by ~70%, 857-2 by 50% and 221-20 by just 37%. The impact on migration was not related to mAb binding affinity, as 857-2's K_D is 20-fold lower than 227-2's (0.6 versus 10.6 nM), yet 227-2 was more effective than 857-2 at inhibiting bacterial migration. When tested at 1 μg mL^{-1}, none of the mAbs had any significant effect on spirochete migration (**Figure S4**).

To examine the relationship between transmigration inhibition and agglutination in the extended panel of mAbs, the volumes in the lower chamber of the Transwell units were collected at 20 h and subjected to flow cytometry, as described above. A total of five mAbs induced notable spirochete agglutination (**Figure 5B**): three from Bin 3 (LA-2, 3-24 and 319-33) and two from Bin 1 (221-5 and 221-20). These results demonstrate that inhibition of transmigration inhibitory can occur independent of agglutination, as defined by flow cytometry.

Selected OspA Human MAbs inhibit transmigration of B. burgdorferi with different OspA serotypes

In the United States, Lyme disease is principally associated with *B. burgdorferi* sensu stricto carrying OspA serotype A [41]. However, in Europe and Asia, *Borrelia* genospecies carrying six additional OspA serotypes (ST2-7) are associated with disease [42]. The serotypes are particularly polymorphic towards the C-terminus of OspA corresponding to epitope Bin 3 and more conserved within Bin 1. We therefore tested 221-7, a Bin 1

MAb, along with LA-2 as a control, for its ability to impact the transmigration of *B. burgdorferi* HB19-R1 strains, individually carrying each of the seven OspA serotypes. As expected, LA-2 inhibited the migration of HB19-R1 strain expressing OspA serotype 1 (~92%) but not the other serotypes. 221-7 at 10 µg mL^{-1} inhibited the migration of all seven serotypes. The strongest effect was seen on the migration of HB19-R1 expressing OspA serotype 5, which represents *B. garinii*.

Impact of OspA camelid-derived V_HHs *B. burgdorferi* transmigration

In addition to the human MAbs described above, we also have a collection of single-domain camelid antibodies (V_HHs) that target a diversity of epitopes on OspA and a range of binding affinities as monovalent V_HHs or bivalent VHH-IgG1 Fc [43]. We tested three V_HHs and their corresponding V_HH-Fcs each from Bins 1 and 3. At 10 µg mL^{-1}, none of the monovalent V_HHs had any significant impact on spirochete transmigration (**Figure 7A, C**). Similarly, at 10 µg mL^{-1}, none of the VHH- IgG1 Fc had inhibitory activity except for L8H8-IgG1 Fc, which reduced spirochete transmigration by ~75% (**Figure 7A**). L8H8- IgG1 Fc also promoted spirochete agglutination (~12%) but at rather low levels (**Figure 7B**). It is probably not a coincidence that L8H8 has the highest binding affinity among all the OspA V_HHs we identified.

OspA immune sera inhibits *B. burgdorferi* transmigration *in vitro*

In light of the effect of OspA mAbs on *B. burgdorferi* transmigration, we were prompted to evaluate OspA immune sera in the same set-up. Towards this end, we pooled hyper-immune sera from mice that had received several doses of a heptavalent mRNA vaccine formulations (kindly provided by Dr. Meredith Finn, Moderna) was diluted 1:100, mixed with *B. burgdorferi* B31-A and seeded into the lower well of a Transwell unit. The addition of the OspA immune serum resulted in >99% reduction in spirochete migration over an 18 h period relative to control cells (**Figure 8A**). Analysis by dark-field microscopy of the volume in the lower chambers revealed numerous borrelia aggregates of varying sizes, consistent with antibody-induced agglutination (**Figure 8B**). Serial dilutions of the OspA hyperimmune sera (1:200 - 1:2000) revealed a significant inhibition of transmigration at 1:400 dilution, but not beyond that. When the volume in the lower chamber of the Transwell was subject to flow cytometry, we found observed

significant macro-agglutination of spirochetes at the 1:200 and 1:400 dilutions, but not at lower doses of the serum (**Figure 8C**). We conclude that OspA polyclonal sera inhibits *B. burgdorferi* transmigration in vitro at concentrations associated with spirochete agglutination.

DISCUSSION

The observation that OspA antibodies entrap *B. burgdorferi* within the tick midgut during the course of a bloodmeal was one of several pivotal findings that contributed to the eventual development of OspA-based Lyme disease vaccines [11,13,18,19,44]. Subsequent reports demonstrated that transmission blocking activity of OspA antibodies does not require host complement or impact spirochete viability [15,22]. Since those seminal studies were conducted more than 20 years ago, few if any reports have sought to address how it is that OspA antibodies interfere with *B. burgdorferi* egress from the tick midgut without impacting spirochete viability.

In this study, we investigated the effect of OspA monoclonal antibodies (mAbs) on *B. burgdorferi* migration across a porous (3-micron) polycarbonate membrane in a Transwell unit, as a proxy for spirochete movement out of the tick midgut. The transmigration assay was a modification of a method developed for the purpose of identifying *B. burgdorferi* chemoattractants in tick salivary glands [23]. In that study, the Transwell membrane was overlaid with 6% gelatin to function as a diffusion barrier and chemoattractant gradient. We did not use gelatin in our transmigration assays because it confounded downstream flow cytometry assays (see Results). In the absence of gelatin, we expected that solute diffusion between the upper and lower compartments of the Transwell unit would be virtually instantaneous. Nonetheless, we observed that *B. burgdorferi* transmigration was dependent on differential media in the upper and lower chambers (i.e., BSK II in upper and PBS in lower). We speculate that the addition of BSK II to the upper chamber results in passive adsorption of BSK II constituents to the polycarbonate membrane, thereby creating a pseudo-chemoattractant gradient sufficient to drive spirochete migration against gravity in both a time and dose-dependent manner.

Using this set-up, we demonstrated that LA-2 IgG was a potent inhibitor of *B. burgdorferi* transmigration when seeded into the lower chamber along with the spirochetes. LA-2 is arguably one of the most well-characterized transmission blocking OspA mAbs in the field [17,27,30,32,33,45]. LA-2 targets the C-terminus of OspA, including the exposed loops between β-strands 16-17, 18-19, and 20-21 [33]. From the standpoint of Lyme disease vaccines, LA-2 is highly significant as a threshold of LA-2 equivalents, based on competition ELISAs, was shown to correlate with protection from *B. burgdorferi* challenge in animal models [46,47]. An LA-2 equivalence assay was subsequently employed in human clinical trials of the original OspA-based vaccines [18].

In this respect it is interesting that 10 μg mL^{-1} was the concentration of LA-2 associated with significant inhibition of spirochete transmigration in the Transwell unit. This number (10 μg mL^{-1}) correlates with the *in vivo* transmission-blocking activity of an LA-2-like mAb known as C3.78 IgG [15]. C3.78 completely prevents tick-host transmission of *B. burgdorferi* at serum antibody concentrations roughly equal to 10 μg mL^{-1}. Moreover, the transmission-blocking activity of C3.78 occurs in the absence of host (mouse) complement. Based on the collective observations, we postulate that LA-2, if present in sufficient quantities in blood, would impede migration of *B. burgdorferi* out of the midgut during tick feeding.

Examination of a well-characterized collection of human mAbs targeting different epitopes along the length of OspA revealed that virtually all of them could inhibit *B. burgdorferi* movement across the Transwell membrane to varying degrees. As a rule, mAbs that targeted epitopes near or overlapping with LA-2 (so called Bin 3) were more potent at inhibiting transmigration than mAbs that recognize epitopes elsewhere on the molecule (Bins 1 and 2). The Bin 3 mAbs like LA-2 were also the most effective at promoting spirochete agglutination, as determined by flow cytometry and dark field microscopy of the volume in the lower chambers. This agrees with previous observations from our group in which we demonstrated that LA-2 (epitope Bin 3) was significantly more effective than Bin 1 mAbs at inducing *B. burgdorferi* agglutination in culture [30]. Obviously, large (macroscopic) bacterial aggregates are not conducive to passage through a 3-micron porous membrane. However, in that same report, we noted

that agglutination was likely the culmination of a series of antibody-induced changes in the behavior of *B. burgdorferi* that initiate with the formation of “lariats,” in which one pole of a spirochete loops back onto itself. While lariat formation did not affect spirochete motility per se, as the bacteria continued to wriggle under these conditions, the spirochetes seemingly lost their directionality [4,30] [4]. Work by others has suggested that OspA antibodies drive OspA to localize at the spirochete poles, possibly as a result of alterations in OspA fluidity within cholesterol rafts [48]. The exact impact OspA mis-localization has on *B. burgdorferi* motility is yet to be determined,

As a final note, we observed that mouse serum from animals vaccinated with a multivalent OspA mRNA vaccine was effective in a dose-dependent manner at blocking *B. burgdorferi* transmigration in the Transwell chamber. Inhibition of transmigration was inversely correlated with spirochete agglutination, similar to what was observed with LA-2. While it is difficult to glean much from a solitary observation, we are intrigued by the possibly that the transmigration assay may have value in as a functional test in assessing vaccine efficacy for *B. burgdorferi* and other genospecies expressing different OspA serotypes. Towards that end, we are actively investigating the wider impact of immune sera on *B. burgdorferi* in the Transwell assay. up

Funding

This work was supported by the National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health, Department of Health and Human Services, Contract No. 75N93019C00040 (PI/PD Mantis). This content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Acknowledgements

We are grateful to Drs. Taylor Van Gundy and Meghan Lybecker at the Centers for Disease Control and Prevention (Fort Collins, CO) for assistance in establishing the transmigration assay. We thank Dr. Graham Willsey for providing *B. burgdorferi* strains,

Carol Lyn Piazza for assistance with flow cytometry protocols, and Dr. David Vance for guidance on antibody selection. We thank Dr. Meredith Finn (Moderna, Inc) for sharing mouse sera. We acknowledge Drs. Renjie Song and Jennifer Yates of the Wadsworth Center's Immunology Core for assistance with flow cytometry and the Media and Cell Culture core for BSK-II medium and *Borrelia* motility buffer. We thank Ms. Elizabeth Cavosie (Wadsworth Center) for administrative assistance. The authors have no conflicts of interest to declare.

References

1. Steere, A.C., Strle, F., Wormser, G.P., Hu, L.T., Branda, J.A., Hovius, J.W., Li, X., and Mead, P.S. (2016). Lyme borreliosis. Nat Rev Dis Primers *2*, 16090. 10.1038/nrdp.2016.90.
2. Goldstein, S.F., Charon, N.W., and Kreiling, J.A. (1994). Borrelia burgdorferi swims with a planar waveform similar to that of eukaryotic flagella. Proc Natl Acad Sci U S A *91*, 3433-3437. 10.1073/pnas.91.8.3433.
3. Charon, N.W., and Goldstein, S.F. (2002). Genetics of Motility and Chemotaxis of a Fascinating Group of Bacteria: The Spirochetes. Annual Review of Genetics *36*, 47-73. https://doi.org/10.1146/annurev.genet.36.041602.134359.
4. Harman, M.W., Dunham-Ems, S.M., Caimano, M.J., Belperron, A.A., Bockenstedt, L.K., Fu, H.C., Radolf, J.D., and Wolgemuth, C.W. (2012). The heterogeneous motility of the Lyme disease spirochete in gelatin mimics dissemination through tissue. Proc Natl Acad Sci U S A *109*, 3059-3064. 10.1073/pnas.1114362109.
5. Harman, M., Vig, D.K., Radolf, J.D., and Wolgemuth, C.W. (2013). Viscous dynamics of Lyme disease and syphilis spirochetes reveal flagellar torque and drag. Biophys J *105*, 2273-2280. 10.1016/j.bpj.2013.10.004.
6. Bockenstedt, L.K., Gonzalez, D., Mao, J., Li, M., Belperron, A.A., and Haberman, A. (2014). What ticks do under your skin: two-photon intravital imaging of Ixodes scapularis feeding in the presence of the lyme disease spirochete. Yale J Biol Med *87*, 3-13.
7. De Silva, A.M., and Fikrig, E. (1995). Growth and migration of Borrelia burgdorferi in Ixodes ticks during blood feeding. Am J Trop Med Hyg *53*, 397-404. 10.4269/ajtmh.1995.53.397.
8. Dunham-Ems, S.M., Caimano, M.J., Pal, U., Wolgemuth, C.W., Eggers, C.H., Balic, A., and Radolf, J.D. (2009). Live imaging reveals a biphasic mode of dissemination of Borrelia burgdorferi within ticks. J Clin Invest *119*, 3652-3665. 10.1172/jci39401.
9. Sultan, S.Z., Manne, A., Stewart, P.E., Bestor, A., Rosa, P.A., Charon, N.W., and Motaleb, M.A. (2013). Motility is crucial for the infectious life cycle of Borrelia burgdorferi. Infect Immun *81*, 2012-2021. 10.1128/iai.01228-12.

10. Hyde, J.A. (2017). Borrelia burgdorferi Keeps Moving and Carries on: A Review of Borrelial Dissemination and Invasion. Front Immunol *8*, 114. 10.3389/fimmu.2017.00114.
11. Fikrig, E., Barthold, S.W., Kantor, F.S., and Flavell, R.A. (1990). Protection of mice against the Lyme disease agent by immunizing with recombinant OspA. Science *250*, 553-556. 10.1126/science.2237407.
12. Fikrig, E., Telford, S.R., 3rd, Barthold, S.W., Kantor, F.S., Spielman, A., and Flavell, R.A. (1992). Elimination of Borrelia burgdorferi from vector ticks feeding on OspA-immunized mice. Proc Natl Acad Sci U S A *89*, 5418-5421.
13. de Silva, A.M., Telford, S.R., 3rd, Brunet, L.R., Barthold, S.W., and Fikrig, E. (1996). Borrelia burgdorferi OspA is an arthropod-specific transmission-blocking Lyme disease vaccine. J Exp Med *183*, 271-275.
14. de Silva, A.M., Zeidner, N.S., Zhang, Y., Dolan, M.C., Piesman, J., and Fikrig, E. (1999). Influence of outer surface protein A antibody on Borrelia burgdorferi within feeding ticks. Infect Immun *67*, 30-35. 10.1128/IAI.67.1.30-35.1999.
15. Gipson, C.L., and de Silva, A.M. (2005). Interactions of OspA monoclonal antibody C3.78 with Borrelia burgdorferi within ticks. Infect Immun *73*, 1644-1647. 10.1128/IAI.73.3.1644-1647.2005.
16. Srivastava, S.Y., and de Silva, A.M. (2008). Reciprocal expression of ospA and ospC in single cells of Borrelia burgdorferi. J Bacteriol *190*, 3429-3433. 10.1128/jb.00085-08.
17. Schaible, U.E., Kramer, M.D., Eichmann, K., Modolell, M., Museteanu, C., and Simon, M.M. (1990). Monoclonal antibodies specific for the outer surface protein A (OspA) of Borrelia burgdorferi prevent Lyme borreliosis in severe combined immunodeficiency (scid) mice. Proc Natl Acad Sci U S A *87*, 3768-3772. 10.1073/pnas.87.10.3768.
18. Steere, A.C., Sikand, V.K., Meurice, F., Parenti, D.L., Fikrig, E., Schoen, R.T., Nowakowski, J., Schmid, C.H., Laukamp, S., Buscarino, C., and Krause, D.S. (1998). Vaccination against Lyme disease with recombinant Borrelia burgdorferi outer-surface lipoprotein A with adjuvant. Lyme Disease Vaccine Study Group. N Engl J Med *339*, 209-215. 10.1056/NEJM199807233390401.
19. Gomes-Solecki, M., Arnaboldi, P.M., Backenson, P.B., Benach, J.L., Cooper, C.L., Dattwyler, R.J., Diuk-Wasser, M., Fikrig, E., Hovius, J.W., Laegreid, W., et al. (2020). Protective Immunity and New Vaccines for Lyme Disease. Clin Infect Dis *70*, 1768-1773. 10.1093/cid/ciz872.
20. Wormser, G.P. (2022). A brief history of OspA vaccines including their impact on diagnostic testing for Lyme disease. Diagn Microbiol Infect Dis *102*, 115572. 10.1016/j.diagmicrobio.2021.115572.
21. Pal, U., de Silva, A.M., Montgomery, R.R., Fish, D., Anguita, J., Anderson, J.F., Lobet, Y., and Fikrig, E. (2000). Attachment of Borrelia burgdorferi within Ixodes scapularis mediated by outer surface protein A. J Clin Invest *106*, 561-569. 10.1172/JCI9427.
22. Rathinavelu, S., Broadwater, A., and de Silva, A.M. (2003). Does host complement kill Borrelia burgdorferi within ticks? Infect Immun *71*, 822-829. 10.1128/iai.71.2.822-829.2003.

23. Van Gundy, T.J., Ullmann, A.J., Brandt, K.S., and Gilmore, R.D. (2021). A transwell assay method to evaluate Borrelia burgdorferi sensu stricto migratory chemoattraction toward tick saliva proteins. Ticks Tick Borne Dis *12*, 101782. 10.1016/j.ttbdis.2021.101782.
24. Burgdorfer, W., Barbour, A.G., Hayes, S.F., Benach, J.L., Grunwaldt, E., and Davis, J.P. (1982). Lyme disease-a tick-borne spirochetosis? Science *216*, 1317-1319. 10.1126/science.7043737.
25. Takacs, C.N., Kloos, Z.A., Scott, M., Rosa, P.A., and Jacobs-Wagner, C. (2018). Fluorescent Proteins, Promoters, and Selectable Markers for Applications in the Lyme Disease Spirochete Borrelia burgdorferi. Appl Environ Microbiol *84*. 10.1128/AEM.01824-18.
26. Sădziene, A., Rosa, P.A., Thompson, P.A., Hogan, D.M., and Barbour, A.G. (1992). Antibody-resistant mutants of Borrelia burgdorferi: in vitro selection and characterization. J Exp Med *176*, 799-809. 10.1084/jem.176.3.799.
27. Haque, H.M.E., Ejemel, M., Vance, D.J., Willsey, G., Rudolph, M.J., Cavacini, L.A., Wang, Y., Mantis, N.J., and Weis, D.D. (2022). Human B Cell Epitope Map of the Lyme Disease Vaccine Antigen, OspA. ACS Infect Dis. 10.1021/acsinfecdis.2c00346.
28. Zhang, K., and Li, C. (2018). Measuring Borrelia burgdorferi Motility and Chemotaxis. In Borrelia burgdorferi: Methods and Protocols, U. Pal, and O. Buyuktanir, eds. (Springer New York), pp. 313-317. 10.1007/978-1-4939-7383-5_23.
29. Blainey, P., Krzywinski, M., and Altman, N. (2014). Replication. Nature Methods *11*, 879-880. 10.1038/nmeth.3091.
30. Frye, A.M., Ejemel, M., Cavacini, L., Wang, Y., Rudolph, M.J., Song, R., and Mantis, N.J. (2022). Agglutination of Borreliella burgdorferi by Transmission-Blocking OspA Monoclonal Antibodies and Monovalent Fab Fragments. Infect Immun, e0030622. 10.1128/iai.00306-22.
31. Kimsey, R.B., and Spielman, A. (1990). Motility of Lyme disease spirochetes in fluids as viscous as the extracellular matrix. J Infect Dis *162*, 1205-1208. 10.1093/infdis/162.5.1205.
32. Wang, Y., Kern, A., Boatright, N.K., Schiller, Z.A., Sadowski, A., Ejemel, M., Souders, C.A., Reimann, K.A., Hu, L., Thomas, W.D., Jr., and Klempner, M.S. (2016). Pre-exposure Prophylaxis With OspA-Specific Human Monoclonal Antibodies Protects Mice Against Tick Transmission of Lyme Disease Spirochetes. J Infect Dis *214*, 205-211. 10.1093/infdis/jiw151.
33. Ding, W., Huang, X., Yang, X., Dunn, J.J., Luft, B.J., Koide, S., and Lawson, C.L. (2000). Structural identification of a key protective B-cell epitope in Lyme disease antigen OspA. J Mol Biol *302*, 1153-1164. 10.1006/jmbi.2000.4119.
34. Jiang, W., Gorevic, P.D., Dattwyler, R.J., Dunn, J.J., and Luft, B.J. (1994). Purification of Borrelia burgdorferi outer surface protein A (OspA) and analysis of antibody binding domains. Clin Diagn Lab Immunol *1*, 406-412.
35. Luft, B.J., Dunn, J.J., and Lawson, C.L. (2002). Approaches toward the directed design of a vaccine against Borrelia burgdorferi. J Infect Dis *185 Suppl 1*, S46-51. 10.1086/338463.

36. Sadziene, A., Thompson, P.A., and Barbour, A.G. (1993). In vitro inhibition of Borrelia burgdorferi growth by antibodies. J Infect Dis *167*, 165-172. 10.1093/infdis/167.1.165.
37. Sears, J.E., Fikrig, E., Nakagawa, T.Y., Deponte, K., Marcantonio, N., Kantor, F.S., and Flavell, R.A. (1991). Molecular mapping of Osp-A mediated immunity against Borrelia burgdorferi, the agent of Lyme disease. J Immunol *147*, 1995-2000.
38. Wilske, B., Luft, B., Schubach, W.H., Zumstein, G., Jauris, S., Preac-Mursic, V., and Kramer, M.D. (1992). Molecular analysis of the outer surface protein A (OspA) of Borrelia burgdorferi for conserved and variable antibody binding domains. Med Microbiol Immunol *181*, 191-207. 10.1007/bf00215765.
39. Rudolph, M.J., Chen, Y., Vorauer, C., Vance, D.J., Piazza, C.L., Willsey, G.G., McCarthy, K., Muriuki, B., Cavacini, L.A., Guttman, M., and Mantis, N.J. (2024). Structure of a Human Monoclonal Antibody in Complex with Outer Surface Protein C of the Lyme Disease Spirochete, Borreliella burgdorferi. J Immunol. 10.4049/jimmunol.2400247.
40. Schiller, Z.A., Rudolph, M.J., Toomey, J.R., Ejemel, M., LaRochelle, A., Davis, S.A., Lambert, H.S., Kern, A., Tardo, A.C., Souders, C.A., et al. (2021). Blocking Borrelia burgdorferi transmission from infected ticks to nonhuman primates with a human monoclonal antibody. J Clin Invest *131*. 10.1172/JCI144843.
41. Wilske, B., Preac-Mursic, V., Gobel, U.B., Graf, B., Jauris, S., Soutschek, E., Schwab, E., and Zumstein, G. (1993). An OspA serotyping system for Borrelia burgdorferi based on reactivity with monoclonal antibodies and OspA sequence analysis. J Clin Microbiol *31*, 340-350. 10.1128/jcm.31.2.340-350.1993.
42. Lee, J.T., Li, Z., Nunez, L.D., Katzel, D., Perrin, B.S., Jr., Raghuraman, V., Rajyaguru, U., Llamera, K.E., Andrew, L., Anderson, A.S., et al. (2024). Development of a sequence-based in silico OspA typing method for Borrelia burgdorferi sensu lato. Microb Genom *10*. 10.1099/mgen.0.001252.
43. Vance, D.J., Basir, S., Piazza, C.L., Willsey, G.G., Haque, H.M.E., Tremblay, J.M., Rudolph, M.J., Muriuki, B., Cavacini, L., Weis, D.D., et al. (2024). Single-domain antibodies reveal unique borrelicidal epitopes on the Lyme disease vaccine antigen, outer surface protein A (OspA). Infect Immun *92*, e0008424. 10.1128/iai.00084-24.
44. Plotkin, S.A., and Shapiro, E.D. (2024). The Current and Future State of Vaccines for Lyme Disease. Clin Infect Dis. 10.1093/cid/ciae639.
45. Shandilya, S., Kurt Yilmaz, N., Sadowski, A., Monir, E., Schiller, Z.A., Thomas, W.D., Jr., Klempner, M.S., Schiffer, C.A., and Wang, Y. (2017). Structural and molecular analysis of a protective epitope of Lyme disease antigen OspA and antibody interactions. J Mol Recognit *30*. 10.1002/jmr.2595.
46. Johnson, B.J., Sviat, S.L., Happ, C.M., Dunn, J.J., Frantz, J.C., Mayer, L.W., and Piesman, J. (1995). Incomplete protection of hamsters vaccinated with unlipidated OspA from Borrelia burgdorferi infection is associated with low levels of antibody to an epitope defined by mAb LA-2. Vaccine *13*, 1086-1094. 10.1016/0264-410x(95)00035-y.
47. Golde, W.T., Piesman, J., Dolan, M.C., Kramer, M., Hauser, P., Lobet, Y., Capiau, C., Desmons, P., Voet, P., Dearwester, D., and Frantz, J.C. (1997). Reactivity

with a specific epitope of outer surface protein A predicts protection from infection with the Lyme disease spirochete, Borrelia burgdorferi. Infect Immun *65*, 882-889.

48. Toledo, A., Crowley, J.T., Coleman, J.L., LaRocca, T.J., Chiantia, S., London, E., and Benach, J.L. (2014). Selective association of outer surface lipoproteins with the lipid rafts of Borrelia burgdorferi. mBio *5*, e00899-00814. 10.1128/mBio.00899-14.

Figure Legends

Figure 1. An *in vitro* Transwell system optimized to measure the impacts of OspA antibodies on spirochete migration. (A) Fluorescent-labeled spirochetes were suspended in a low-nutrient *Borrelia* motility buffer (Dulbecco's PBS, 2% recrystallized bovine serum albumin, 0.1 mM EDTA, pH 7.4) and seeded into the Transwell lower chamber and allowed to migrate to the upper chamber containing gelatin- and Phenol Red-free BSK-II media. Migration was measured by collecting upper chamber sample and counting fluorescence events through flow cytometry using effective gating strategies for size and fluorescence. **(B)** Data represents migration of spirochetes (GFP events/min) relative to a range of spirochete concentrations seeded in the Transwell lower chamber. **(C)** Data represents migration of spirochetes (GFP events/min) relative to a range of Transwell incubation periods. **(D)** Migration of spirochetes (GFP events/min) relative to a chemical gradient. Spirochetes were either suspended in gelatin-free BSK-II media or *Borrelia* motility buffer (Bb mot) and seeded in the lower chamber (LC), with upper chamber (UC) containing either gelatin- and Phenol Red-free BSK-II media or *Borrelia* motility buffer (Bb mot). **(E)** Migration of spirochetes (GFP events/min) in the presence of a range of gelatin concentrations layered over the polycarbonate Transwell membrane, compared to the absence of gelatin. Data in all charts are representative, acquired from a single biological replicate with three technical replicates. Error bars in all charts represent SD of the mean. Statistical significance was determined by one-way ANOVA followed by Tukey's (B, C, D) or Dunnett's (E) *post hoc* multiple comparisons test, as applicable. Pink shading indicates $p \leq 0.05$.

Figure 2. LA-2 significantly inhibits the migration of *B. burgdorferi* B31-A and agglutinates spirochetes in the Transwell assay. 2×10^7 GFP-tagged spirochetes were either untreated or treated with 100 µg/mL of LA-2 or an isotype control mAb in the Transwell assay (Refer to Materials and Methods). Data was acquired from three independent biological replicates. **(A)** Data represents migration (GFP events/min) normalized to untreated spirochetes and error bars represent standard deviation of the mean. **(B)** Data represents percent agglutination of spirochetes in the lower Transwell chamber normalized to untreated spirochetes and error bars represent standard

deviation of the mean. Percent agglutination was calculated by summing increased size (FSC) and granularity (SSC) events relative to the total number of events collected, that represent antibody-mediated aggregation of live spirochetes. Statistical significance was determined by one-way ANOVA followed by Tukey's *post hoc* multiple comparisons test. Asterisks indicate $p \leq 0.05$ (**) and $p \leq 0.001$ (***) and ns=not significant compared to isotype mAb-treated group. **(C)** Dark field microscopy images of spirochetes in the Transwell lower chamber after overnight incubation. All images have been captured at 20X objective magnification.

Figure 3. LA-2 inhibits the migration of *B. burgdorferi* B31-A in a dose-dependent manner and migration inhibition correlates with antibody-mediated spirochete agglutination. 2×10^7 GFP-tagged spirochetes were either untreated or treated with a range of LA-2 concentrations or 10 µg/mL of isotype control mAb in the Transwell lower chamber. The Transwell migration assay was performed as described in Materials and Methods. Data was acquired from three independent biological replicates. **(A)** Data represents migration (GFP events/min) normalized to untreated spirochetes and error bars represent standard deviation of the mean. **(B)** Data represents percent agglutination of spirochetes in the lower Transwell chamber normalized to untreated spirochetes and error bars represent standard deviation of the mean. Percent agglutination was calculated as described in Materials and Methods. Statistical significance was determined by one-way ANOVA followed by Dunnett's *post hoc* multiple comparisons test. Pink shading indicates $p \leq 0.05$ compared to isotype mAb-treated group.

Figure 4. LA-2 monovalent Fab' fragments fail to significantly inhibit the migration of *B. burgdorferi* B31-A and agglutinate spirochetes in Transwell assay. 2×10^7 GFP-tagged spirochetes were either untreated or treated with 10 µg/mL of LA-2 Fab', mAb, or an isotype control mAb in the Transwell assay (Refer to Materials and Methods). Data was acquired from three independent biological replicates. **(A)** Data represents migration (GFP events/min) normalized to untreated spirochetes and error bars represent standard deviation of the mean. **(B)** Data represents percent agglutination of spirochetes in the lower Transwell chamber normalized to untreated

spirochetes and error bars represent standard deviation of the mean. Statistical significance was determined by one-way ANOVA followed by Dunnett's *post hoc* multiple comparisons test. Pink shading indicates $p \leq 0.05$ compared to isotype mAb-treated group.

Figure 5. A panel of OspA mAbs inhibit the migration of *B. burgdorferi* B31-A in an agglutination-dependent and -independent manner. 2×10^7 GFP-tagged spirochetes were either untreated or treated with 10 µg/mL of individual OspA mAbs or an isotype control mAb in the Transwell assay (Refer to Materials and Methods). Data was acquired from three independent biological replicates, excluding 319-33 and 3-24 which were done twice. **(A)** Data represents migration (GFP events/min) normalized to untreated spirochetes and error bars represent standard deviation of the mean. **(B)** Data represents percent agglutination of spirochetes in the lower Transwell chamber normalized to untreated spirochetes and error bars represent standard deviation of the mean. Statistical significance was determined by one-way ANOVA followed by Dunnett's *post hoc* multiple comparisons test. Pink shading indicates $p \leq 0.05$ compared to isotype mAb-treated group.

Figure 6. Selected Bin 1 mAbs inhibit the migration of Borrelia with different OspA serotypes while LA-2, a Bin 3 mAb, only inhibits migration of serotype 1. 2×10^6 mScarlet-tagged spirochetes were either untreated or treated with 10 µg/mL of 221-7 or LA-2 in the Transwell lower chamber. The Transwell migration assay was performed as described in Materials and Methods. Data was acquired from three independent biological replicates. Data represents migration (mScarlet events/min) of *B. burgdorferi* HB19-R1 strains (lacks lp54, Δ*ospAB*), with expression plasmids for OspA serotypes 1-7 (ST 1-7), normalized to spirochetes carrying the empty vector (EV), and error bars represent standard deviation of the mean. Statistical significance was determined by one-way ANOVA followed by Dunnett's *post hoc* multiple comparisons test. Pink shading indicates $p \leq 0.05$ when compared to empty vector.

Figure 7. Impact of Bin 1 and Bin 3 representative V_HH and V_HH-IgG constructs on the migration of *B. burgdorferi* B31-A and its correlation with spirochete agglutination. 2×10^7 GFP-tagged spirochetes were either untreated or treated with 10

μg/mL of either V_HH or V_HH-IgGs or an isotype control mAb in the Transwell lower chamber. LA-2 at 10 μg/mL was used as the positive control. The Transwell migration assay was performed as described in Materials and Methods. Data was acquired from three independent biological replicates. **(A, C)** Data represents migration (GFP events/min) of spirochetes treated with Bin 1 and Bin 3 V_HH and V_HH-IgGs, respectively, normalized to untreated spirochetes, and error bars represent standard deviation of the mean. **(B, D)** Data represents percent agglutination of spirochetes treated with Bin 1 and Bin 3 V_HH and V_HH-IgGs, respectively, in the lower Transwell chamber normalized to untreated spirochetes and error bars represent standard deviation of the mean. Statistical significance was determined by one-way ANOVA followed by Dunnett's *post hoc* multiple comparisons test. Pink shading indicates $p \leq 0.05$ when compared to isotype mAb-treated group.

Figure 8. Polyclonal sera from immunized mice significantly inhibits the migration of *B. burgdorferi* B31-A, in a dose-dependent manner which correlates with antibody-mediated spirochete agglutination. (A) 2×10^7 GFP-tagged spirochetes were either untreated or treated with 1:100 dilutions of naïve or immune serum. The Transwell migration assay was performed as described in Materials and Methods. Data were acquired from three independent biological replicates. Data represents migration (GFP events/min) normalized to untreated spirochetes and error bars represent standard deviation of the mean. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc multiple comparisons test. Asterisks indicate $p \leq 0.05$ (*) and $p \leq 0.01$ (**) and ns=not significant. **(B)** Dark field microscopy images of spirochetes in the Transwell lower chamber after overnight incubation with 1:100 serum dilution. All images have been captured at 20X objective magnification. **(C)** 2×10^7 GFP-tagged spirochetes were either untreated or treated with a range of immunized serum dilutions or 1:200 dilution of naïve serum. The Transwell migration assay was performed as described in Materials and Methods. Data were acquired from three independent biological replicates. Data represents migration (GFP events/min, in violet) and percent agglutination (in orange), normalized to untreated spirochetes and error bars represent standard deviation of the mean. Statistical significance was determined by one-way

ANOVA followed by Dunnett's post hoc multiple comparisons test. Pink shading indicates $p \leq 0.05$ when compared to naïve serum-treated group.

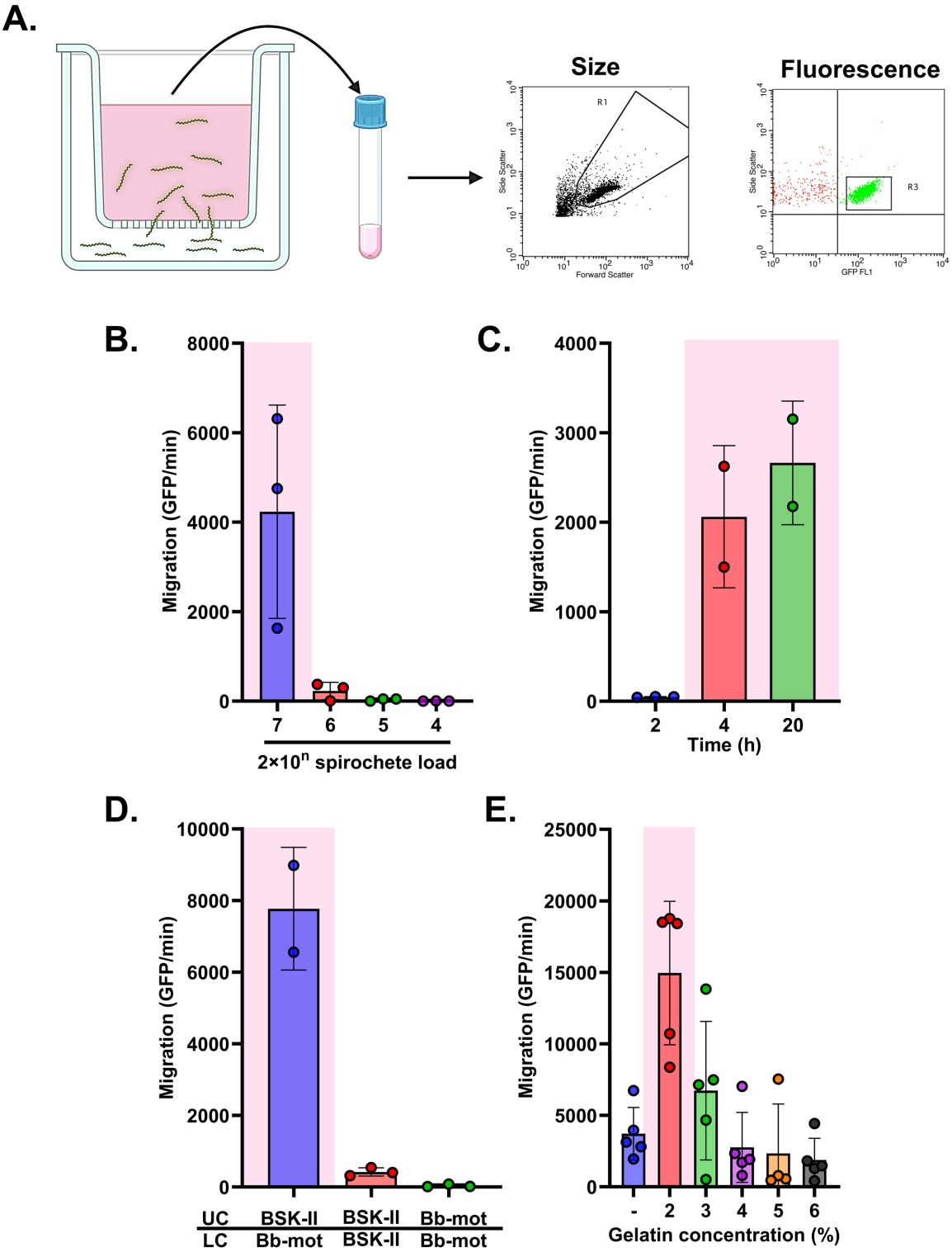
Tables

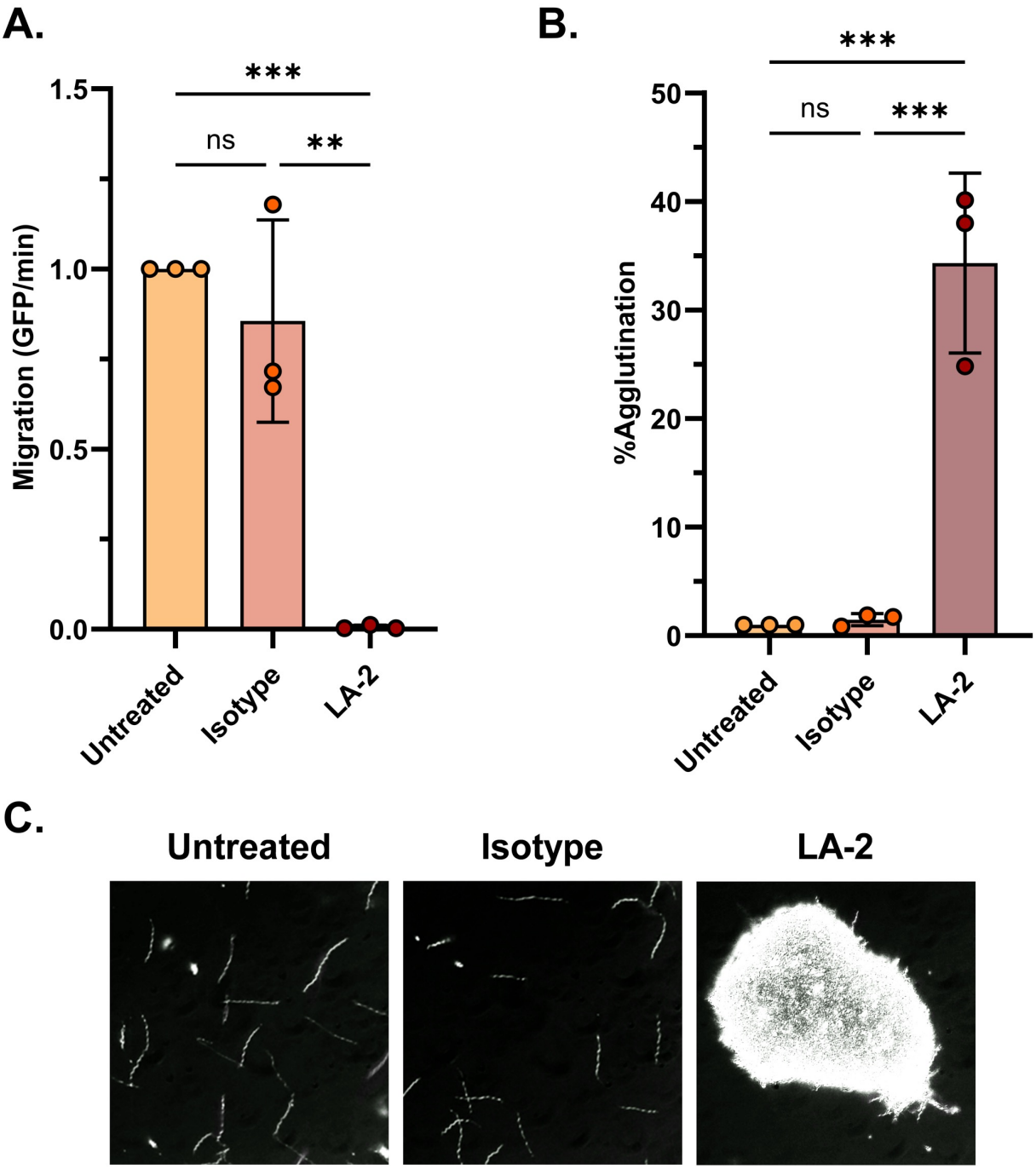
Table 1: Characteristics of mAbs used in this study

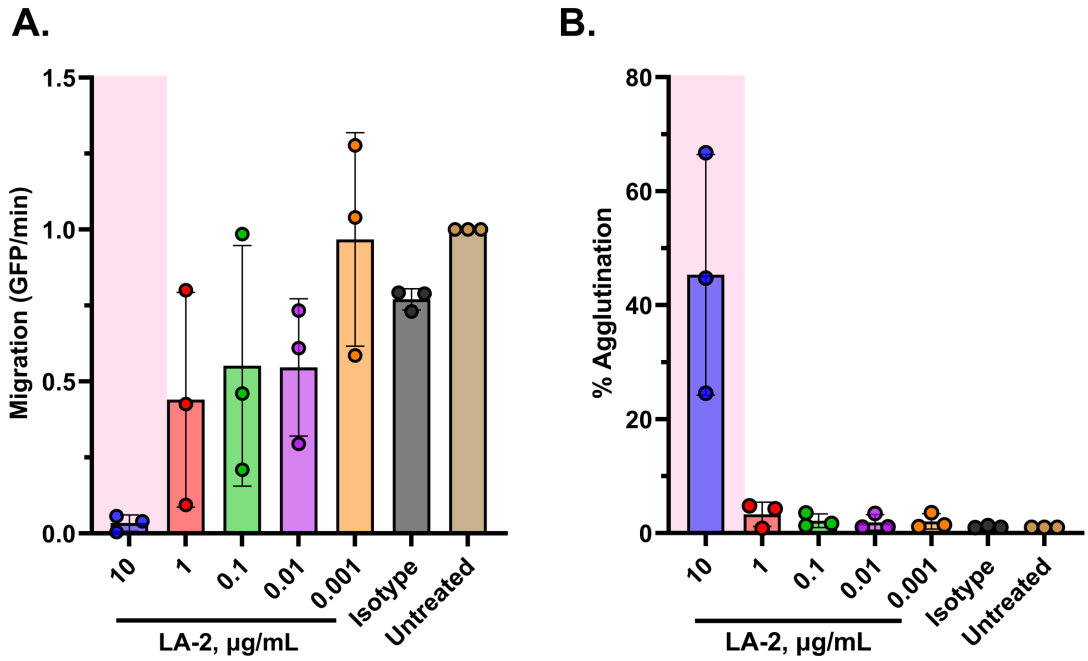
Bin	mAb	migration inhibition (%)	agglutination (%)
1	857-2	46 ± 5	4 ± 0.8
1	221-7	62 ± 13	4 ± 2.2
1	221-5	55 ± 7	16 ± 5
1	221-11	61 ± 9	2 ± 0.4
1	221-20	37 ± 25	23 ± 11
1	223-5	50 ± 10	1 ± 0.2
1	227-2	70 ± 3	2 ± 0.3
2	212-55	67 ± 10	3 ± 2
2	319-28	33 ± 12	2 ± 0.4
3	LA-2	98 ± 1	15 ± 6
3	319-44	89 ± 4	6 ± 4
3	319-33	83 ± 3	34 ± 10
3	3-24	93 ± 4	32 ± 11

*Underline, mAbs shown to have transmission blocking activity in mouse model; migration inhibition and agglutination values mean ± SD of three independent replicates.

Table 2: V_HHs used in this study		
V_HH/VHH-Fc	Bin	Epitope
L8H8	**1**	*β*-Strands 10-14
L8D3	**1**	*β*-Strands 11-13
L8G12	**1**	*β*-Strands 11-14
L8A9	**3**	*β*-Strands 16-18
L8C3	**3**	*β*-Strands 16-18
L8D9	**3**	*β*-Strands 16-18


A.
Size
Fluorescence
R1
R3
Side Scatter
Forward Scatter
GFP FL1
B.
Migration (GFP/min)
2×10^n spirochete load
C.
Time (h)
D.
UC
LC
BSK-II
Bb-mot
E.
Gelatin concentration (%)


A.
***
ns
**
1.5
1.0
0.5
0.0
Migration (GFP/min)
Untreated
Isotype
LA-2
B.
***
ns
***
50
40
30
20
10
0
%Agglutination
Untreated
Isotype
LA-2
C.
Untreated
Isotype
LA-2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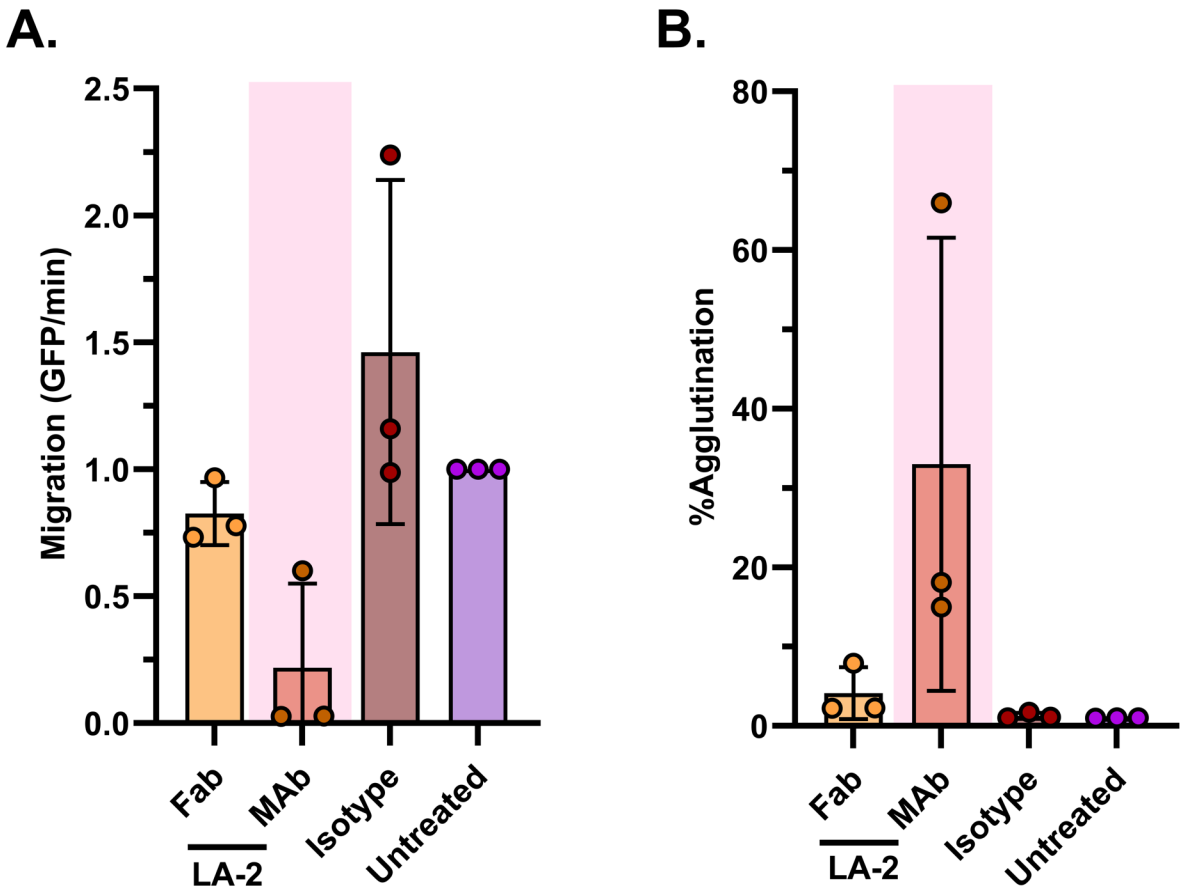
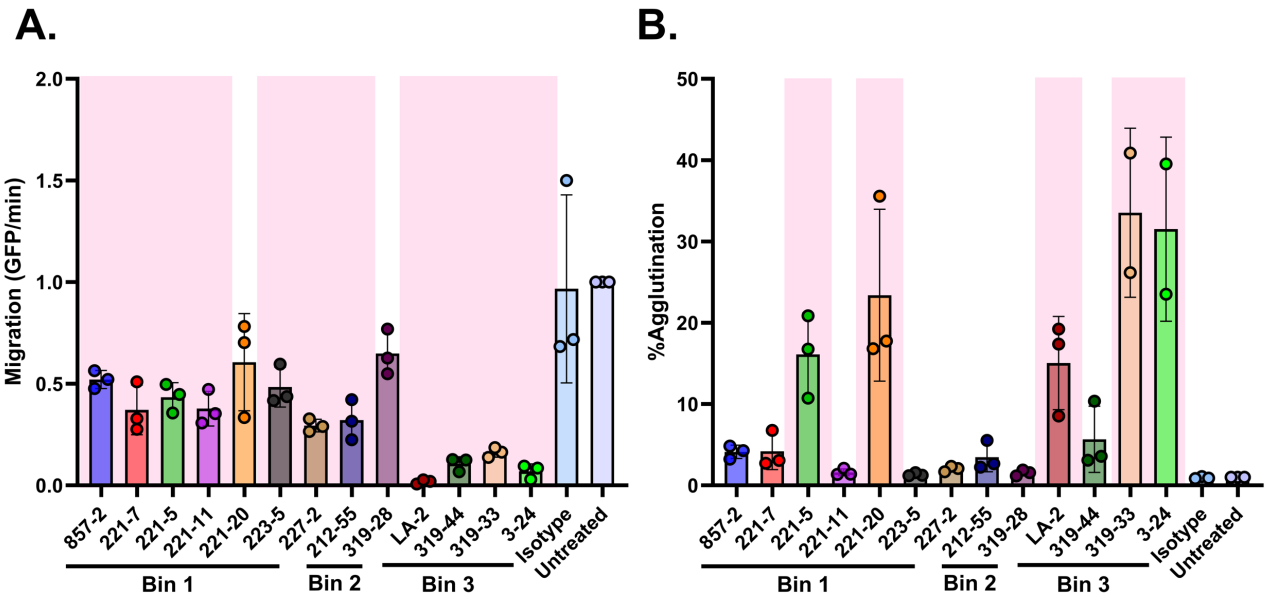
Migration (GFP/min)
1.5
1.0
0.5
0.0
10
1
0.1
0.01
0.001
Isotype
Untreated
LA-2, µg/mL
B.
% Agglutination
80
60
40
20
0
10
1
0.1
0.01
0.001
Isotype
Untreated
LA-2, µg/mL


A.
Migration (GFP/min)
2.5
2.0
1.5
1.0
0.5
0.0
Fab
MAb
LA-2
Isotype
Untreated
B.
%Agglutination
80
60
40
20
0
Fab
MAb
LA-2
Isotype
Untreated


A.
Migration (GFP/min)
2.0
1.5
1.0
0.5
0.0
857-2
221-7
221-5
221-11
221-20
223-5
227-2
212-55
319-28
LA-2
319-44
319-33
3-24
Isotype
Untreated
Bin 1
Bin 2
Bin 3
B.
%Agglutination
50
40
30
20
10
0
857-2
221-7
221-5
221-11
221-20
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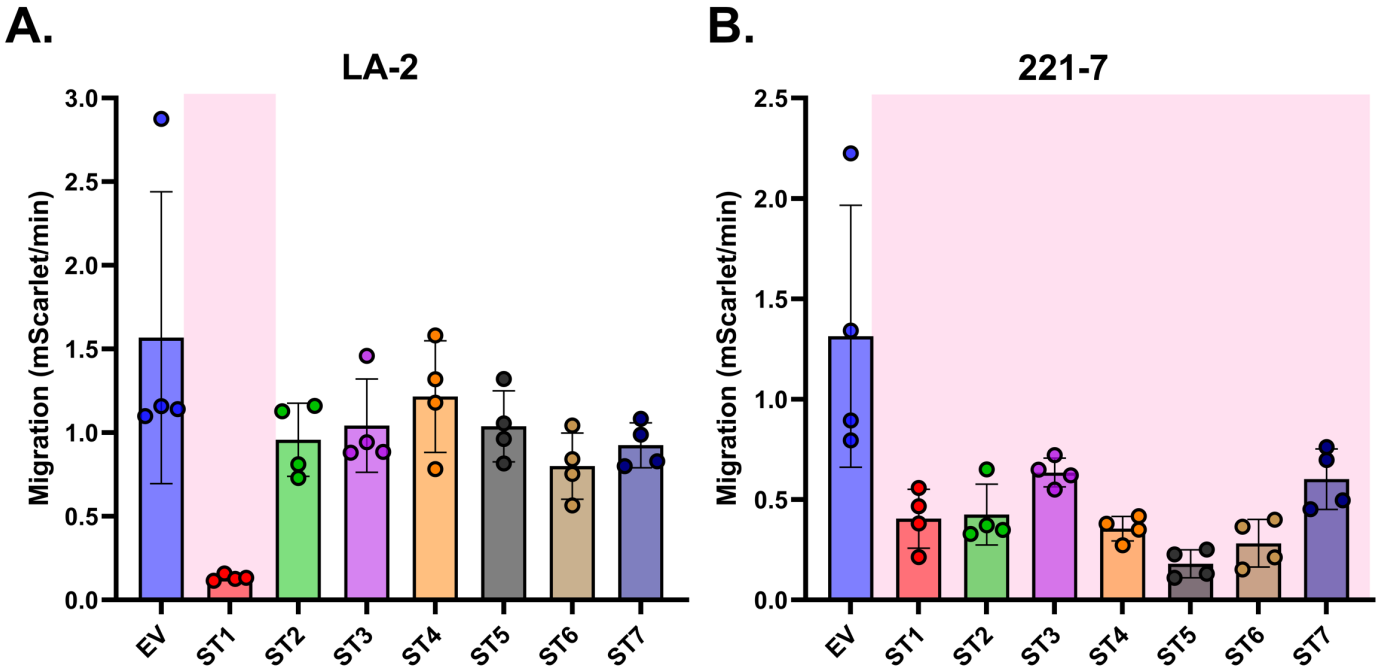
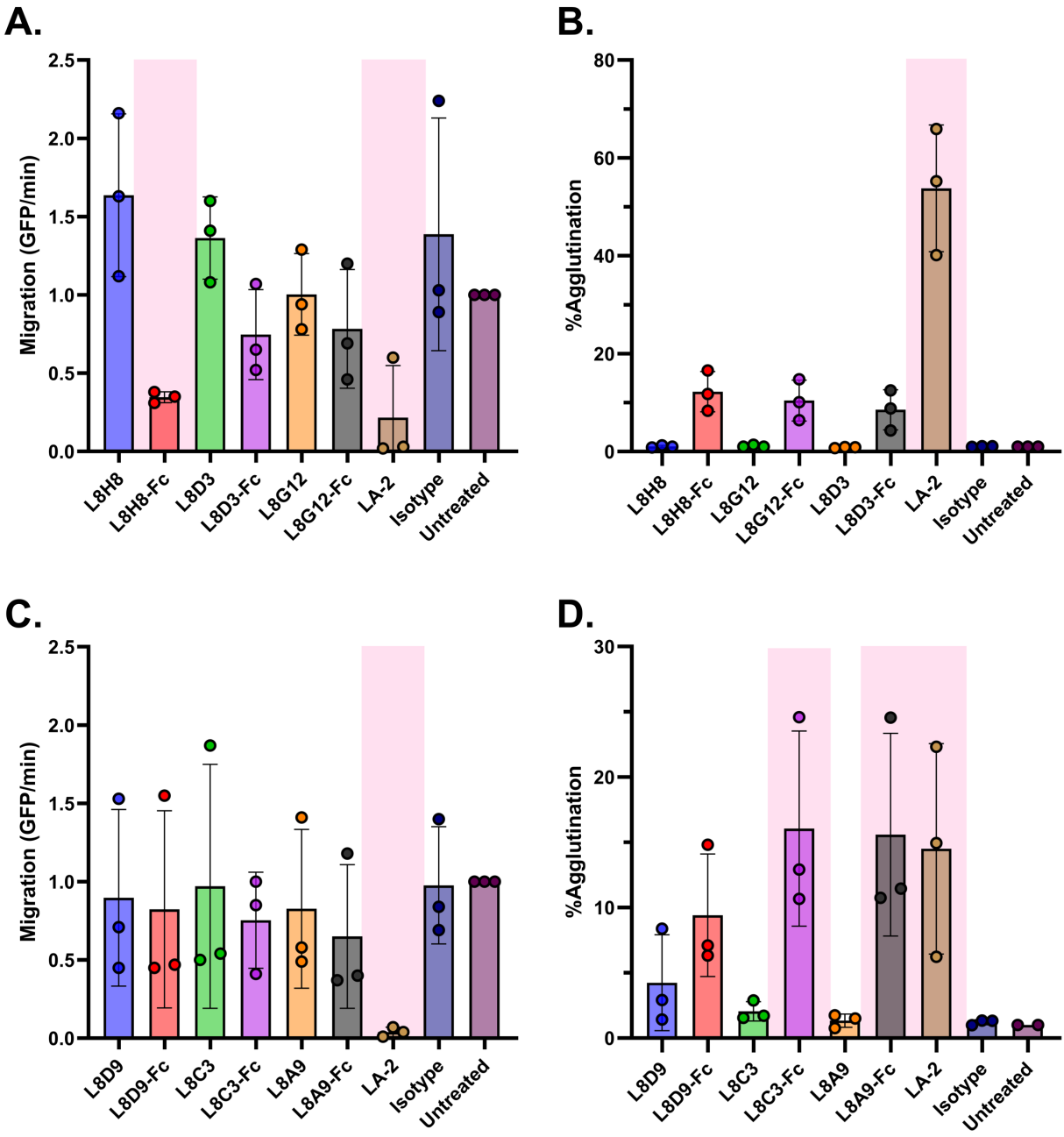
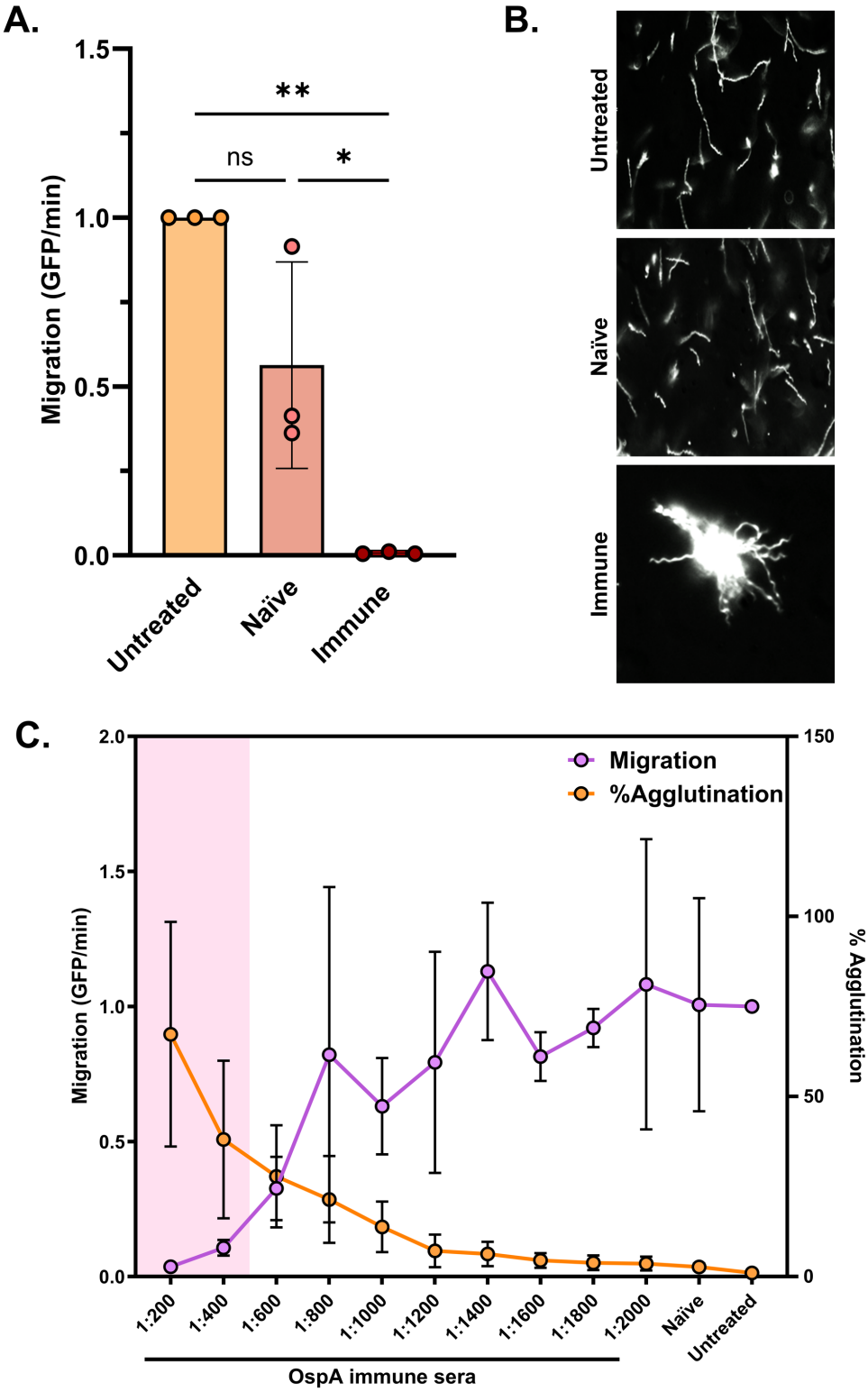

A.
LA-2
Migration (mScarlet/min)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
EV
ST1
ST2
ST3
ST4
ST5
ST6
ST7
B.
221-7
Migration (mScarlet/min)
2.5
2.0
1.5
1.0
0.5
0.0
EV
ST1
ST2
ST3
ST4
ST5
ST6
ST7


A.
Migration (GFP/min)
2.5
2.0
1.5
1.0
0.5
0.0
L8H8
L8H8-Fc
L8D3
L8D3-Fc
L8G12
L8G12-Fc
LA-2
Isotype
Untreated
B.
%Agglutination
80
60
40
20
0
L8H8
L8H8-Fc
L8G12
L8G12-Fc
L8D3
L8D3-Fc
LA-2
Isotype
Untreated
C.
Migration (GFP/min)
2.5
2.0
1.5
1.0
0.5
0.0
L8D9
L8D9-Fc
L8C3
L8C3-Fc
L8A9
L8A9-Fc
LA-2
Isotype
Untreated
D.
%Agglutination
30
20
10
0
L8D9
L8D9-Fc
L8C3
L8C3-Fc
L8A9
L8A9-Fc
LA-2
Isotype
Untreated


A.
Migration (GFP/min)
1.5
1.0
0.5
0.0
**
ns
*
Untreated
Naïve
Immune
B.
Untreated
Naïve
Immune
C.
Migration
%Agglutination
Migration (GFP/min)
% Agglutination
2.0
1.5
1.0
0.5
0.0
150
100
50
0
1:200
1:400
1:600
1:800
1:1000
1:1200
1:1400
1:1600
1:1800
1:2000
Naïve
Untreated
OspA immune sera