

# Heritably immunizing white-footed mice against tick-borne disease

## Project Narrative

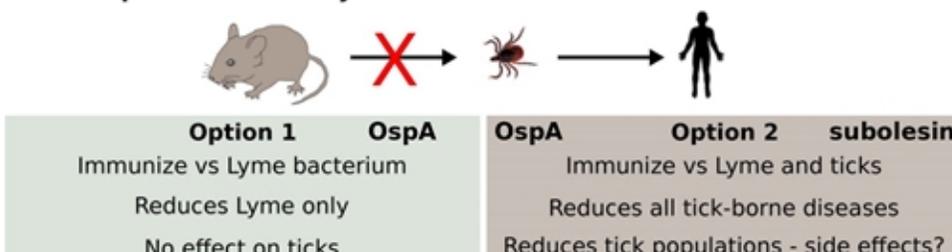
### Background

Tick-borne diseases are serious and growing health problems in the northeastern United States. The CDC has estimated that over 300,000 Americans are infected with Lyme disease each year, making it the most common vector-borne disease in the United States [1]. The problem has worsened due to the creation of additional fragmented woodland habitat over the past few decades, especially around human dwellings. This change has dramatically increased the number of white-footed mice, white-tailed deer, and ticks over the past few decades. In the eastern United States, the white-footed mouse *Peromyscus leucopus* is considered to be the primary reservoir of tick-borne disease because it is infected by and infects the black-legged tick *Ixodes scapularis* more effectively than any other vector [2]. With more larval ticks biting mice instead of less efficient small mammal reservoirs, the infection rate in the ticks, the mice, and even the other reservoirs increases, resulting in many more human infections.

The severity of the problem is particularly apparent on the islands of Martha's Vineyard and Nantucket: from 2010 to 2014, the town of Chilmark on Martha's Vineyard and the island of Nantucket had the highest per capita rates of confirmed and probable Lyme disease infections in Massachusetts according to the Massachusetts Department of Public Health [3]. Sixty three percent of respondents to an online survey conducted by the Vineyard Gazette this summer reported that they or a family member had contracted Lyme disease or another tick-borne illness on Martha's Vineyard [4]. Nearly 40 percent of Nantucket's population has suffered from Lyme disease according to the chair of Nantucket's Board of Health [5]. Even these staggering statistics do not likely reflect the total number of individuals that contract tick-borne diseases on Martha's Vineyard and Nantucket. Hundreds of thousands of tourists from around the world visit these islands during the summer months when ticks are most active. Island visitors are arguably the most at risk population because they know the least about prevention, and are the most statistically underrepresented as they are likely to receive medical attention elsewhere.

Proven interventions have been met with local resistance. Culling the deer herds on Martha's Vineyard and Nantucket is one such option. With over 40 deer per square mile of wooded habitat, both islands support deer populations that far exceed the threshold density below which the tick population is suppressed, maintaining low levels of infection [6, 7]. Past kills have prompted local opposition and the prospect of future culls remains divisive. In February of 2004, a special hunt on Nantucket provoked "public opposition [that] led to the cancellation of future hunts"[7]. Alternative solutions such as the reintroduction of the human vaccine and use of acaricides are even less publicly acceptable. Given the growing rate of infections, the shortage of acceptable solutions, and the increasing severity of the problem, new and more effective prevention strategies are urgently needed. If the ecological transmission cycle between white-footed mice and ticks could be broken, the number of infected ticks and therefore human infections in the eastern United States would be dramatically reduced. One way to disrupt the transmission cycle involves immunizing *P. leucopus* (Fig. 1).

**Concept: Permanently immunize the mice that infect most ticks**



**Fig. 1:** We propose to disrupt the transmission cycle by heritably immunizing populations of *P. leucopus*, the primary reservoir of tick-borne pathogens in the eastern United States, against *B. burgdorferi* (OspA) and against the tick salivary protein subolesin.

Previous research has demonstrated that vaccinating even a fraction of a wild mouse population against Lyme disease can significantly reduce the number of infected ticks, even if immunity is weak [8-11]. In their 2004 PNAS publication, Tsao and colleagues reported that by-hand immunization of wild white-footed mice in open forest against the *B. burgdorferi* outer surface protein A (OspA) reduced the prevalence of *B. burgdorferi* in the blood of mice by 42% and nymphal infections by 25%. While they trapped and vaccinated almost one thousand mice, they could not vaccinate every mouse, nor prevent unvaccinated mice migrating into the test area. Moreover, their vaccine was unable to provide complete immunity to every vaccinated mouse.

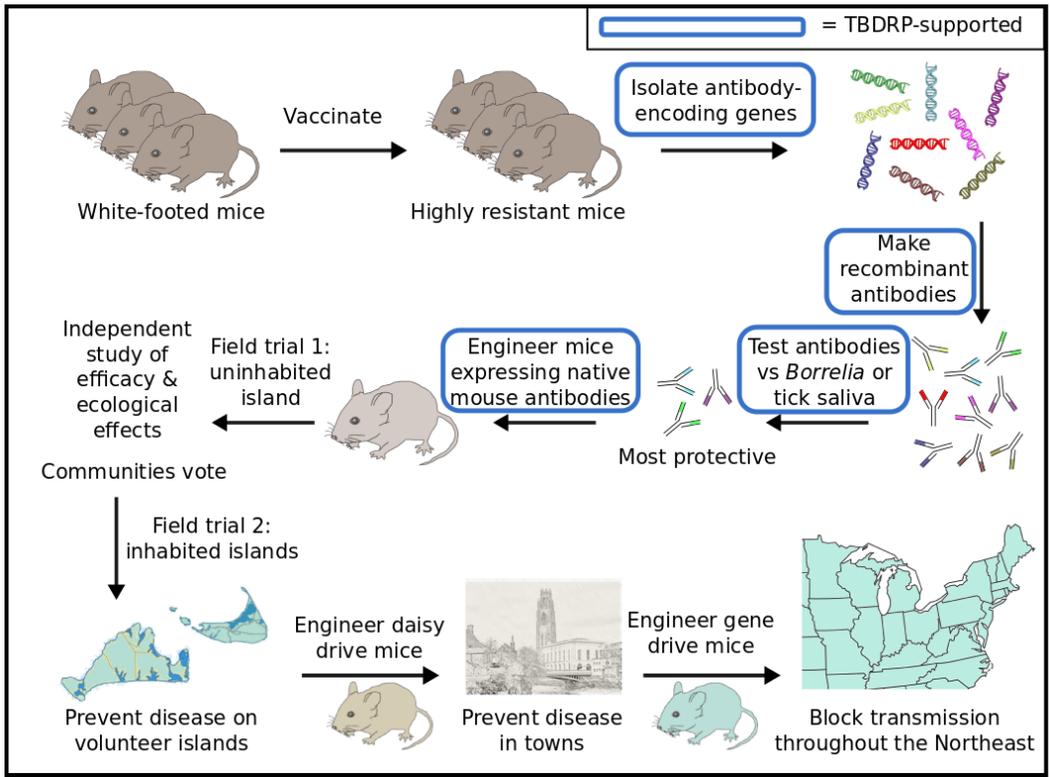
One can only imagine the profound effect of perfectly immunizing every mouse in a contained population against tick-borne pathogens. If, for example, every white-footed mouse on Martha's Vineyard or Nantucket produced antibodies conferring effective immunity from birth, the reservoirs of tick-borne *Borrelia* would likely collapse: fewer infected nymphs in the next generation would infect fewer secondary reservoir mammals, resulting in even fewer infected nymphs in subsequent generations [12-13]. If the mice were also immunized to interrupt tick feeding, as has been demonstrated by Linden Hu and colleagues in the laboratory by vaccinating them against the tick salivary protein subolesin, transmission of other tick-borne diseases would be similarly disrupted [14-15]. Moreover, interrupted feeding would likely reduce the total number of ticks, further reducing infectious bites. The positive feedback cycle resulting from the increase in mice and ticks would go into reverse, returning infection rates and human cases to the levels of many decades ago or even below. Best of all, once enacted in a given area, the preventive effects would last for many decades and synergize with other ecological approaches

## Objectives

The aim of our Idea Award is to genetically alter white-footed mice to be immune to tick-borne disease. We plan to endow mice with naturally occurring mouse antibodies derived from the native mouse population. Studies of gene therapy approaches in laboratory mice have demonstrated that continuous production of highly protective antibodies from muscle cells can block numerous diseases at least as effectively as a standard vaccine [16-20]. By inserting multiple copies of highly protective antibody-encoding genes into the genomes of mouse reproductive cells, we will create mice that are resistant from birth and transmit their immunity to subsequent generations.

Subsequent research beyond the scope of this award would test the ability of these mice to reduce tick infection rates on a small uninhabited island (Fig. 2). Subject to federal and state regulatory approval, community approved safeguards and continuing community support from the nearby islands of Nantucket and Martha's Vineyard, we would release large numbers of engineered resistant mice on a small uninhabited island at the low point in the population cycle in early spring. Because mouse populations normally fluctuate by several-fold over the course of the year and the population could be simultaneously reduced by trapping, ecological effects from mouse introduction are unlikely. Since mice reproduce every 2.5 months, our intervention would quickly transfer Lyme immunity to the entire white-footed mouse population, theoretically reducing the population of infected ticks in the following generation.

Should our approach be deemed safe and effective by an independent data safety monitoring board and broadly supported by the citizens of Nantucket and/or Martha's Vineyard, we would initiate a similar release program on one or both of these islands.



**Fig. 2.** Proposal for long-lasting prevention of tick-borne disease throughout eastern North America. TBDP-funded generation of heritably immune white-footed mice would disrupt the ecological transmission cycle. Immune mice can be 100% mouse due to germline incorporation of protective antibodies from other mice. Immunity to both the OspA antigen of the Lyme pathogen and to subolesin, a tick saliva component, could block transmission of most diseases.

If successful on Nantucket or Martha's Vineyard, the effect could subsequently be extended to mainland communities, including military bases and training sites, using mice with hundreds of copies of the antibody cassette or mice that spread resistance using a “daisy drive”, a local CRISPR-based gene drive system that we are developing. Eventually, our approach could permanently prevent most cases of tick-borne disease throughout eastern North America (Fig. 2).

Our proposal represents a highly innovative and potentially long-lasting ecological solution to a seemingly intractable problem. In contrast to efforts to cull deer, reintroduce a human vaccine, or spray acaricides, our proposal received strong initial support from both public health officials and local citizens at public meetings on Martha’s Vineyard [21] and Nantucket[5]. Our success can be attributed to our novel solution and (perhaps equally novel) commitment to community-driven science. During our presentations this summer, we asked citizens of Martha's Vineyard whether they would prefer to selectively immunize mice

Event	Status
Initial consultation with citizens of affected island communities, NGOs, ecologists, state/federal regulators	Completed
Town hall meetings and Board of Health briefings on Martha's Vineyard and Nantucket	First completed Many more to come
 Community governance agreement established	Nantucket: Yes Martha's Vineyard: Pending
<i>P. leucopus</i> genome sequencing 	Underway
Mice vaccinated against OspA, against subolesin	Underway
Isolation of highly protective antibodies Generation of heritably Lyme-resistant mice	<b>TBDP Idea Award</b>

against Lyme disease (OspA) or against ticks (subolesin) in general, which could prevent many tick-borne diseases. Overwhelmingly, citizens voted to endow mice with every available protection (Option 2 in Fig. 1).

The current status of the project is summarized in Figure 3.

**Fig. 3.** Project history and current status.

## Specific Aims

We have already commissioned Dovetail Genomics to sequence and assemble the *P. leucopus* genome for subsequent CRISPR editing, while our collaborators have begun immunizing white-footed mice against OspA and subolesin. To generate engineered mice with heritable immunity to Lyme and/or ticks, we aim to:

- 1) *Identify protective P. leucopus antibodies against OspA and tick subolesin,*
- 2) *Test efficacy via gene therapy into P. leucopus muscle and infected tick challenges, and*
- 3) *Generate highly resistant mice without foreign DNA that produce antibodies from birth*

## Focus Area

Our proposed research is directly relevant to the FY16 TBDRP Focus area of prevention. Immunizing white-footed mouse populations would interrupt the natural cycle of the pathogens that cause Lyme disease, Anaplasmosis and Babesiosis. The pathogens responsible for all these diseases are maintained in the environment in host reservoirs and in the *Ixodes* ticks. But if the white-footed mouse, the primary reservoir, became immune, many fewer ticks would become infected in the next generation. This would decrease the fraction of infected shrews, chipmunks, and other secondary reservoirs, leading to still fewer infected ticks, and so on until transmission stabilized at a far lower level [23-25]. Far fewer infected ticks would equate to many fewer human infections. Because this natural form of antibody-based immunity would be inherited by subsequent generations of mice with little impact on fitness, it would likely be stable for many decades absent further intervention.

Our geographic areas of focus include the islands of Nantucket and Martha's Vineyard. Our intervention on these islands could prevent hundreds of thousands of individuals from suffering from tick-borne diseases. Other East Coast islands with high rates of tick-borne disease may also be interested in this preventative approach. In the longer term, mainland East Coast communities could similarly decrease the rates of infection by spreading the same antibodies. If we are able to generate healthy mice with hundreds of copies of protective antibody genes, sufficiently few mice would need to be released in any given area that it might be feasible to directly extend the island-based approach to the mainland. Alternatively, mainland communities might employ the daisy drive currently being developed in our lab, which can protect hundreds of mice in future generations for every engineered mouse released. In short, our TBDRP proposal is the first step towards significantly reducing the incidence of tick-borne disease across eastern North America.

## New Investigator

Dr. Kevin Esvelt, an assistant professor at the Massachusetts Institute of Technology, helped develop CRISPR genome editing and invented CRISPR-based gene drive systems capable of altering wild populations. His research focuses on developing novel and eco-friendly methods of solving environmental problems in an open and community-responsive manner. Because tick-borne diseases feature an ecological transmission cycle between reservoirs and vectors, they are uniquely amenable to technological solutions involving the alteration of wild organisms.

Dr. Esvelt's highly interdisciplinary research on ecological engineering demands far more community engagement than is typically encouraged by more conventional departments. He is incredibly fortunate to be located at the MIT Media Lab, where he is actively supported in any and all efforts to meaningfully improve the world. Judging from the positive responses of the communities on Nantucket and Martha's Vineyard to date, his

open, community-guided approach is particularly well-suited to the sometimes-controversial field of Lyme disease.

While his long experience with CRISPR genome editing is uniquely suited to engineering mice, the project also requires a deep technical knowledge of the ecology and molecular biology of tick-borne disease transmission as well as the immunology of white-footed mice. Dr. Esvelt is deeply grateful to have Dr. Sam Telford and Dr. Linden Hu as collaborators in this project.

Dr. Sam Telford, of the Cummings School of Veterinary Medicine at Tufts University, is a leading tick ecologist who has been working on Nantucket and Martha's Vineyard for more than twenty years and played a key role in developing the OspA-based vaccine[26]. Dr. Linden Hu, of the Sackler School of Graduate Biomedical Sciences at Tufts University, developed the subolesin-based vaccine[10]. Both have been key players in the project since the first planning workshop convened by Dr. Esvelt at MIT in December 2015, which was attended by numerous scientists, regulators, environmental NGOs, and island community leaders (Fig. 3).

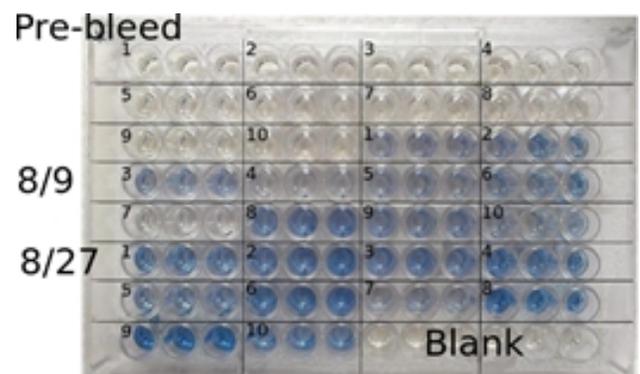
For this project, Dr. Telford will repeatedly vaccinate mice with the *Borrelia burgdorferi* outer surface protein A (OspA) to elicit antibodies capable of neutralizing the bacteria in the tick gut [22,25]. Dr. Hu will vaccinate mice with the tick salivary protein subolesin to generate antibodies that block transmission of numerous tick-borne diseases by interrupting tick feeding[14-15]. They will also advise the design and performance of assays testing the genetic immunity of mice.

While Dr. Esvelt has a great deal of experience with phage-based selections and directed evolution, yeast surface display is superior to phage-display for antibody-based selections. Dr. Dane Wittrup's group at MIT, which invented yeast surface display, will advise and guide experiments aiming to identify genes encoding the best antibody binders to OspA and subolesin.

Finally, we will leverage our own internal expertise with CRISPR to insert antibody-encoding genes into the *P. leucopus* genome. The TBDRP Idea Award would support (1) selections to identify the best antibody binders, (2) tests of antibody gene efficacy by “vectored immunoprophylaxis gene therapy and infected tick challenges in mice, (3) the development of transgenesis in white-footed mice via lentiviral delivery of CRISPR into spermatogonial cells as has been demonstrated for other rodents[27-28], or by standard oocyte injection and embryo implantation in pseudopregnant mice, and (4) the generation of heritably disease-resistant *P. leucopus* that express protective antibodies from birth.

## Research Strategy

Our collaborators have already begun repeatedly vaccinating white-footed mice against OspA and subolesin. Sam Telford has confirmed that his OspA-immunized mice have produced a positive antibody response by ELISA (Fig. 4). Similar tests by Linden Hu using recombinant subolesin will be completed in the next two months. We aim to subsequently 1) isolate protective antibodies that bind OspA or subolesin, 2) test *in vivo* efficacy by transducing mouse muscle cells to secrete candidate antibodies from a muscle-specific promoter and challenging the mice with infected ticks, and 3) insert the antibody-expressing cassettes in the mouse germline through viral transduction or oocyte microinjection.



**Fig. 4.** Preliminary ELISA data from mice vaccinated against OspA by the Telford lab. Five of ten mice displayed a strong immune response.

### ***Aim 1) Identify protective *P. leucopus* antibodies against *OspA* and tick subolesin***

The best current method of identifying antibody binders to a given antigen is yeast surface display, which was first pioneered by K. Dane Wittrup's laboratory in 1997 [29-30]. Single-chain variable fragment (scFv) antibodies comprising linked heavy and light chains are displayed on the surface of the yeast by fusing them to the Aga2p protein. Antigen binding is quantitatively measured and the best binders isolated through exposure to biotinylated soluble antigen, labeling with a streptavidin fluorophore, and fluorescence-activated cell sorting.

Because the community of Martha's Vineyard has voiced a strong preference for mice without any foreign DNA, we will identify a native mouse linker sequence for scFv construction. We will replace the linker sequences of the *Mus musculus*-derived anti-*OspA* LA-2 antibody with linkers derived from the mouse genome. Tests of binding (via yeast surface display FACS) will identify an adequate native mouse linker sequence.

Advised by the Wittrup lab, we will generate scFv (single-chain variable fragment) libraries in electrocompetent *E. coli* by separately amplifying genes encoding heavy and light chains from the spleens of vaccinated mice supplied by Dr. Sam Telford (*OspA*, Fig. 4) and Dr. Linden Hu (subolesin), and combining them via overlap extension PCR with the native linker. Our initial attempts to amplify these genes from control *P. leucopus* spleens supplied by Sam Telford were successful (not shown).

We will begin by ensuring that all yeast cells display equivalent levels of all library members by labeling the c-Myc tag adjacent to the scFv with an anti-c-Myc fluorescent antibody. Briefly, a tenfold excess of yeast cells will be induced to express the scFv, pelleted and washed, and labeled with chicken anti-c-Myc IgY at 1:250 dilution, then pelleted and washed against and fluorescently labeled with Alexa Fluor 488-goat anti-chicken IgG. After verifying negative and positive controls on the flow sorter, the yeast population will be sorted using gates to remove debris and improperly expressing cells. The resulting population will be re-induced and labeled with biotinylated recombinant *OspA* or recombinant subolesin (GenScript), maintaining a ten-fold antigen concentration excess, and streptavidin-phycoerythrin as well as Alexa Fluor, then conservatively sorted for double-positive cells. Enriched cells will be re-labeled and sorted again for 3-5 rounds, always changing secondary reagents to avoid unwanted selection for binding. Clones will be sequenced, the most abundant scFvs individually retransformed, then binding verified and affinities estimated by labeling at different antigen concentrations and repeating flow cytometry.

**Deliverables:** Identification of the top 10 *P. leucopus* antibodies of each type by affinity to the target antigen.

**Troubleshooting:** If we encounter difficulties with yeast surface display, we will attempt phage display (Dr. Esvelt has a great deal of experience with phage-based selections [31]) and high-throughput immunome sequencing [32, 33], which can pull down tight binders and identify highly abundant antibodies in vaccinated animals (B. DeKosky, personal communication).

### ***Aim 2) Test efficacy via gene therapy into *P. leucopus* muscle and infected tick challenges***

Vectored immunoprophylaxis, or vaccination by gene therapy, involves viral transduction of genes encoding highly protective antibodies into mouse muscle cells, which subsequently secrete high levels of the antibody. It has been demonstrated to robustly immunize humanized mice against HIV[16,18], influenza[17], malaria[19], and hepatitis C[20] more effectively than standard vaccination, and can even serve as a contraceptive[34]. We will evaluate the efficacy of isolated antibodies through vectored immunoprophylaxis in *P. leucopus*. For all animal experiments, assays will be performed by researchers blinded to the nature of the

treatment. The resulting effects should be sufficiently dramatic to render statistical testing superfluous; if there is not an order-of-magnitude difference, the experiment has failed.

#### Identifying the most protective antibodies in vivo

AAV vectors expressing the top 10 candidate antibodies against each antigen from CMV promoters will be constructed as in previous studies. Each vector will be injected into the gastrocnemius muscles of 4 *P. leucopus* mice. Another 16 mice will receive an empty AAV vector or buffer only, while 16 more will receive previously developed OspA or subolesin vaccines [14, 22]. Levels of antibody production will be assayed by Western blot using biotinylated antigen.

**Effects on tick survival:** Resistance to subolesin should impair the feeding and survival of larval ticks. After two weeks, mice will be infested with 150 larval ticks, which will be allowed to feed to repletion, then collected and placed in a 95% humidity chamber, allowed to molt, and survivorship measured.

**Effects on tick-to-mouse transmission:** Four weeks post-injection, mice will be challenged by allowing 5 *B. burgdorferi*-infected nymphal ticks to feed on them to repletion. To determine whether produced antibodies can kill spirochetes in the tick gut, ticks will be recovered, homogenized, and assayed by qPCR or cultured in BSK media and examined by microscope to determine whether they are still infected. Mouse infection status will be monitored by cultures of ear punches at 2 and 3 weeks. After 4 weeks, mice will be sacrificed and heart, joint, and spleen tissue similarly assayed to detect *B. burgdorferi*. Any positive culture from any mouse will be considered indicative of infection. As for all animal experiments, all tests will be performed by researchers blinded to the nature of the experiment.

Anti-subolesin antibodies will be rank-ordered by their ability to protect mice from infection and their lethality to larval ticks. Anti-OspA antibodies will be rank-ordered by their ability to protect mice from infection and their lethality to *B. burgdorferi* in nymphal ticks.

**Deliverables:** A list of the top antibodies of each type ranked by efficacy in vectored immunoprophylaxis relative to existing OspA and subolesin vaccines.

**Troubleshooting:** If no antibodies are obviously highly protective (e.g. at least three of the four mice are not infected and/or tick lethality is >90% for subolesin), we will subject the enriched yeast surface display library to *in vitro* mutagenesis and selection for binders with at least 10-fold higher affinity, then repeat the testing.

#### Evaluating the protective efficacy of antibody combinations

Next, we will test the effect of combining the best 5 antibodies of each type when expressed from a muscle-specific promoter identified in Aim 3, below. Four groups of twenty mice will be injected with the top 5 anti-subolesin antibody vectors, the top 5 anti-OspA antibody vectors, both, or an equivalent amount of empty vector. Levels of antibody production will be assayed by Western blot and ELISA.

**Effects on tick survival and mouse-to-tick transmission:** 10 mice from each group will be infected with *B. burgdorferi* by subcutaneous injection. After two weeks, mice will be infested with 150 larval ticks, which will be allowed to feed to repletion, then collected and placed in a 95% humidity chamber, allowed to molt, and survivorship measured.

**Effects on tick-to-mouse transmission:** Four weeks post-injection, mice will be challenged by allowing 5 *B. burgdorferi*-infected nymphal ticks to feed on them to repletion. To determine whether produced antibodies can kill spirochetes in the tick gut, ticks will be recovered, homogenized, cultured in BSK media, and examined by microscope to determine whether they are still infected. Mouse infection status will be monitored by cultures of ear punches at 2 and 3 weeks. After 4 weeks, mice will be sacrificed and heart, joint, and spleen tissue cultured for *B. burgdorferi*. Any positive culture from any mouse will be considered indicative of infection.

**Deliverables:** Efficacy of antibody combinations as determined by vectored immunoprophylaxis in *P. leucopus*.

**Troubleshooting:** If antibody combinations are no more protective than individual antibodies, the difference is probably due to the muscle-specific promoter. This difference should be apparent by Western blot. If necessary, fresh mice will be injected with CMV-promoter and muscle-specific promoter AAVs and expression levels compared. Additional muscle-specific promoters or even constitutive promoters will be tested by creating new AAV vectors as needed until levels are comparable.

### ***Aim 3) Generate highly resistant mice without any foreign DNA that produce antibodies from birth***

#### **Identifying suitable promoters for antibody expression**

Immunizing populations will be easiest if we can insert many copies of the resistance cassette into the genome per engineering attempt. Ideally, we will be able to generate mice with hundreds of copies, only one of which is required for immunity. This would permit many fewer mice to be released in order to alter the wild population, potentially avoiding all use of gene drive on the mainland. However, any such attempt requires a feedback loop governing gene expression so that the initial mice do not express hundreds of times as much antibody.

To investigate potential methods of accomplishing this goal in a more tractable system, we will identify four repeated sequences in the *Mus musculus* genome with varying numbers of copies scattered throughout the genome: 1, 5-10, 20-100, and 500-1000. Working with the MIT transgenesis facility and employing CRISPR-based oocyte injection, we will attempt to deliver a DNA cassette containing one of three candidate muscle-specific promoters. MEF2c, myogenin, and MyoD all display extensive feedback loops with a number of other proteins that should prevent changes in copy number from greatly affecting overall expression[35]. In the AAV, they will drive expression of the proven murine monoclonal anti-OspA antibody LA-2 [36].

As a positive control and test of 2A peptide processing, we will construct two AAV vectors in which the CMV promoter drives LA-2 expression. One of this will express GFP fused to LA-2 by a 2A-equivalent peptide sequence identified in the *P. leucopus* genome [37]. Wild-type negative control mice will not receive treatment. 10 mice in each group will be challenged with 5 *B. burgdorferi*-infected nymphs 4 weeks following injection of the AAV vector and infection status monitored by ear punch at 6 weeks, 7 weeks, and heart/joints/spleen at 8 weeks. Note that mice are likely to have varying copy numbers of the antibody cassette; results will be plotted relative to number of copies.

**Deliverables:** Data on the feasibility of DNA insertion into repeats in mice, the efficacy of heritable antibody production from various promoters, and identification of a *P. leucopus* 2A peptide suitable for expressing multiple antibodies from a single promoter.

**Troubleshooting:** A lack of LA-2 efficacy is likely due to insufficient expression; a comparison of titers between transgenics and CMV positive control mice by Western blot should assist in identifying any problems.

If needed, the experiment will be repeated using constitutive whole-body promoters instead of muscle-specific ones. In the worst-case scenario, we will perform directed of the antibodies by yeast surface display to identify more protective versions.

### Making and testing heritably resistant white-footed mice

We will design a *P. leucopus* insertion strategy by analyzing the genome sequence for repeated sequences with desirable CRISPR insertion sites that are within the desired copy number range as determined in the *Mus musculus* experiments above. We will synthesize integration cassettes encoding the top-performing antibodies from Aim 2 expressed from *P. leucopus* equivalents of the promoters determined by the *Mus musculus* experiments in Aim 3. Each promoter will drive multiple antibodies, ideally ten fused to one another by the effectively self-cleaving 2A peptide identified earlier. We will make two different versions designed to integrate into two different repeated regions on the genome using flanking homology arms. Each integration cassette will be packaged into a lentiviral vector. Two other vectors will encode *S. pyogenes* Cas9 and multiple *P. leucopus* U6 promoters driving guide RNAs that target the two repeated regions.

Lentivirus pairs will be injected into the gonads of five male *P. leucopus* mice. Males will be allowed to recover for 1 week, then mated to four different females. Pups will be genotyped to identify cisgenic insertions and the number of cassettes in each mouse quantified by qPCR. The mice will be mated to maximize predicted insertion counts and the offspring similarly evaluated.

To quantify the level of resistance conferred, we will perform tests measuring mouse-to-tick and tick-to-mouse transmission for wild-type and engineered mice as described above.

**Deliverables:** Transgenic mice containing many copies of antibody-encoding cassettes and quantification of transmission-blocking relative to the number of copies. Mice will similarly be assessed for overall health and fertility as a means of evaluating probable fitness in the wild.

**Troubleshooting:** We may experience problems with transgenesis or with fitting all of our antibodies into a lentiviral vector. If so, we will work with the MIT transgenesis facility to accomplish an alternative method of transgenesis by oocyte injection. This will involve ovarian hyperstimulation and oocyte harvesting, oocyte injection and fertilization, and embryo implantation into pseudopregnant mice, much as is done for *Mus musculus*, *Rattus rattus*, and other mammals.

### **Conclusion**

Our TBDRP New Investigator Idea Award proposal encompasses a key step towards durably breaking the cycle of tick-borne disease transmission across eastern North America. The project is novel in proposing to lastingly immunize an animal reservoir, in deliberately making acquired immunity heritable, and in involving local communities in decision-making from the earliest stages of the project before any experiments began. At the same time, every experimental method required by our proposal has been demonstrated in other organisms, substantially reducing risks. A fully assembled *P. leucopus* genome sequence and established transgenesis methods will also benefit tick-borne disease more generally. We are deeply grateful to our collaborators for their extreme generosity, to the broader community of Lyme disease researchers and patients for their interest in our unorthodox proposal, and to the citizens of Nantucket and Martha's Vineyard for their open-mindedness, enthusiasm, and heartfelt concerns. We look forward to working towards a future without tick-borne disease.

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