Tick proteins in Borrelia transmission and tick feeding: t(r)ick or treat?
Schuijt, T.J.

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Chapter 7

The Noduler-like Protein in Ixodes scapularis is Essential for Borrelia Acquisition and Tick Molting

Tim J. Schuijt, Sukanya Narasimhan, Jeroen Coumou, Kathleen DePonte, Tom van der Poll, Joppe W. Hovius, Sirlei Daffre and Erol Fikrig

Manuscript in preparation
The Lyme diseases agent, *Borrelia burgdorferi*, resides in the gut of *Ixodes scapularis* ticks during its life cycle. We now demonstrate, by subtractive hybridization, that the presence of *Borrelia*, alters the expression of selected genes in the tick gut. One of the upregulated genes was a homolog of Noduler, an immune-induced nodulation facilitating protein found in diverse invertebrates, and henceforth referred to as the *I. scapularis* Noduler-like protein (ISNP). ISNP was preferentially expressed in the gut of both larval and nymphal ticks and quantitative RT-PCR confirmed that ISNP expression was increased in *Borrelia*-infected ticks. RNA interference-mediated silencing of ISNP, as well as active immunization with rISNP impaired successful acquisition of *Borrelia* from the vertebrate host by the tick. *In vitro* experiments showed that recombinant ISNP was able to bind *B. burgdorferi*. These findings reveal new insights into the interaction of *Borrelia* with the tick immune system and suggest that *Borrelia* exploit components of the tick immune system to its advantage.
**INTRODUCTION**

*Ixodes scapularis* transmit several human pathogens, including the Lyme borreliosis agent *Borrelia burgdorferi* [1]. Lyme borreliosis is the most common tick-borne human disease in the northern hemisphere with an annual incidence of over 100 cases per 100,000 people per year in several European countries and endemic areas in the US [2]. There is no commercial vaccine currently available to prevent Lyme borreliosis in humans despite of a great public demand. Both *Borrelia* and tick antigens could be utilized as vaccine candidates [3]. In order to identify proper antigens for vaccination it is mandatory to understand the differential expression of *Borrelia* genes as well as tick genes that play a crucial role in successful spirochete acquisition and transmission from the mammalian host to the tick vector and vice versa. [3]. *B. burgdorferi* is maintained in a mammal-tick cycle and regulates its outer surface proteins differently in response to host conditions [4]. Interestingly, several tick genes have also been shown to be differentially expressed in the presence of *Borrelia* [5,6], and may contribute to *Borrelia* survival.

Infection by pathogens upregulates the expression of genes involved in the innate immune system of ticks and other invertebrates [7,8,9]. Ticks have a well-developed innate immune system enabling them to combat infection, but they also vector several micro-organisms such as *Borrelia*. The innate immune system of ticks consist of cellular and humoral immune responses which function cooperatively [7,10]. The humoral response involves induced production and secretion of antimicrobial peptides, lysozymes, lectins and activation of the prophenoloxidase (PPO) system. Hemocytes play a crucial role in the cellular response to invading pathogens and are present in the hemolymph of ticks, where they are involved in phagocytosis, nodulation and encapsulation [7,10,11]. Very little is known of the various innate immune responses of the tick that confront pathogens such as *Borrelia* and how *Borrelia* circumvents these immune responses [7].

*Borrelia* engages in interactions with the nymphal gut and salivary gland during the process of acquisition and transmission. The molecular understanding of these interactions is only beginning to emerge. We now examine, by subtractive hybridization [12,13], the changes in the transcriptome of the nymphal gut during *Borrelia* transmission. We identified several genes upregulated in the midguts of *Borrelia*-infected *I. scapularis*, including a gene that encodes for a secreted protein homologous to Noduler, a protein shown to be involved in the nodulation response against various microorganisms in the silkmoth *Antheraea mylitta* [14]. We examined the role of *Ixodes scapularis* nodular-like protein (ISNP) in *Borrelia* survival in ticks while entering and colonizing ticks as well as migrating and transmission to the murine host and demonstrate a novel role for ISNP in facilitating *Borrelia* acquisition and colonization.
Subtractive hybridization of pathogen-free versus *Borrelia*-infected *I. scapularis* midguts.

Suppression subtractive hybridization was used to examine *I. scapularis* genes preferentially expressed when *B. burgdorferi* are present in ticks. Only clones that provided high quality sequences were selected and ones that appeared at least 3 times were considered specific and shown in Table 1. Most of the upregulated genes were mitochondrial and genes encoding secreted proteins (Fig.1A).

Clones that encoded for mitochondrial Cytochrome Oxidase subunits I and III appeared at least 5 times, suggesting that these genes were abundantly expressed during the presence of *Borrelia* growth in the tick gut. Cytochrome Oxidase is a key mitochondrial enzyme in the respiratory chain critical for energy balance. Besides mitochondrial genes, other genes with various functions were upregulated in the presence of *Borrelia* (Fig.1B). We found several clones...
encoding for the short chain dehydrogenase family of proteins induced by the presence of *Borrelia* in the tick gut. In addition, several secreted proteins paralogous to secreted salivary proteins including Salp26A were observed to be upregulated. Several genes encoded hypothetical proteins or proteins with unknown functions. Further research efforts would help to clarify their functions. We observed two immune response genes increased during *Borrelia* growth, the Scapularisin and the Reeler-domain containing protein (protein ISCW005667). Scapularisin belongs to the defensin family of antibacterial peptides [18]. The protein ISCW005667 is homologous to an immune-induced Noduler response protein from the hemolymph of silk moth *Antheraea mylitta* [14]. Since little is known on tick immune responses against *Borrelia*, we next examined the physiological significance of the upregulation of ISCW005667 - from here on referred to as *I. scapularis* Noduler-like protein (ISNP) - in *Borrelia* acquisition and transmission.

Figure 2. ISNP is expressed in both salivary glands and midguts of *I. scapularis* nymphs and upregulated in the presence of *Borrelia*. Quantitative PCR performed on RNA isolated from salivary glands (SG) and midguts (MG) of non-infected (A,B) and *Borrelia*-infected (B) nymphs 72 hours post-feeding. Results represent mean ± SEM of values. (C) Coomassie blue staining of recombinant ISNP produced and purified by means of a *Drosophila* Expression System and nickel chromatography. Proteins were separated on a 12% SDS polyacrylamide gel. (C) Nymphal midgut extract was probed with mouse rISNP-antiserum.
Table 1. List of genes that are upregulated in midguts of *Borrelia*-infected *Ixodes scapularis* nymphs

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Protein Encoded</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISCW022329</td>
<td>Transmembrane protein (Steroidogenic Acute Regulatory STAR)</td>
<td>Possibly involved in lipid transfer</td>
</tr>
<tr>
<td>ISCW09548</td>
<td>Hypothetical protein</td>
<td>Unknown</td>
</tr>
<tr>
<td>ISCW019584</td>
<td>Phospholipid-hydroperoxide Glutathione peroxidase</td>
<td>Reduction of lipid and hydroperoxides, possible ROS quenching</td>
</tr>
<tr>
<td>ISCW016022</td>
<td>Cytochrome C Oxidase subunit I</td>
<td>Heme-Cu oxidase I superfamily, key enzyme in proton pump coupled to ATP generation</td>
</tr>
<tr>
<td>ISCW07844</td>
<td>Short chain alcohol dehydrogenase</td>
<td>Detoxification of alcohol</td>
</tr>
<tr>
<td>ISCW00814</td>
<td>Salp26A, secreted protein</td>
<td>Unknown</td>
</tr>
<tr>
<td>ISCW020757</td>
<td>Cytochrome Oxidase III</td>
<td>Key enzyme in proton pump coupled to ATP generation</td>
</tr>
<tr>
<td>ISCW004817</td>
<td>Similar to <em>Apis mellifera</em> Exocyst complex subunit- Sec15</td>
<td>Vesicular traffic of proteins from the golgi apparatus</td>
</tr>
<tr>
<td>ISCW003205</td>
<td>Hypothetical protein</td>
<td>Unknown</td>
</tr>
<tr>
<td>ISCW005926</td>
<td>Scapularinin preprotein</td>
<td>Antibacterial peptide</td>
</tr>
<tr>
<td>ISCW011165</td>
<td>Probable short chain dehydrogenase</td>
<td>Acyl carrier protein reductase, conversion of alcohols to aldehydes and ketones</td>
</tr>
<tr>
<td>ISCW013959</td>
<td>Putative secreted salivary protein with basic tail</td>
<td>Unknown</td>
</tr>
<tr>
<td>ISCW012492</td>
<td>Hypothetical protein</td>
<td>Fibronectin 3 domain containing</td>
</tr>
<tr>
<td>ISCW014240</td>
<td>5’Nucleotidase/Adenosine 5’-phosphosulfate ribohydrolase</td>
<td>Putative anticancer peptide</td>
</tr>
<tr>
<td>ISCW019584</td>
<td>Glutathione peroxidase similar to <em>L. ricinus</em></td>
<td>Reduction of hydroperoxides, ROS quenching</td>
</tr>
<tr>
<td>ISCW024071</td>
<td>Secreted salivary gland protein</td>
<td>Unknown</td>
</tr>
<tr>
<td>ISCW018909</td>
<td>Hypothetical protein</td>
<td>Unknown</td>
</tr>
<tr>
<td>ISCW014515</td>
<td>35% identity with secreted salivary protein</td>
<td>Unknown</td>
</tr>
<tr>
<td>ISCW015983</td>
<td>Legumin, Aspartyl peptidase also called hemoglobinase</td>
<td>Peptidase</td>
</tr>
<tr>
<td>ISCW012432</td>
<td>Short chain alcohol dehydrogenase</td>
<td>Conversion of alcohols to ketones and aldehydes</td>
</tr>
<tr>
<td>ISCW003426</td>
<td>Glutathione S-Transferase (Thioredoxin superfamily)</td>
<td>Detoxification of peroxides and products of oxidative stress</td>
</tr>
<tr>
<td>ISCW005667*</td>
<td>Secreted protein</td>
<td>Contains the Reeler domain and the Cohesin domains, and Thrombospondin-1 domain</td>
</tr>
<tr>
<td>ISCW018909</td>
<td>Putative secreted protein</td>
<td>Unknown</td>
</tr>
<tr>
<td>ISCW03983</td>
<td>Oxyesterase binding protein</td>
<td>Lipid transport and signaling</td>
</tr>
<tr>
<td>ISCW007199</td>
<td>Villa (contains gelsoxin domains that sever actin filaments)</td>
<td>Actin binding to regulate cell-cell signaling</td>
</tr>
<tr>
<td>ISCW013337</td>
<td>Conserved hypothetical protein</td>
<td>Unknown</td>
</tr>
<tr>
<td>ISCW007338</td>
<td>Erlik-2, putative protein</td>
<td>Lipid raft associated protein, modulates signal transduction by cytoskeleton rearrangement</td>
</tr>
<tr>
<td>ISCW017045</td>
<td>Conserved hypothetical protein (Beta-Catenin-like)</td>
<td>Cell-cell communication and regulation of epithelial layers</td>
</tr>
</tbody>
</table>

* encodes for a secreted protein homologous to “Noduler”, a protein shown to be involved in the nodulation response against various microorganisms in the silkworm *Antheraea mylitta* [14].

Characterization of *I. scapularis* Noduler-like protein. *ISNP* was expressed both in salivary glands and midguts of pathogen-free *I. scapularis* nymphs during feeding, although a higher expression of *ISNP* was seen in midguts after the nymphs had fed to repletion (Fig.2A). In line with the subtractive hybridization data presented in Fig.1 and Table 1, increased expression of *ISNP* was found in midguts of feeding *I. scapularis* nymphs infected with *Borrelia* compared to pathogen-free nymphs, as detected by qRT-PCR (Fig.2B). *ISNP* was expressed in a *Drosophila*
ISNP is essential for Borrelia acquisition and tick molting expression system, produced as recombinant protein and purified using a Ni-NTA Superflow column (Fig.2C). When *I. scapularis* midgut extract (MGE) was probed with mouse rISNP-antiserum, a ~15kDa band appeared on the blot (Fig.2D), demonstrating native ISNP is recognized by IgG antibodies present in the rISNP-antiserum. *In silico* analysis of ISPN revealed homology with Noduler (GenBank no. ABG72705), a protein involved in immunity in *Antheraea mylitta* [14] (Fig.3A). Other homologues of ISPN and Noduler were found in *Aedes aegypti* (GenBank no. EAT35239), *Anopheles gambiae* (GenBank no. XP_315224), *Culex quinquefasciatus* (GenBank no. XP_001849402) and *Pediculus humanus* (GenBank XP_002426361) with 46-52% similarity (Fig.3A), demonstrating that ISNP is conserved in invertebrates. Additionally, the predicted protein structure of ISPN using the structure prediction tool PHYRE [19] was identical to Noduler and the other homologues (data not shown). Protein ISNP, as well as the homologues, showed a predicted signal cleavage site using SignalP (Fig.3A), indicating ISPN is most likely a secreted protein. The major part of the mature protein consists of a reeler domain (Fig.3B), with ~140 amino acid residues long domain located at the N-terminus of a variety of secreted and surface proteins, including Noduler and the other homologues [14]. Noduler has been shown to be upregulated upon bacterial infection [20], and was shown to bind several micro-organisms in order to control and clear infection [14]. In line with these observations recombinant ISNP bound to the surface of *Borrelia* (Fig.3C).

**ISNP is required for successful *Borrelia* acquisition by ticks and is pivotal for larvae to molt to the nymphal stage.** Having established that rISNP binds the outer surface of *Borrelia* spirochetes and is homologues to Noduler - a protein crucially important in the cellular response against various pathogens in insects - we studied the role of ISNP in *Borrelia* survival in the tick. We hypothesized that by RNAi silencing ISNP the cellular response against *Borrelia* would be abrogated resulting, in increased spirochetal loads in ticks after acquiring *Borrelia* from *Borrelia*-infected mice. A body injection of dsRNA for ISNP in pathogen-free *I. scapularis* nymphs resulted in a significant decrease of ISNP expression in the midguts (Fig.4A) but not in the salivary glands (data not shown) after 72 hours of feeding on *Borrelia*-infected mice. ISNP-silenced nymphs were significantly lighter than mock injected nymphs after feeding for 72 hours of feeding (Fig.4C; $1.1 \pm 0.2$ mg and $1.8 \pm 0.2$ mg, respectively, p=0.0057). ISNP -silenced nymphs were not able to acquire *Borrelia* from *Borrelia*-infected mice (Fig.4D). We next studied *Borrelia* acquisition from *Borrelia*-infected rISNP-immunized mice by larval ticks. *Borrelia* loads in larvae were markedly reduced after feeding on rISNP-immune mice infected with *Borrelia burgdorferi* (Fig.5A).
Figure 3. ISNP and its homologues in several invertebrates. (A) Multiple sequence alignment of *I. scapularis* ISNP with the amino acid sequences of several homologues identified in *Antheraea mylitta* ("Noduler", GenBank no. ABG72705), *Aedes aegypti* (GenBank no. EAT35239), *Anopheles gambiae* (GenBank no. XP_315224), *Culex quinquefasciatus* (GenBank no. XP_001849402) and *Pediculis humanus* (GenBank XP_002426361). Amino acids in white on a black background are identical; residues in white on a grey background are similar. Region inside the grey box shows the predicted signal peptide sequence. Similarities and identities to ISNP are presented at the end of the amino acid sequences. (B) Schematic overview of ISNP showing that the mature protein for the greater part consists of a reeler domain. Bar scale represents number of amino acids. (C) *B. burgdorferi* N40 was incubated with 0.1 ng/ul recombinant Salp15 (positive control), rTSLPI (negative control) or rISNP. *Borrelia* was pelleted and the pellet and supernatant were separated. Unbound recombinant proteins were washed away and the pellet was resuspended in the same volume as the supernatant. Equal volume of supernatant (sup) and pellet (*Borrelia*) was used to run on a SDS gel and the presence of recombinant protein was detected using an HRP conjugated anti-V5 antibody.

Interestingly, larvae that had their blood meal on rISNP-immune, *Borrelia*-infected mice were less able to molt to the nymphal stage (Fig.5B). No difference was found in *Borrelia* transmission – from the tick to the host - when *Borrelia* infected nymphs fed on rISNP-immune mice compared to OVA-immunized mice (data not shown), indicating that the presence of ISNP-antibodies specifically affects *Borrelia* migration from mice to ticks, but not from ticks to mice.
We and others have shown that selected tick salivary genes are differentially expressed when *Ixodes* ticks acquire and transmit *Borrelia* spirochetes during feeding and that these differentially expressed genes play a pivotal role in the life cycle of *Borrelia* [5,6,17,21]. The role of tick midgut genes in the context of *Borrelia* growth and transmission is less understood [7]. We utilized subtractive hybridization approach to define genes preferentially expressed in the midguts of *Borrelia*-infected *I. scapularis* nymphs.

Several immune-related tick genes were upregulated in *Borrelia*-infected midguts indicating *Ixodes* ticks sense the presence of *Borrelia* and respond by activation of their immune system. Nevertheless, *Borrelia* survives the *Ixodes* immune response and is able to migrate successfully from the tick midgut, via the hemolymph to the salivary glands in order to be introduced into the host's skin. In other tick species such as *Dermacentor variabilis*, *Borrelia* cannot survive and is cleared rapidly by the immune system of the tick. One hour post hemocoelic inoculation of *Borrelia* spirochetes, a ~3-fold increase of hemocytes was detected in hemolymph of both *Dermacentor* and *Ixodes* ticks [7,22] which further demonstrates the *Ixodes* immune system recognizes *Borrelia*.

We showed that ISNP was upregulated in *I. scapularis* midguts in the presence of *Borrelia*, which encodes for a protein that is homologues to Noduler [14]. Noduler has shown to be required in the nodulation response against various pathogens in the silkmoth *Antheraea mylitta* [14]. Nodulation is an important defense mechanism against microbial infections in insects [23].
Figure 5. Impaired larval molting and decreased *Borrelia* loads in larvae fed on ISNP-immune *Borrelia*-infected mice (A) *Borrelia flaB* levels in pooled larvae after feeding on OVA- (control) or rISNP-immune mice (B) A second group of larvae were allowed to molt to the nymphal stage and the ability of molting was determined. Each dot represents the amount of molted larvae after feeding on a ISNP-immune mouse. Horizontal bars represent the mean ± SEM. Asterisks indicate a statistically significant difference.

Hemocyte nodule formation starts with the formation of hemocyte microaggregates and the entrapment of pathogens by these cells. Additional hemocytes aggregate and nodules are formed facilitating rapid clearance of pathogens [11]. Interestingly, ISNP and Noduler are homologues to proteins expressed in several blood-feeding insects, suggesting an important role of Noduler-like proteins in the immune response of a wide range of invertebrates. Nodulation has been described in ticks as well, demonstrating to play a role in the clearance of *Escherichia coli* within *Dermacentor variabilis* ticks [24]. Consistent with our data, Noduler was upregulated in response to a wide range of bacteria and yeasts [14]. By binding to both the outer membrane components of micro-organisms as well as hemocytes Noduler was shown to be critical in the formation of nodules and pathogen clearance [23]. In line with these observations, recombinant rISNP bound to the outer surface of *Borrelia*.

Since ISNP-expression in midguts was upregulated in *Borrelia*-infected ticks we determined if RNAi silencing of ISNP would result in increased *Borrelia* loads in tick midguts after having a bloodmeal on *Borrelia*-infected mice. Unexpectedly, RNAi silencing of ISNP in midguts of *Borrelia*-free nymphs completely blocked *Borrelia* acquisition by these ticks. In the natural transmission cycle of *Borrelia*, larval ticks acquire the spirochetes and nymphal ticks transmit *Borrelia* to the mammalian host during their subsequent blood meal [25], thus we further studied *Borrelia* acquisition and the role of ISNP in larval ticks. Consistent with the silencing studies in nymphs, *Borrelia* acquisition from mice by larval ticks was impaired when mice were immunized with rISNP. These findings suggest that *Borrelia* is not eradicated by the nodulation response of *Ixodes* ticks, but conversely take advantage of this response to successfully...
ISNP is essential for *Borrelia* acquisition and tick molting. Further research is necessary to investigate the exact function of ISNP and mechanism of *Borrelia* acquisition.

The fact that several tick immune-related genes are upregulated in the presence of *Borrelia*, suggest that *Ixodes* ticks sense *Borrelia* invasion but are not able to clear infection. It is possible that ISNP upregulation in the tick midgut affects the gut microflora in a way that can provide favorable conditions for *Borrelia*. Not only *Borrelia* acquisition was impaired when larvae fed on *Borrelia*-infected rISNP-immunized mice but also fewer ticks were able to molt to the nymphal stage when they had a blood meal on those mice. It is possible that the immune response against several tick pathogens is impaired by neutralizing ISNP in a way that tick pathogens are able to survive and grow in the midguts disadvantageous for both *Borrelia* survival and successful tick molting. Currently we investigate the role of ISNP on the *Ixodes* microflora and determine how RNAi silencing of ISNP changes the tick microbiome. Interestingly, proteins that play an important role in either acquisition of *Borrelia* by ticks or in tick feeding/molting, could potentially be used for vaccination of wildlife in order to block the life cycle of *Borrelia*, a strategy similar to that previously described by Tsao *et al.* [26].

*Borrelia* is killed rapidly after injection into *D. variabilis* by defensins that are released by hemocytes in the hemolymph [27]. Interestingly, a defensin-like peptide was identified in *I. scapularis* as well which is now known as scapularisin. Scapularisin was shown to be 78.9 percent similar to the defensin produced by *D. variabilis* [18]. We found that *I. scapularis* scapularisin mRNA was upregulated in *Borrelia*-infected midguts. However, the peptide could not be identified after *Borrelia* challenge [18] and *Borrelia* was not eradicated after inoculation into the *I. scapularis* hemocoel cavity or with incubation with *I. scapularis* hemolymph, in contrast in which was observed in *D. variabilis* [27]. It is possible that *I. scapularis* scapularisin mRNA is not translated or that the precursor protein is not processed to a mature protein. A similar phenomenon was demonstrated in mosquitoes and it has been suggested that defensin could have different functions in the immune response against pathogens [28].

Pathogen transmission and acquisition is impaired in tick-resistant animals, which makes vaccination against tick proteins an attractive approach to target tick-borne pathogens [3]. Previously we have shown that the tick salivary protein Salp25D facilitates the success of *Borrelia* transmission from host to ticks by quenching oxygen radicals produced by neutrophils at the tick bite site [15]. In this study we report a protein that is differentially expressed in *Borrelia*-infected ticks and plays a pivotal role in both tick feeding and *Borrelia* acquisition, but not transmission. ISNP would be an interesting vaccine candidate since this protein not only blocks *Borrelia* acquisition but also tick development.
Ticks and animals. *I. scapularis* nymphs and larvae were obtained from a tick colony at the Connecticut Agricultural Experiment Station in New Haven CT, USA. Ticks were maintained at 23°C and 85% relative humidity under a 14 hour light, 10 hour dark photoperiod. *Borrelia* infected nymphs were generated by placing larvae on *B. burgdorferi* infected C3H mice and fed larvae were molted to nymphs. For active immunization studies, 4-6 weeks of age, female C3H/HeJ mice (Jackson Laboratory) were used. The protocol for the use of mice was reviewed and approved by the Yale Animal Care and Use Committee.

Subtractive Hybridization. At least 15-20 *Borrelia*-infected unfed *I. scapularis* nymphs were individually tested by DNA-PCR for *Borrelia* infection using the *flab* primers that amplify the *flagellin* gene essentially as described earlier [15]. A batch of nymphs with an infection rate of 90-95% was chosen as the tester group for subtractive hybridization. An age-matched (nymphs molted around the same time as the *Borrelia*-infected nymphs) batch of clean nymphs was chosen as the driver group for subtractive hybridization. At least 30-50 nymphs from each group were allowed to feed on pathogen-free C3H/HeN mice for 72 hours, nymphs collected, midguts dissected and processed for total RNA extraction as described earlier [16]. cDNA was generated from 2 μg of total RNA from clean and *Borrelia*-infected midguts using the Super SMART™ PCR cDNA synthesis kit according to the manufacturer’s instructions (Clontech, CA). The cDNA was then directly utilized in the forward subtraction procedure using the PCR-Select cDNA Subtraction kit manual (Clontech, CA). This method utilizes a patented technique that overcomes the traditional subtraction methods and allows rapid enrichment of unique and rare message RNAs. cDNA prepared from the *Borrelia*-infected guts served as the “Tester” and cDNA prepared from the clean guts served as the “Driver” in the subtraction procedure to enrich for cDNAs preferentially upregulated in the *Borrelia*-infected tick gut. The cDNAs were then cloned into a T/A cloning vector and plated on LB-Ampicillin plates. At least 500 single colonies were picked and dot-blot nitrocellulose membrane arrays of the clones generated and screened using labeled probes of the Tester (*Borrelia*-infected gut cDNA) and Driver (clean gut cDNA) cDNAs as described in the PCR-Select Differential Screening kit (Clontech, CA). About 100 clones that hybridized preferentially with the Tester and not the Driver probes were considered differentially expressed in the *Borrelia*-infected guts. Positive clones were then picked, plasmid purified and insert DNA sequenced at the W.M Keck Sequencing Facility at Yale University using the nested PCR primers flanking the inserts. In silico analysis of the DNA sequences was performed using the Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/blast) to search for homology with genes described in the public database.

Tick RNA isolation and quantitative RT-PCR. Ticks were allowed to feed for to repletion (between 80 and 96 h after initiation of tick feeding, ticks were monitored twice a day and recovered after detachment from the hosts) on experimental and control animals. Nymphs were dissected and salivary glands and midguts were pooled (3 ticks), homogenized and RNA was extracted using the RNeasy minikit (Qiagen, CA). The same procedure was performed with unfed ticks. cDNA was synthesized using the iScript RT-PCR kit (Biorad, CA) and analyzed by quantitative PCR for the expression of tick actin and *ISNP*, using the iQ Syber Green Supermix (Biorad, CA) on a MJ cycler (MJ Research, CA) and designed primers were as follows: sense *ISNP* 5'–TCACGGATACATTGCCAAGA–3', antisense *ISNP* 5'–ACCGTGACCTTGAAGTGGAC–3'. sense *tick actin* 5'–GGCGACGTAGCAG–3' and antisense *tick actin* 5’–GGTATCGTGCTCGACTC–3’. 

RNAi silencing of *ISNP* in *Borrelia*-infected *I. scapularis* nymphs. Primers were designed by addition of a T7 promoter site (TAATACGACTCACTATAGGGAGA) at the 5’ end of the forward (GCTACCGTCTGCTCCAAAAC) and reverse (TTCTTTGAGGGTGCTGGTAT) primers. dsRNA complementary to the *ISNP* gene was synthesized by using the MEGAscript RNAi kit (Ambion). The dsRNA was purified and quantified spectrophotically. dsRNA (0.5 μl)
was injected into the body of *Borrelia* infected nymphs using 10 μl microdispensers (Drummond Scientific, Broomall, PA) drawn to fine-point needles by using a micropipette puller (Sutter Instruments, Novato). The needles were loaded onto a micromanipulator (Narishige, Tokyo) connected to a Nanojet microinjector (Drummond Scientific). At least 30 nymphs were used in each group. The ticks were allowed to rest for 3 hours before placement on mice. Ticks were allowed to feed for 72 hours, were weighed and dissected for mRNA isolation and quantitative RT-PCR as described above.

**Production of recombinant protein ISNP.** Cloning and expression of *ISNP* was performed as described before [16]. Briefly, *ISNP*-cDNA were inserted into the pMT/Bip/V5-HisA plasmid (Invitrogen, CA), and validated by sequencing. *Drosophila melanogaster* S2 cells were transfected with the plasmids containing *ISNP* and stable transfectants were generated. Protein expression was induced with copper sulfate as described by the manufacturer (Invitrogen, CA). The supernatant was filtered using a 0.22-μm filter (Millipore, MA) and rISNP were purified from the supernatant using Ni-NTA Superflow column chromatography (Qiagen, CA) and eluted with 250 mM imidazole. The eluted fractions were sterilized using a 0.22-μm filter, concentrated with a 5-kDa concentrator (Sigma-Aldrich, MO) and dialyzed against PBS. The purity of rISNP was checked by Coomassie blue staining after electrophoreses on SDS 12% polyacrylamide gel and the concentration was determined by BCA protein assay kit (Thermo Fisher Scientific inc., IL).

**Borrelia pull-down assay.** The *Borrelia* pull-down assay was performed as described before [17]. Briefly, *B. burgdorferi* N40 (2.5 X 10^7) was incubated with 0.1 ng/μl recombinant Salp15, rTSLPI or rISNP in PBS/0.1% BSA for 1 hour at room temperature. After centrifugation at 6000 g for 15 min at 4ºC the pellet and supernatant were separated. The pellet was washed twice in PBS/0.1% BSA and was resuspended in the same volume as the supernatant. Equal volumes of supernatant and pellet was submitted to SDS gel and transferred to nitrocellulose membranes. The membranes were blocked with PBS containing 5% milk powder and the immunoblots were probed with an HRP conjugated anti-V5 antibody. Immunoreactive bands were visualized using horseradish peroxidase conjugated goat anti-rabbit secondary antibodies (Sigma-Aldrich, MO) and the enhanced chemiluminescence Western Blotting Detection System (GE Healthcare, NJ).

**Active immunization with recombinant ISNP.** C3H/HeN mice were immunized subcutaneously with 3 doses containing 10 μg of rISNP emulsified with Complete Freund’s Adjuvant (first dose) and two subsequent booster injections emulsified in Incomplete Freund’s Adjuvant at 3-week intervals. Control mice were inoculated with adjuvant and OVA (10 μg). To address *Borrelia* acquisition, at least 30 pathogen free *I. scapularis* larvae were placed on each *B. burgdorferi* (N40)-infected rISNP-immune mouse and allowed to feed to repletion. At least three mice were used in each experiment. Larvae were analyzed in pools of 5. Alternatively, 6 pathogen free *I. scapularis* nymphs were placed on *B. burgdorferi*-infected rISNP-immune mouse and allowed to feed for 72 h and nymph weights were recorded. Ticks were gently removed, and midguts and salivary glands were dissected and processed in pools of three salivary glands and two midguts for quantitative RT-PCR analysis as described above. To assess *Borrelia* transmission to mice a group of 5 mice were immunized with rISNP and 6 *B. burgdorferi*-infected nymphs were placed on each mouse, were allowed to feed for 72 hr. Tick weights were measured and salivary glands and midguts were dissected as described above. After 5, 7, 11 and 18 days post engorgement skin samples from each mouse were collected and assessed for spirochete burden by RT-PCR analysis. Equal amounts (2 μg) of nymphal midgut extract (MGE) were electrophoresed on a SDS 12% polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked with PBS containing 5% milk powder and the immunoblots were probed with a 1:250 dilution of mouse ISNP antiserum and non-immune mouse antiserum. Immunoreactive bands were visualized using horseradish peroxidase conjugated goat anti-rabbit secondary antibodies (Sigma-Aldrich, MO) and the enhanced chemiluminescence Western Blotting Detection System (GE Healthcare, NJ).
Statistical analysis. The significance of the difference between the mean values of the samples was analyzed using a two-tailed student t test with Prism 5.0 software (GraphPad Software, USA) and the p value is indicated by asterisks in the figures.

REFERENCES

ISNP is essential for Borrelia acquisition and tick molting.


