Scavenger Receptor Mediates Systemic RNA Interference in Ticks

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Abstract

RNA interference is an efficient method to silence gene and protein expressions. Here, the class B scavenger receptor CD36 (SRB) mediated the uptake of exogenous dsRNAs in the induction of the RNAi responses in ticks. Unfed female Haemaphysalis longicornis ticks were injected with a single or a combination of H. longicornis SRB (HlSRB) dsRNA, vitellogenin-1 (HlVg-1) dsRNA, and vitellogenin receptor (HlVgR) dsRNA. We found that specific and systemic silencing of the HlSRB, HlVg-1, and HlVgR genes was achieved in ticks injected with a single dsRNA of HlSRB, HlVg-1, and HlVgR. In ticks injected first with HlVg-1 or HlVgR dsRNA followed 96 hours later with HlSRB dsRNA (HlVg-1/HlSRB or HlVgR/HlSRB), gene silencing of HlSRB was achieved in addition to first knockdown in HlVg-1 or HlVgR, and prominent phenotypic changes were observed in engorgement, mortality, and hatchability, indicating that a systemic and specific double knockdown of target genes had been simultaneously attained in these ticks. However, in ticks injected with HlSRB dsRNA followed 96 hours later with HlVg-1 or HlVgR dsRNAs, silencing of HlSRB was achieved, but no subsequent knockdown in HlVgR or HlVg-1 was observed. The Westernblot and immunohistochemical examinations revealed that the endogenous HlSRB protein was fully abolished in midguts of ticks injected with HlSRB/HlVg-1 dsRNAs but HlVg-1 was normally expressed in midguts, suggesting that HlVg-1 dsRNA-mediated RNAi was fully inhibited by the first knockdown of HlSRB. Similarly, the abolished localization of HlSRB protein was recognized in ovaries of ticks injected with HlSRB/HlVgR, while normal localization of HlVgR was observed in ovaries, suggesting that the failure to knock-down HlVgR could be attributed to the first knockdown of HlSRB. In summary, we demonstrated for the first time that SRB may not only mediate the effective knock-down of gene expression by RNAi but also play essential roles for systemic RNAi of ticks.

Introduction

Ticks are obligate hematophagous ectoparasites of wild and domestic animals and humans. They are considered to be second to mosquitoes as vectors of human diseases and are the most important arthropods transmitting pathogens to domestic animals [1]. Double-stranded RNA (dsRNA)-mediated gene silencing, commonly referred to as RNA interference (RNAi), has been extensively used for the analysis of gene functions in ticks [2]. Long dsRNAs have been successfully and regularly applied in Haemaphysalis longicornis [3] and other tick species (e.g., Amblyomma, Isodes, Rhipicephalus, and Dermacentor spp.) for targeted gene knockdown in various stages of tick life, with evidence of systemic RNAi spread into subsequent stages [2,4]. Four different methods have been used to deliver dsRNA for RNAi in ticks to date: injection, soaking, feeding, and virus production of dsRNA [2]. We have confirmed that RNAi can be a powerful tool for gene silencing of the hard tick, H. longicornis, by the injection [3,5–8] and soaking methods [9].

Direct injection of the dsRNA into target tissues or developmental stages is the most common method of delivering dsRNA to arthropods, such as insects and ticks [2,10]. Injection of the exogenous dsRNAs into the insect’s hemocoel can provide transient knockdown of the target endogenous genes, since the dsRNA in the hemolymph can circulate systemically through the open circulatory system, in which dsRNA is taken up by a cell from the environment [11]. It is known that there are at least two pathways for exogenous dsRNA uptake in insects [12]. One is based on the transmembrane SID-1 channel protein, as well known from the Caenorhabditis elegans nematode. The second possible mechanism is based on the endocytosis-mediated pathway because it shares several components of its machinery with the dsRNA uptake mechanism. Herein, vacuolar H\textsuperscript{+}ATPase is considered to play an important role [13]. However, the participation of scavenger receptors (SRs) already known to play a key role in microbe phagocytosis as “pattern recognition receptors” [14] is not well-established in dsRNA uptake.
SRs are known to potentially act as receptors for dsRNA molecules in an endocytosis-mediated uptake mechanism in the Tribolium castaneum beetle [13] and Drosophila melanogaster by [13]. However, the involvement of SRs in dsRNA uptake and processing in the gene silencing of arthropods, including ticks, are not understood.

In a previous study, the gene encoding putative class B scavenger receptor (designated as HISRBB) was identified and characterized from H. longicornis [16]. The HISRBB had overall 30% identity to both mammalian and insect SRB membrane proteins. The mRNA transcripts of HISRBB were expressed in multiple organs of adult females but with varying levels in the different developmental stages of ticks. The recombinant HISRBB was expressed in Escherichia coli as the His-tagged protein, and anti-mouse recombinant HISRBB serum elucidated the localization of the endogenous protein in the midgut, salivary gland, ovary, fat body, and hemocytes of partially fed H. longicornis females. Gene silencing of HISRBB in female ticks resulted in a significant reduction of engorged body weights [16].

In this study, we elucidated the crucial role of HISRBB induction of knock-down of other endogenous genes via microinjections of a different combination of dsRNAs into the hemocoel of female ticks. RNAi has been proposed to have application possibilities for the autocidal control of tick populations [17] and the characterization of tick-borne pathogens [18,4]. Therefore, a better understanding of the dsRNA uptake mechanism in tick RNAi will provide a comprehensive contribution to studies linked with the development of control measures for ticks and tick-borne diseases.

Materials and Methods

Ticks and animals

The parthenogenetic Okayama strain of the hard tick H. longicornis has been maintained by feeding on Japanese white rabbits (Kyudo, Kumamoto, Japan) in our laboratory [19]. Rabbit care was approved by the Animal Care and Use Committee of Kagoshima University (Approval no. A08010).

Construction of dsRNA and microinjection of dsRNA into adult ticks

The dsRNA construction of H. longicornis HISRBB [16], H. longicornis HVgR [6], and H. longicornis HVg-1 [7] and firefly luciferase (luc) as a control [16] were performed as described previously. The dsRNAs were injected into the hemocoel of unfed female ticks as described by Aung et al. [16]. The HISRBB-, HVgR-, HVg-1-, and luc dsRNA-injected ticks were allowed to rest at 25°C and 90% humidity regulated in an incubator for 96 hours to complete knock-down of these genes [16,7], and the mortality rate was then checked every 12 hours. Ninety-six hours after the first injection, three ticks were collected from the incubator in order to confirm gene-specific silencing by RT-PCR [16,7]. The remaining dsRNA-treated ticks were subjected to a second injection of dsRNAs.

Twelve tick groups injected with a single dsRNA or a combination of dsRNAs are as shown in Table 1. Each tick received a total of 0.5 µl dsRNA with a different concentration (for single dsRNA-injected groups, 1 µg/tick; for a combination of dsRNA(s)-injected groups, 1 µg/gene for a dose equal to the injected dsRNA at 2 µg/tick). The ticks injected with these dsRNAs were infested on the ear of rabbits 12 hours after the first or the second dsRNA injection. Four days after infestation, a total of 16 attached ticks were removed and collected from rabbits for the subsequent experiments including four ticks for RNA extraction, four ticks for protein lysate preparation, and eight ticks for tissue collection. The remaining ticks were allowed to feed until engorgement. To assess the effects of RNAi in ticks after the first and the second injections, we measured the number of ticks attached on a rabbit 2 days after attachment, the engorged body weight of ticks 5–6 days after attachment, the mortality rates, fecundities, and oocyte development of engorged ticks 20 days after engorgement, and the hatching rate to larvae 60 days after the first dsRNA injections.

Collection of different tissues of dsRNA-injected ticks

Partially engorged female ticks 4 days post-infestation were removed from rabbits and dissected out for the subsequent tissue collection [19]. Midguts were collected from ticks injected with a single HISRBB or HVg-1 dsRNA and a combination of HISRBB/HVG-1, HVgR/HVG-1/HISRBB, or HVgR/HVG-1 dsRNAs, ovaries were collected from ticks injected with a single HISRBB or HVgR dsRNA and a combination of HISRBB/HVG-1, HVgR/HVG-1/HISRBB, or HVgR/HVG-1 dsRNAs, and salivary glands and the fat bodies were collected from ticks injected with a single HISRBB or luc dsRNA and a combination of HISRBB/luc or luc/HISRBB dsRNAs.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Whole bodies and dissected tissues from female ticks of each dsRNA-injected group fed for 4 days were subjected to total RNA extraction using a TRIzol reagent (Invitrogen, CA, USA). The RT-PCR analysis was performed using a one-step RNA PCR kit (Takara, Otsu, Japan) with the primer sets of HISRBB [16], HVgR [6], and HVg-1 [7] genes. For all experiments, control amplification was carried out using the H. longicornis β-actin-specific primers (accession no. AY254898). The PCR products were subjected to electrophoresis in a 1.5% agarose gel in a TAE buffer; the DNA was visualized by ethidium bromide staining and analyzed using Quantity One 1-D Analysis Software (Quantity One Version 4.5, Bio-Rad Laboratories, Milan, Italy).

Protein expression analysis by Western blotting

The tick proteins from the lysates of whole bodies and the dissected tissues (about 1000 ng/lane) from female ticks of each

| Table 1. Female tick groups injected with a single and a combination of dsRNA(s). |
|---|
| **Tick groups** | dsRNA used for the first injection | dsRNA used for the second injection |
| HISRBB | HISRBB | – |
| HVgR | HVgR | – |
| HVg-1 | HVg-1 | – |
| HISRBB/HVG-1 | HISRBB | HVgR |
| HISRBB/HVG-1 | HVG-1 | HISRBB |
| HVG-1/HISRBB | HVG-1 | HISRBB |
| luc/HISRBB | luc | HISRBB |
| HVgR/HVG-1 | HVgR | HVgR |
| HVgR/HVG-1 | HVgR | HISRBB |
| luc | luc | – |

The first and second dsRNA injections were carried out at 96-hours interval. doi:10.1371/journal.pone.0028407.t001
dsRNA-injected group fed for 4 days were separated by 5-12% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, MA, USA). The membrane was blocked with 5% skim milk in PBS-T (157 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 0.05% Tween-20, pH 7.4) and then incubated with 1:100 dilution of anti-recombinant HlSRB (rHlSRB) [16], 1:250 dilution of anti-rHlVgR [6], and 1:200 dilution of anti-HlVg-1 mouse sera [7] or anti-actin serum [8] as a first antibody. After the incubation of peroxidase-conjugated sheep anti-mouse IgG as a second antibody (1:2000 dilution; GE Healthcare, Little Chalfont, UK), the specific protein bands were detected using 0.5 mg/ml 3,3’-diaminobenzidine tetrahydrochloride.

Indirect immunofluorescent antibody test (IFAT)
The midguts, ovaries, salivary glands, and fat bodies dissected out from female ticks of each dsRNA-injected group fed 4 days were separately fixed with 4% paraformaldehyde in PBS including 0.1% glutaraldehyde at 4°C overnight. After washing with a sucrose series in PBS overnight, samples were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, CA, USA) and frozen at −80°C. Frozen sections (12 μm) were cut with a cryostat (Leica CM 1850; Leica Microsystems, Wetzlar, Germany) and placed on micro-glass slides and then blocked with 5% skim milk in PBS overnight at 4°C. Sections were incubated for 30 minutes at 37°C with a 1:100 dilution of an anti-rHlSRB, a 1:200 dilution of anti-rHlVgR mouse sera. After washing three times with PBS, Alexa 488- or Alexa 594-conjugated goat anti-mouse immunoglobulin (1:1000; Invitrogen) was applied as a second antibody at 37°C for 1 hour. After washing three times with PBS, samples were mounted in a mounting medium with DAPI or Propidium Iodide (Vectashield; Vector Laboratories, Burlingame, CA, USA) and then covered with a cover glass. The images were photographed and recorded using a fluorescence microscope (Olympus, Tokyo, Japan).

Statistical analyses
All statistical analyses were done with the Student’s t-test. Results are presented as the means ± SD. *P < 0.05 values were considered significant.

Results
dsRNA-mediated gene silencing of HlSRB, HlVg-1, and HlVgR
To investigate whether HlSRB knockdown has an effect on RNAi of other endogenous genes in ticks, we selected the HlVg-1 and HlVgR genes. HlVg-1 was transcribed only in the midgut [7], and the HlVgR gene, only in the ovary [6]. It was previously demonstrated that HlVg-1, one of the tick multiple vitellogenins (Vgs), is a crucial yolk protein precursor for oocyte development in ticks [7] and that HlVgR, a receptor localized on the surface of oocytes, plays a critical role for the specific binding with Vgs and the resultant Vgs transfer from hemolymph into oocytes via receptor-mediated endocytosis [6]. In this study, unfed female ticks were injected with a single HlSRB, HlVgR, or HlVg-1 dsRNA for 96 hours later with luc/HlSRB, HlVgR/HlSRB, HlVg-1/HlSRB, or luc/HlSRB dsRNAs, apparent mRNA and protein knock-down of HlSRB was attained in all groups regardless of whether HlVgR dsRNA was used for the first or second injection (Fig. 2A and B). In ticks injected with a combination of HlVgR/HlSRB, HlVgR/HlVg-1, HlVg-1/HlSRB, or luc/HlSRB dsRNAs, clear double knockdown of endogenous HlVg-1 and HlVgR genes was observed in both groups (Fig. 2A and B). Similar double gene knockdown was detected in ticks injected with a combination of HlVgR/HlVg-1 or HlVgR/HlVg-1/HlSRB dsRNAs (Fig. 2A and B). However, in ticks injected with HlSRB dsRNA followed 96 hours later with HlVg-1 or HlVgR dsRNAs, no knockdown of HlVgR and HlVg-1 was achieved, although clear gene silencing of HlSRB was attained, as described above (Fig. 2A and B), suggesting that RNAi mediated by the second HlVgR or HlVg-1 dsRNA injection was inhibited by the first gene knockdown of HlSRB.

mRNA and protein knock-down of HlSRB, HlVgR, and HlVg-1 in different tissues of female ticks injected with a single dsRNA or a combination of dsRNA(s)
RT-PCR and Western blot analysis were performed to elucidate whether the mRNA and protein level in major internal organs, such as midguts, ovaries, salivary glands, and fat bodies, of female ticks attained single or double gene knockdown after various dsRNA injections (Fig. 3). As a result, mRNA and protein knock-down of HlSRB, HlVgR, and HlVg-1 in whole bodies of female ticks injected with a single dsRNA or a combination of dsRNA(s) (Table 1). As shown in Fig. 2A and B, the β-actin gene and protein levels did not change in all dsRNA-injected groups. A clear mRNA and protein knock-down of HlSRB, HlVgR, or HlVg-1 was observed in ticks injected with a single dsRNA of HlSRB, HlVgR, or HlVg-1 (Fig. 2A and B). In ticks injected with a combination of HlSRB/HlvR, HlSRB/HlVgR, HlSRB/HlVg-1, HlVgR/HlSRB, HlVgR/HlVg-1, or HlVg-1/HlSRB dsRNAs, clear double knockdown of endogenous HlVg-1 and HlVgR genes was observed in both groups (Fig. 2A and B). Similar double gene knockdown was detected in ticks injected with a combination of HlVgR/HlVg-1 or HlVgR/HlVg-1/HlSRB dsRNAs (Fig. 2A and B). However, in ticks injected with HlSRB dsRNA followed 96 hours later with HlVg-1 or HlVgR dsRNAs, no knockdown of HlVgR and HlVg-1 was achieved, although clear gene silencing of HlSRB was attained, as described above (Fig. 2A and B), suggesting that RNAi mediated by the second HlVgR or HlVg-1 dsRNA injection was inhibited by the first gene knockdown of HlSRB.

Gene silencing of HlSRB, HlVgR, and HlVg-1 in whole bodies of female ticks injected with a single dsRNA or a combination of dsRNA(s)}
In the salivary glands and fat bodies, mRNA and protein knockdown of HlSRB was observed after dsRNA injections with HlSRB, HlSRB/luc, and luc/HlSRB (data not shown).

Immunofluorescent staining of the midguts, ovaries, salivary glands, and fat bodies

An immunohistochemical examination using an indirect fluorescent antibody test (IFAT) was conducted to illustrate the localization of the endogenous protein in the midguts, ovaries, salivary glands, and fat bodies of female ticks that exhibited single or double gene knockdown after the various dsRNA injections (Fig. 4).

As shown in Fig. 4A, no localization of HlSRB protein in midguts was observed after dsRNA injections with HISRB, HISRB/HlVg-1, or HlVg-1/HISRB (panel a, c, and e). Similarly, no localization of HlVg-1 in midguts was observed after dsRNA injections with HlVg-1, HlVg-1/HlVgR, HlVg-1/HISRB, or HlVgR/HlVg-1 (panel h, j, k, and l). Neither HlVg-1 nor HlSRB protein localized in the midguts of ticks exhibited double gene knockdown by an injection of a combination of HlVg-1/HISRB dsRNAs (panel e and k). Normal localization of the HlVg-1 protein in midguts, attributed to the failure to knock-down HlVg-1 in HISRB/HlVg-1 injection, was also confirmed by IFAT (panel i).

Figure 1. Gene silencing and mortality rate of H. longicornis 96 hours after a single dsRNA injection. dsRNA complementary to HISRB, HlVgR, and HlVg-1 was injected into H. longicornis adult females. The injected ticks were allowed to rest at 25 °C in an incubator for four days to check mortality rates and gene silencing. RT-PCR analysis (A). PCR was performed using cDNA synthesized from three ticks injected with HISRB, HlVgR, HlVg-1, or luc dsRNA with primer sets specific to HISRB, HlVgR, HlVg-1, and the β-actin gene. Lane 1, HISRB dsRNA-injected ticks; lanes 2, 4, and 6, luc dsRNA-injected ticks; lane 3, HlVgR dsRNA-injected ticks; lane 5, HlVg-1 dsRNA-injected ticks. Mortality rates (B). Each panel represents treatment with one gene-specific dsRNA. Mortality rates were calculated by the percentage of number of dead ticks to the number of ticks used at the beginning of experiment in a different time course. HISRB, HISRB dsRNA-injected ticks; HlVgR, HlVgR dsRNA-injected ticks; HlVg-1, HlVg-1 dsRNA-injected ticks; luciferase, luciferase dsRNA-injected ticks.

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In the ovaries, no localization of the native HlSRB protein was observed after dsRNA injections with HlSRB, HlSRB/HlVgR, or HlVgR/HlSRB (Fig. 4B, panel a, c, and e). No expression of HlVgR in ovaries was detected after dsRNA injections with HlVgR, HlVg-1/HlVgR, HlVgR/HlSRB, or HlVgR/HlVg-1 (Fig. 4B, panel h, j, k, and l). Neither HlVgR nor HlSRB proteins localized in the ovaries of ticks exhibited double gene knock-down by an injection of a combination of HlSRB/HlVgR dsRNAs (panel e and k). The localization of HlVgR expressed in ovaries, thought to result from the failure to knock-down HlVgR with HlSRB/HlVgR injection, was also observed by IFAT (panel i).

In the salivary glands and fat bodies, no localization of native HlSRB protein was detected after dsRNA injections with HlSRB, HlSRB/luc, and luc/HlSRB, while HlSRB localization was observed after a single dsRNA injection of luc (data not shown).

Engorgement, mortality and fecundity of female ticks injected with a single dsRNA or a combination of HlSRB, HlVgR, HlVg-1, and luc dsRNA(s)

Table 2 shows the phenotypic changes of adult female ticks injected with HlSRB, HlVgR, and HlVg-1 dsRNA individually or in combination as well as luc dsRNA as a control (Table 1).

Table 2 shows the phenotypic changes of adult female ticks injected with HlSRB, HlVgR, and HlVg-1 dsRNA individually or in combination as well as luc dsRNA as a control (Table 2). It was evident that the engorged body weights of HlVg-1 knockdowned ticks was conspicuously lower (Table 2).

The mortality rates 20 days after engorgement in ticks injected with a combination of HlVg-1/HlVgR, HlVgR/HlSRB, HlVg-1/HlSRB and HlVgR/HlVg-1 dsRNAs, in which a double knockdown of targeted two genes was achieved (Fig. 2), were significantly higher, 86.4%, 60.5%, 71.0%, and 91.3%, respectively (Table 2). Most of the ticks from these groups died 18 hours after engorgement, and very few died within 5 days of engorgement. Mortality rates of 7.5%, 5.0%, and 27.5% were observed in ticks injected with a single dsRNA of HlSRB, HlVgR, and HlVg-1 (Table 1). Similar lower mortality rates of 7.5%, 5.0%, 27.5%, 7.8%, 7.6%, 7.6%, and 7.8% were found in ticks injected with a single HlSRB, HlVgR, and HlVg-1 dsRNA, respectively, or a combination of HlSRB/HlVgR, HlSRB/HlVg-1, HlSRB/luc, and luc/HlSRB dsRNAs, respectively (Table 2). These low mortality rates were observed in tick groups in which only a single knockdown of targeted genes was obtained (Fig. 2). No mortality was found in ticks injected with control luc dsRNA.

The fecundity of female ticks was estimated from the ratio of egg weight to engorged body weight. The ticks did not lay eggs in tick groups injected with a combination of HlVg-1/HlVgR, HlVgR/HlSRB, HlVg-1/HlSRB and HlVgR/HlVg-1 dsRNAs, in which double knockdown of targeted two genes was achieved, because the ticks could not achieve oviposition (Table 2). However, normal fecundity ratios were observed in tick groups with single gene knockdown, as described above (Table 2). The lower fecundity...
observed in ticks injected with a single Hlvg-1 dsRNA (Table 2) was attributed to the crucial involvement of Vgs in oocyte development in the ovaries.

The hatching rates from eggs to larvae were also examined at 25°C in an incubator for 60 days (Table 2). All eggs from ticks injected with a single dsRNA of luc succeeded in hatching to larvae (Table 2). In tick groups injected with a single dsRNA of HisRB and a combination of HisRB/luc/HlvgR, HisRB/HlvgR, HisRB/luc, and luc/HisRB dsRNAs, in which single knockdown of HisRB was achieved (Fig. 2), ca. 80% of hatching rates were observed (Table 2). The hatching rates of eggs laid by ticks showing single gene knockdown of HlvgR or Hlvg-1 after a respective dsRNA injection were evidently low, and eggs from the HlvgR dsRNA-injected ticks died without hatching (Table 2).

Discussion

There are at least two pathways for exogenous dsRNA uptake in insects, such as the transmembrane channel protein-mediated and the endocytosis-mediated mechanisms [12]. In the latter endocytosis-mediated dsRNA uptake mechanism, the participation of SRs, known to be a key component of endocytosis [14], in dsRNA uptake was previously suggested in T. castaneum [15] and D. melanogaster insects [13]. However, it was unknown if SRs could have an important role in the dsRNA-mediated RNAi of ticks. To elucidate the role of SRs in dsRNA uptake in ticks, we used dsRNA of HisRB, the class B scavenger receptor of H. longicornis ticks [16]. In the current study, dsRNA of HisRB was injected into female ticks individually or in combination with different exogenous dsRNAs of ticks, namely Hlvg-1, a yolk protein precursor expressed only at the midgut of H. longicornis [7], and HlvgR, a receptor localized only in the oocyte surface of H. longicornis [6], as well as firefly luciferase (luc) as a control [16].

As shown in Fig. 1A, the individual gene expression of the HisRB, HlvgR, and Hlvg-1 was clearly inhibited in ticks 96 hours after a single dsRNA injection, indicating that mRNA knockdown had been successfully attained in H. longicornis ticks within 96 hours of the exogenous dsRNA injection. We found higher mortalities, from 44.4% to 62.2% in ticks 96 hours after a single dsRNA injection with HisRB, HlvgR, or Hlvg-1 (Fig. 1B), while the mortality of control ticks injected with a luc dsRNA was 22.2%, which is not negligible. This result suggests that increased mortality in ticks injected with dsRNAs has been substantially attributed to the knockdown of targeted genes but partially associated with the possible external injuries, off-target effects associated with long dsRNA in RNAi screens, and functional impairments caused by microinjections in recipient ticks.

As shown in Fig. 2A and B, in the whole bodies of ticks injected with a combination of Hlvg-1/HlvgR or HlvgR/Hlvg-1 dsRNAs, a clear and systemic double knockdown of endogenous Hlvg-1 and HlvgR genes was attained in both tick groups. Similar systemic double gene knockdown of targeted genes was observed in ticks injected with a combination of HlvgR/HisRB or Hlvg-1/HisRB dsRNAs. However, in the whole bodies of ticks injected with a combination of HisRB/HlvgR or HisRB/Hlvg-1 dsRNAs, no knockdown of HlvgR and Hlvg-1 was achieved, although clear systemic gene silencing of HisRB was attained. These results suggest that systemic RNAi mediated by the second HlvgR or Hlvg-1 dsRNA injection was dramatically inhibited by the first systemic gene knockdown of HisRB. No conclusion can yet be drawn, but it is speculated that SID-1, SRs, vacuolar H+ATPase, RDS-5, and RdRp could be possible components of the dsRNA uptake mechanism in several insects [12]. With regard to SRs, two scavenger receptors of D. melanogaster, SR-CI and Eater, account for more than 90% of dsRNA uptake by S2 cells [13]. Our results clearly indicate that HisRB, class B scavenger receptor...
of *H. longicornis* ticks is essential for systemic RNAi/effective knockdown of gene expression by RNAi.

Boldbaatar et al. [6,7] demonstrated that the transcription and translation of *HlVg-1* are midgut-specific and those of *HlVgR* are ovary oocyte-specific. In the current study, we examined the expression and localization of *HlVg-1* in midguts and *HlVgR* in ovaries of female ticks showing a single or double systemic RNAi. As a result, normal expression of the gene and protein of *HlVg-1* in midguts and *HlVgR* in ovaries were clearly shown in female ticks with a systemic RNAi of *HlSRB* (Figs. 3 and 4).

Systemic RNAi can only take place in multicellular organisms because it includes processes in which a silencing signal is transported from one cell to another or from one tissue type to another [12]. In multicellular organisms, such as ticks, the *HlVg-1* or *HlVgR* dsRNA internalized through injection into hemocoel must be taken up from the hemolymph to the midgut cells or ovary oocytes in order to silence the target genes. We conclude that the dysfunction of receptor-mediated endocytosis was introduced in ticks by the systemic RNAi of *HlSRB*, resulting in the uptake abrogation of *HlVg-1* or *HlVgR* dsRNA in midguts or ovaries and leading to normal protein expression.

Most of our understanding of tick RNAi is mediated systemic delivering of RNAi effect and the literature demonstrated that a systemic RNAi silencing mechanism is active in ticks [3–9]. Results obtained in this study might explain that the SR-mediated dsRNA uptake mechanism is evolutionarily conserved in ticks and plays a crucial role in controlling the induction of systemic gene silencing in ticks. However, other factors, such as proteins of the vesicle-mediated transport, conserved oligomeric Golgi complex family, cytoskeleton organization and protein transport are directly and/or indirectly involved in endocytosis and known to play roles in dsRNA uptake and processing [20]. Further studies...

**Figure 4. Confirmation of RNAi on the endogenous HlSRB, HlVg-1, and HlVgR in the different tissues of *H. longicornis*.** The dissected tissues from the dsRNA-injected 4-days-feeding ticks were observed under fluorescence microscopy. The name of each dsRNA group is indicated above. The midguts were stained with anti-rHlSRB and anti-rHlVg-1 antibodies followed by Alexa 488-conjugated mouse anti-IgG with DAPI (A). Arrowheads indicate the native HlSRB and HlVg-1 expressed in the midguts. ML, midgut lumen; MC, midgut cells. The ovaries, staining pattern of anti-rHlSRB and anti-rHlVgR serum were used as first antibodies (B). The mouse anti-IgG conjugated with Alexa 488 was used as a second antibody with DAPI for the upper panels and Alexa 594 with Propidium Iodide for the lower panels. Arrowheads indicate the native HlSRB and HlVgR expressed in the ovaries. OO, oocyte; OD, oviduct. The scale bar represents 20 μm.
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are needed to examine these factors in *H. longicornis*. These results may contribute to the development of a control strategy for ticks and pathogen transmission. The overall results presented in this study show that dsRNAs of *HISRB*, *HlVg-1*, and *HlVgR* introduced into ticks individually or in combination resulted in different but significant phenotypic changes in them (Table 2). These phenotypes in engorgement, mortality, fecundity, and oocyte development of ticks were comparable with those in previous characterization [6,7,16] and changes in them (Table 2). These phenotypes in engorgement, combination resulted in different but significant phenotypic

| dsRNA groups | Number of ticks attached 24 h after infestationa | Average engorged body weight (mg)b | Mortality rate (%)c,d | Egg weight/body weight (%)c,d | Hatching rate (%)c,d |
|--------------|-----------------------------------------------|----------------------------------|----------------------|-----------------------------|---------------------|
| *HISRB* 40   | 142.10±30.30*                                  | 7.5                              | 41.95±11.25*         | 83.7                        |
| *HlVgR* 40   | 172.18±19.92*                                  | 5                                | 11.62±12.41*         | 0                            |
| *HlVg-1* 40  | 81.56±18.16*                                   | 27.5                             | 8.16±30.45*          | 13.7                        |
| *HISRB/HlVgR* 38 | 143.61±14.38*                                 | 7.8                              | 42.15±15.08*         | 82.8                        |
| *HISRB/HlVg-1* 39 | 141.57±45.39*                                 | 7.6                              | 42.10±16.32*         | 83.3                        |
| *HISRB/luc* 39 | 142.81±17.12*                                 | 7.6                              | 41.75±11.21*         | 83.3                        |
| *HlVg-1/HlVgR* 37 | 76.91±24.37*                                  | 86.4                             | 0                    | 0                            |
| *HlVgR/HISRB* 38 | 150.29±11.32*                                 | 60.5                             | 0                    | 0                            |
| *HlVg-1/HISRB* 38 | 85.81±64.14*                                  | 71                               | 0                    | 0                            |
| luc/HISRB    38 | 143.15±32.63*                                 | 7.8                              | 42.87±24.13*         | 82.8                        |
| *HlVgR/HlVg-1* 37 | 98.45±68.26*                                  | 91.8                             | 0                    | 0                            |
| *luc* 40     | 245.75±37.35                                  | 0                                | 50.70±21.31          | 100                          |

aSixteen ticks were collected from the host for the subsequent experiments at 4 days after attachment.
bThese ratios show the percentage of number of dead ticks 20 days after drop-off to the total number of engorged ticks per treatment.
cThese mortality rates show the percentages of number of dead ticks 20 days after drop-off to the total number of engorged ticks per treatment.
dThese ratios show the fecundity of engorged females. Values are the means of ±SD.

**P**<0.05, luc dsRNA-injected group vs. *HISRB*, *HlVgR*, *HlVg-1*, *HISRB/HVgR*, *HISRB/HlVg-1*, *HISRB/luc*, *HlVg-1/HlVgR*, *HlVgR/HISRB*, *HlVg-1/HISRB*, *luc/HISRB*, and *HlVgR/HlVg-1* dsRNA-injected groups.

**P**<0.05, luc dsRNA-injected group vs. *HISRB*, *HlVgR*, *HlVg-1*, *HISRB/HlVgR*, *HISRB/HlVg-1*, *HISRB/luc*, *HlVg-1*, and *luc/HISRB* dsRNA-injected groups.

In summary, research in recent years has given new insights into the dsRNA uptake mechanism in the gene silencing of insects. However, the role of dsRNA uptake in ticks remains to be proven. We demonstrated for the first time in the current study, using *HISRB*, a class B scavenger receptor CD36 of *H. longicornis*, that SRB mediates the effective knock-down of gene expression by RNAi and plays essential roles for the systemic RNAi of ticks.

## Author Contributions
Conceived and designed the experiments: KMA DB KF. Performed the experiments: KMA DB ML. Analyzed the data: KMA XX HS RLG KF. Contributed reagents/materials/analysis tools: KMA RU-S TT. Wrote the paper: KMA RU-S TT KF.

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