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Silencing the Honey Bee (*Apis mellifera*) Naked Cuticle Gene (*nkd*) Improves Host Immune Function and Reduces *Nosema ceranae* Infections

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ABSTRACT

***Nosema ceranae* is a new and emerging microsporidian parasite of European honey bees, *Apis mellifera*, that has been implicated in colony losses worldwide. RNA interference (RNAi), a posttranscriptional gene silencing mechanism, has emerged as a potent and specific strategy for controlling infections of parasites and pathogens in honey bees. While previous studies have focused on the silencing of parasite/pathogen virulence factors, we explore here the possibility of silencing a host factor as a mechanism for reducing parasite load. Specifically, we used an RNAi strategy to reduce the expression of a honey bee gene, *naked cuticle* (*nkd*), which is a negative regulator of host immune function. Our studies found that *nkd* mRNA levels in adult bees were upregulated by *N. ceranae* infection (and thus, the parasite may use this mechanism to suppress host immune function) and that ingestion of double-stranded RNA (dsRNA) specific to *nkd* efficiently silenced its expression. Furthermore, we found that RNAi-mediated knockdown of *nkd* transcripts in *Nosema*-infected bees resulted in upregulation of the expression of several immune genes (*Abaecin*, *Apidaecin*, *Defensin-1*, and *PGRP-S2*), reduction of *Nosema* spore loads, and extension of honey bee life span. The results of our studies clearly indicate that silencing the host *nkd* gene can activate honey bee immune responses, suppress the reproduction of *N. ceranae*, and improve the overall health of honey bees. This study represents a novel host-derived therapeutic for honey bee disease treatment that merits further exploration.**

IMPORTANCE

Given the critical role of honey bees in the pollination of agricultural crops, it is urgent to develop strategies to prevent the colony decline induced by the infection of parasites/pathogens. Targeting parasites and pathogens directly by RNAi has been proven to be useful for controlling infections in honey bees, but little is known about the disease impacts of RNAi silencing of host factors. Here, we demonstrate that knocking down the honey bee immune repressor-encoding *nkd* gene can suppress the reproduction of *N. ceranae* and improve the overall health of honey bees, which highlights the potential role of host-derived and RNAi-based therapeutics in controlling the infections in honey bees. The information obtained from this study will have positive implications for honey bee disease management practices.

European honey bees, *Apis mellifera*, play a critical role in the pollination of important crops. However, honey bee populations have suffered high losses in much of the world (1), coincident with an increase in agricultural demand for honey bee pollination (2). Specifically, honey bee colony losses in the United States have been exacerbated since the report of colony collapse disorder (CCD), a syndrome that comprises large-scale, unexplained losses of managed honey bees (3–9). High levels of parasites and pathogens have been linked to the decline of honey bee colonies (10, 11).

Nosema is a genus of obligate, intracellular microsporidian parasites that infect many diverse animal species, including honey bees (12, 13). For years, *Nosema* disease of European honey bees was exclusively attributed to a single *Nosema* species, *Nosema apis*. Another species, *Nosema ceranae*, was originally detected in Asian honey bees, *Apis cerana* (14), and subsequently found to infect European honey bees, *A. mellifera* (15, 16). Since then, the infection of *A. mellifera* by *N. ceranae* has been reported worldwide (17–20), and nosemosis of *A. mellifera* caused by *N. ceranae* is now far more prevalent than that by its native sympatric congener *N. apis* (17, 21–25). Although there are no outward disease symptoms reported (12), *N. ceranae* infection can cause energetic stress and behavioral changes in worker bees (26–29), leading to a reduced life span of infected bees. As an emerging parasite, *N. ceranae* has often been linked to colony losses worldwide. A study based on Spanish honey bee populations showed that natural in-

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fection by *N. ceranae* could cause colony collapse (30, 31). New evidence has shown that *N. ceranae* interacts synergistically with pesticides, resulting in more complex and severe disease in honey bees (32–35). So far, the only registered treatment for nosemosis in North America is fumagillin. With prolonged use of fumagillin, the issues of drug resistance have arisen (36). As a result, novel parasite-specific and environmentally friendly therapeutic options are urgently needed for *Nosema* treatment.

RNA interference (RNAi), a posttranscriptional gene silencing mechanism, is an efficient and specific method of gene silencing which functions by inducing degradation of homologous mRNAs (37, 38). RNAi technology has been explored to protect honey bees from infection by pathogens and parasites (39). Three honey bee viruses, Israeli acute paralysis virus (IAPV), deformed wing virus (DWV), and Chinese sacbrood virus (CSBV), have been successfully inhibited by RNAi under laboratory conditions by feeding bees with virus-specific double-stranded RNAs (dsRNAs) or small interfering RNAs (siRNAs) (40–43). Moreover, a large-scale field application of IAPV dsRNA improved bee survival, colony size, and honey yield (44). RNAi has also been used to help control the parasitic mite *Varroa destructor* (45). In one previous study, *N. ceranae* ADP/ATP transporter genes were targeted and silenced by corresponding dsRNAs, resulting in the decline of spore loads and alleviation of disease in infected bees (46).

All applications of RNAi in treating honey bee diseases to date have targeted the genes of parasites or pathogens. Nevertheless, disease always involves interactions between hosts and parasites/pathogens, and it is also possible to mitigate infections from the host perspective, i.e., to use RNAi to manipulate host factors that interact with parasites or pathogens. In fact, *Nosema* infection dramatically alters honey bee transcriptional responses (47–49), providing potential targets for host-based RNAi manipulation.

The Wnt signaling pathway is an important regulator of immune function in mammals (50) and has recently been found to function in regulating immune pathways, specifically Toll pathways, in insects (*Drosophila*) (51). There are several genes that serve as antagonists of the Wnt pathway, and thus, upregulated expression of these genes should suppress immune function. One of these genes has been found in *Drosophila*—the *naked cuticle* gene (52). As suppressors of immune function, these genes may serve as excellent targets for parasite manipulation of the hosts' transcriptional pathways: if the parasite can upregulate these antagonists, it can suppress the host's immune response. In light of the fact that *nkd* is a negative regulator of the Wnt signaling pathway, we hypothesized that *nkd* expression might be regulated by *Nosema* infection and thereby could serve as a potential target of RNAi to mitigate *Nosema* infection in honey bees. In the work described here, we confirmed that *nkd* mRNA levels were upregulated when bees were infected by *N. ceranae*. Our RNAi experiments showed that silencing *nkd* led to the enhancement of immune responses through an increase in immune gene expression, reduction in parasite spore load, and improvement in the life expectancy of *N. ceranae*-infected bees. We provide unequivocal evidence that silencing the *nkd* gene is an efficient way to control *N. ceranae* infection and improve honey bee health.

MATERIALS AND METHODS

Honey bees. The honey bees used in this study were collected from colonies of *Apis mellifera ligustica* maintained at the USDA-ARS Bee Research Laboratory, Beltsville, MD. Newly emerged bees were obtained by removing honeycomb frames with sealed brood from strong and healthy colonies that were identified as *Nosema* negative, placing the frames into mesh-walled cages individually, and maintaining the frames in an insect growth chamber at 34 ± 1°C and 55% ± 5% relative humidity (RH) overnight. Emerging adult worker bees were collected the next day (<24 h). In order to make sure that the experimental bees were free of *N. ceranae* infection before proceeding to the experimental inoculation, we confirmed the negative status of *Nosema* infection using a hemocytometer and light microscopy. Briefly, 30 abdomens of newly emerged bees were dissected and ground up thoroughly in 30 ml of deionized H_2O. Ten microliters of the homogenate was loaded onto a hemocytometer, and the presence or absence of spores was determined under a light microscope following a previously described method (53).

Inoculum preparation. *N. ceranae* spores were purified from foragers collected outside the entrance of identified *N. ceranae*-infected colonies. Midguts were pulled out and homogenized in sterile distilled water (dH_2O). The purification of *Nosema* spores from the homogenate was performed as described by Fries et al. (54). The homogenate was filtered through a nylon mesh cloth (65-μm pore size) by centrifuging it for 5 min at 3,000 × *g*. The supernatant was discarded, and the pellet was resuspended in sterile water and centrifuged for 10 min at 5,000 × *g*; this step was repeated twice. Finally, the pellet was resuspended in dH_2O and stored at room temperature for no more than 1 week. The inoculum was obtained by diluting spore solution with sucrose solution, with a final concentration of 2.0×10^7 spores/ml in 50% (mass/vol) sucrose solution.

Inoculation. The newly emerged bees were collected and starved for 3 h in an incubator (32°C and 75% RH) before being inoculated with *Nosema* spores in solution. Individual feeding was performed for each bee by holding two wings of a bee on each side with one hand and feeding the bee with 5-μl inoculum (100,000 spores) with a pipette with the other hand. Thirty bees were then distributed into each cup cage, which is a plastic bee-rearing cup with a top-feeder design (55). A 3-ml syringe filled with 50% (mass/vol) sucrose solution was inserted in the top of the cage to feed the bees, and the solution was changed every 3 days. A small pollen patty was supplied in the bottom of the cage for 6 days. The same number of control bees (without spore inoculation) were transferred into a cup cage as well. There were 4 replicates for each group. All cup cages were maintained in an incubator (32°C and 75% RH). Five bees were sampled from each cage at days 6 (D6), 9 (D9), 12 (D12), 15 (D15), and 18 (D18) postinoculation. The collected bee samples were immediately frozen at −80°C and stored until processing.

Production of dsRNA. Primers were designed from the sequence of the *A. mellifera nkd* gene (GenBank accession no. XM_001120899) by using the E-RNAi web service (56), and primers for *GFP*, which served as the control gene, were used from previous studies (57). All primer sequences were fused with the T7 promoter sequence (Table 1). PCRs were performed using different templates individually: the cDNA of an adult bee was used for the amplification of *nkd*, and the pGFP vector (Clontech) was used for the amplification of *GFP*. The 100-μl PCR mixture contained the following: 78 μl H_2O, 10 μl 10× reaction buffer (Invitrogen), 3 μl $MgCl_2$, 2 μl deoxynucleoside triphosphate (dNTP) mixture (10 mM; Invitrogen), 2 μl forward primer (20 μM), 2 μl reverse primer (20 μM), 1 μl *Taq* polymerase (Invitrogen), and 2 μl DNA template. The PCR program was 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 90 s, and then 72°C for 10 min. After each PCR amplification, the products were verified in 1.0% agarose gels, purified, and used as the templates for the *in vitro* transcription reaction. The production of dsRNAs was carried out by using the MEGAscript RNAi kit (Ambion). The transcription reaction mixtures were assembled according to the manufacturer's instruction, and the time of incubation at 37°C was extended to 15 h. The following steps, such as nuclease digestion, purification, and elution, were performed using the materials associated with the kit. The quality of the dsRNAs was tested using 1.0% agarose gels, and their concentrations were determined with a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Inc.). The products were diluted

TABLE 1 Primer sequences used in this study

Purpose	Gene	Accession no.	Primer	Sequence (5′→3′)[a]	Amplicon Location	Amplicon Length (bp)	Reference or source
RNAi	*nkd*	XM_001120899	nkd-RNAi-F	TAATACGACTCACTATAGGGCGACGCGCTTATGTTCAACCTC	1889–2122	234	This study
			nkd-RNAi-R	TAATACGACTCACTATAGGGCGAGGTCGCGTGTTTCAAATGAT			
	GFP	AF324407	GFP-RNAi-F	TAATACGACTCACTATAGGGCGATTCCATGGCCAACACTTGTCA	173–674	502	57
			GFP-RNAi-R	TAATACGACTCACTATAGGGCGATCAAGAAGGACCATGTGGTC			
qPCR	*nkd*		nkd-F	AGGATGACGGTGAAAATGCG	1365–1540	176	This study
			nkd-R	ATTAGTCGTGAGGAGAGGCG			
	β-*actin*	NM_001185145	actin-F	TGCCAACACTGTCCTTTCTG	1018–1173	156	73
			actin-R	AGAATTGACCCACCAATCCA			
	Abaecin	NM_001011617	Abaecin-F	AGATCTGCACACTCGAGGTCTG	14–214	201	74
			Abaecin-R	TCGGATTGAATGGTCCCTGA			
	Apidaecin	NM_001011613	Apidaecin-F	TTTTGCCTTAGCAATTCTTGTTG	58–137	80	This study
			Apidaecin-R	GCAGGTCGAGTAGGCGGATCT			
	Defensin-1	NM_001011616	Defensin-1-F	TGTCGGCCTTCTCTTCATGG	88–288	201	This study
			Defensin-1-R	TGACCTCCAGCTTTACCCAAA			
	PGRP-S2	NM_001163716	PGRP-S2-F	TTGCACAAAATCCTCCGCC	146–274	129	This study
			PGRP-S2-R	CACCCCAACCCTTCTCATCT			

[a] Underlining shows the T7 promoter sequence.

with sucrose solution to final concentrations of 10 μg/ml, 20 μg/ml, and 40 μg/ml dsRNA in 50% (mass/vol) sucrose solution. The final solutions were stored at −80°C until use.

RNAi treatment. Before the RNA interference (RNAi) treatment, the newly emerged bees were collected, inoculated with *N. ceranae* spores as described above, and then transferred into bee-rearing cages. In each cage, 20 bees were supplied with 1.5 ml of 50% sucrose solution containing *nkd* or *GFP* dsRNA in a 3-ml syringe and a small pollen patty in the bottom of the cage on the same day. Bees were fed with the dsRNA for 15 days; the dsRNA solution was changed daily. Pollen patties were supplied for the first 6 days and changed every 3 days. All cages were incubated at 32°C and 75% RH, and dead bees were removed every day.

To test the efficiency of gene knockdown, three different concentrations of *nkd* dsRNA solution, 10 μg/ml, 20 μg/ml, and 40 μg/ml, were applied to separate cages, and 20 μg/ml *GFP* dsRNA solution was used for control bees. There were three replicates for each treatment. The bees were sampled at D9 and D15 after the ingestion of dsRNA and stored at −80°C until use.

To study the biological responses to the knockdown of *nkd*, 20 μg/ml of *nkd* dsRNA and *GFP* dsRNA were fed to infected bees. Another control group was set up with bees that were not given any treatment and fed only 50% sucrose solution and pollen. Each group contained three replicates. The numbers of dead bees were recorded daily, and dead bees were then removed. All the bees were collected at D15 and stored at −80°C until use.

Spore counting. To evaluate the *Nosema* infection levels in the RNAi-treated bees, the spores were counted in individual bees. First, the abdomens were separated, put into 1.5-ml Eppendorf tubes individually, and homogenized thoroughly in 1 ml dH_2O using a pestle. Then, each homogenate was diluted 100 times. Ten microliters of the diluted solution was loaded onto a hemocytometer, and spores were counted under a light microscope as described by Cantwell (53).

RNA extraction and cDNA synthesis. TRIzol reagent (Invitrogen) was used to extract total RNA from the abdomens of individual bees, following the manufacturer's protocols. Any genomic DNA contamination was removed by treatment with DNase I (DNA-free kit; Ambion). The purity and quantity of RNA samples were examined by using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Inc.). All RNAs were stored at −80°C until use. First-strand cDNA was produced from a 20-μl reverse transcription reaction mixture that contained 2 μl total RNA (approximately 1 μg/μl), 1 μl dNTP mixture (10 mM), 1 μl random primers (0.15 μg/μl), 1 μl dithiothreitol (DTT) (0.1 M), 4 μl 5× first-strand buffer, 1 μl SuperScript III reverse transcriptase (200 U/μl, Invitrogen), and 10 μl nuclease-free water. The reaction program was as follows: 25°C for 5 min and 50°C for 45 min, followed by 70°C for 15 min. The cDNAs were stored at −20°C until use.

qPCR. Quantitative PCRs (qPCRs) were run on a CFX384 Touch real-time PCR system (Bio-Rad, Hercules, CA), and SYBR green was selected as the detection signal. The primers used here were designed with Primer3 (Table 1) (58). β-*actin* served as the reference gene, and all primer pairs were validated as described in reference 59. Each 10-μl PCR mixture was assembled by mixing 5 μl 2× Brilliant III ultrafast SYBR green qPCR mix (Stratagene, La Jolla, CA), 0.25 μl forward primer (20 mM), 0.25 μl reverse primer (20 mM), 0.5 μl cDNA, and 4 μl nuclease-free water. Each reaction was run in duplicate. The PCR program was 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 45 s. Melting curves and no-template-control (NTC) reactions were monitored to evaluate the quality and specificity of amplification. Only a single peak was seen in all melting curves, and no peaks showed for the NTCs. The PCR products were run on a 1% agarose gel to confirm the expected amplification sizes. The threshold cycle (C_T) values were generated by using CFX Manager 3.1 (Bio-Rad). The relative quantification of gene expression was calculated with the comparative C_T ($\Delta\Delta C_T$) method (60). For each gene, the average C_T value of the target was normalized with the corresponding β-*actin* value using the formula ΔC_T = average $C_{T(\text{target})}$ − average $C_{T(\beta\text{-}actin)}$, and the group of bees with the lowest level of gene expression was chosen as a calibrator [$\Delta C_{T(\text{calibrator})}$]. The ΔC_T value of each group was subtracted from the $\Delta C_{T(\text{calibrator})}$ value to generate the $\Delta\Delta C_T$. The concentration of each target in each group was calculated using the formula $2^{-(\Delta\Delta CT)}$ and expressed as the *n*-fold difference relative to the calibrator value.

Bioinformatic and statistical analyses. Multiple alignments of insect *nkd* protein sequences were carried out with ClustalX 2.0 (61). Protein domain identification and secondary structure prediction of *A. mellifera nkd* were performed with the InterProScan tool and EMBOSS: Garnier algorithm of Geneious version 9.1.3 (Biomatters), respectively.

The dynamics of *nkd* gene expression during the course of *Nosema* infection, the levels of immune gene expression, and the spore loads of infected bees after RNAi treatment were analyzed using independent-sample *t* tests. The mRNA levels of *nkd* after dsRNA treatment were analyzed by one-way analysis of variance (ANOVA) with all compared groups passing an equal variance test, and the *post hoc* effects were determined using the Tukey honestly significant difference (HSD) test. Survival anal-

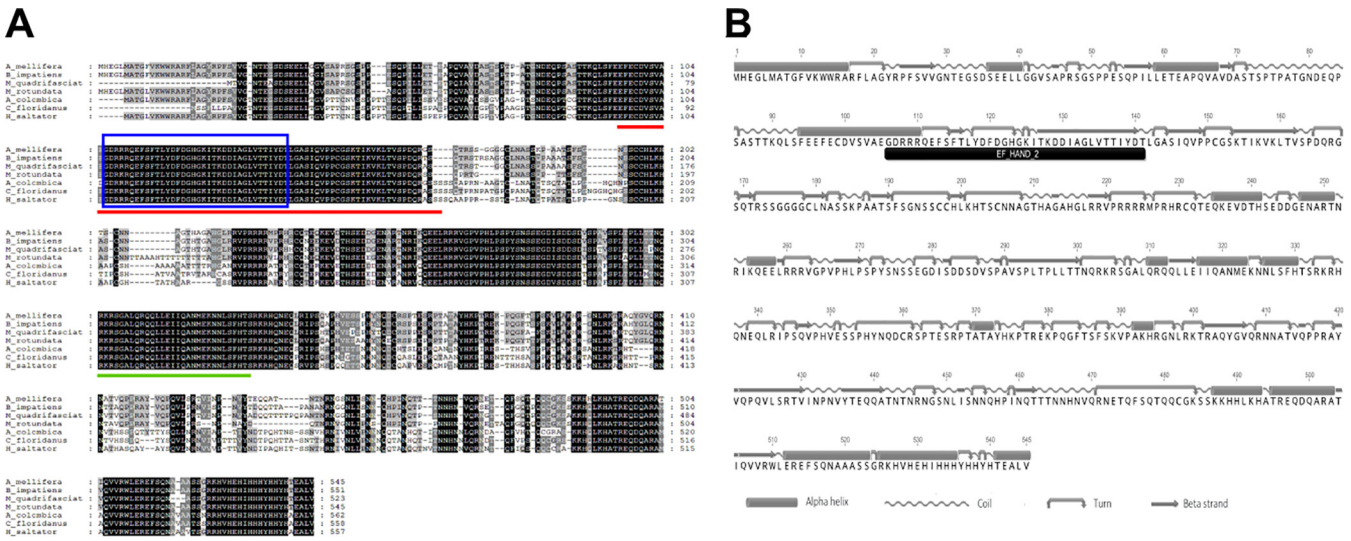

FIG 1 Sequence conservation and predicted secondary structure of *A. mellifera nkd*-encoded protein. (A) Multiple alignment of *A. mellifera* (XP_001120899), *Bombus impatiens* (XP_012249347), *Megachile rotundata* (XP_003702467), *Melipona quadrifasciata* (KOX70301), *Atta colombica* (KYM87061), *Harpegnathos saltator* (XP_011151892), and *Camponotus floridanus* (EFN66676) *nkd* protein sequences. Black and gray shadings indicate identity and a high degree of conservation of amino acids, respectively. The region responsible for interaction with *dsh* is indicated by the red bar. The nuclear localization motif is underlined by the green bar. The blue box highlights the EF-hand domain. (B) Protein domain identification and secondary structure prediction of *A. mellifera nkd*. The conserved domain is shown by a black bar.

ysis was performed using the Kaplan-Meier method, and log rank and Wilcoxon tests were computed to assess the overall homogeneity between the treatment strata. Pairwise comparisons were carried out using Wilcoxon tests. In all cases, a P value of <0.05 was taken to be significant. All analyses were carried out using PASW Statistics 18 (SPSS, Inc.).

RESULTS

Identification of a highly conserved *nkd* gene in honey bees. The identified honey bee *nkd* gene encodes a predicted protein of 545 amino acids (aa), which shares 92.9% and 89.2% sequence identity with the *nkd*-encoded proteins of the bumble bee *Bombus impatiens* and stingless bee *Melipona quadrifasciata*, respectively (Fig. 1). This protein is highly conserved in Hymenoptera, as remarkable sequence identity is also seen in non-Apidae species, ranging from 65.5% (*Camponotus floridanus*) to 85.8% (*Megachile rotundata*) (Fig. 1). A conserved EF-hand domain (InterPro accession number IPR002048), which is known to bind calcium, was identified in the *nkd* proteins (Fig. 1). Additional conserved regions included a region responsible for interaction with *Dishevelled* (*Dsh*), a component of the Wnt signaling pathway (62), and the nuclear localization motif that is required for nuclear localization and inhibition of Wnt signaling (Fig. 1) (63). Although the diversity of *nkd* protein sequences increases dramatically when the comparison is expanded to different insect orders, the sequence features mentioned above are still highly conserved (see Fig. S1 in the supplemental material).

***N. ceranae* infection upregulates the expression of the *nkd* gene.** As shown by the results in Fig. 2, the dynamics of *nkd* gene expression was altered during the *N. ceranae* infection. The expression of *nkd* in the infected bees was significantly upregulated compared with that of control bees at day 6 (D6) and D18 postinoculation (for D6, t test, $t = -2.774$, df $= 9$, $P = 0.022$; and for D18, t test, $t = -3.387$, df $= 10.860$, $P = 0.006$) (Fig. 2). Collectively, *N. ceranae* infection upregulated the *nkd* gene expression (t test, $t = -2.872$, df $= 63$, $P = 0.006$).

Ingestion of dsRNA silences the *nkd* gene expression in *N. ceranae*-infected bees. As shown by the results in Fig. 3, 9 days of feeding *nkd* dsRNA was insufficient to reduce the mRNA levels of the target gene in adult bees (ANOVA, $F_{1,11} = 0.442$, $P = 0.742$), but the gene expression could be significantly silenced by 15 days of ingesting the corresponding dsRNA (ANOVA, $F_{1,11} = 9.1781$, $P = 0.005$). Moreover, the knockdown of *nkd* was dosage dependent (Fig. 3): along with an increase in the dsRNA concentration, the effect of gene silencing increased. When 10 μg/ml of dsRNA was applied, a 40% knockdown of *nkd* mRNA was achieved, while larger amounts of 20 or 40 μg/ml dsRNA resulted in 50% and 70%

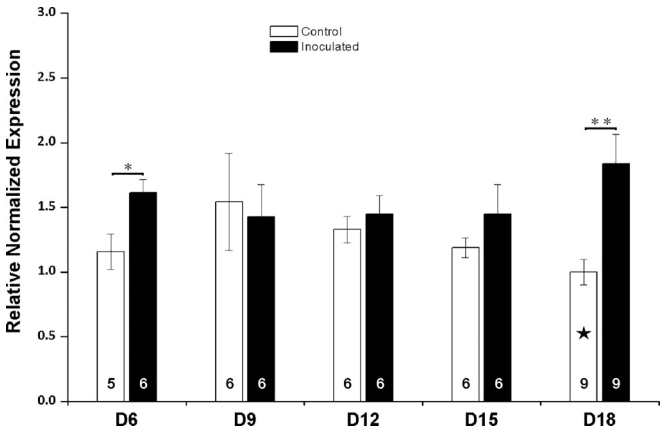

FIG 2 Expression profile of *nkd* during *N. ceranae* infection. The *x* axis indicates the days postinoculation with *Nosema* spores. The relative gene expression levels (*y* axis) are shown as mean values ± standard errors of the means (SEM). The sample size is shown in the bottom of each bar, and the solid five-pointed star indicates the calibrator used to normalize the gene expression. Data were analyzed by the independent-sample t test. Significant differences between groups are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$).

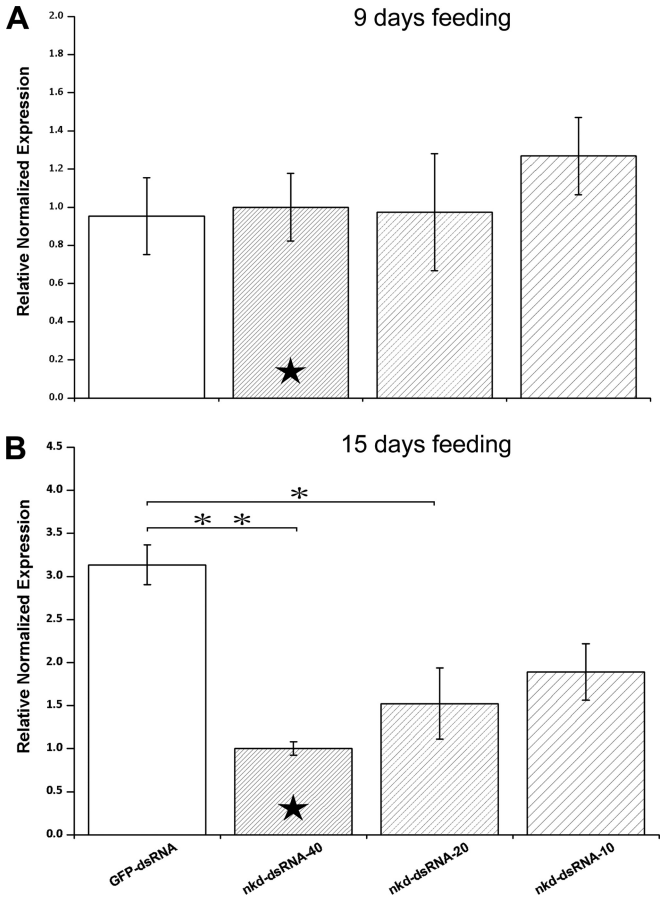

FIG 3 Knockdown of *nkd* gene in adult bees by dsRNA ingestion. All groups of adult bees were inoculated with *Nosema* spores first and then fed with sucrose solution containing dsRNA for 15 days. The silencing effect was examined after 9 days (A) and 15 days (B) of feeding dsRNA. The control bees (GFP-dsRNA) were fed with the dsRNA derived from the *GFP* sequence. For the treatment groups, three different concentrations of *nkd* dsRNA were examined, as follows: 40 μg/ml (nkd-dsRNA-40), 20 μg/ml (nkd-dsRNA-20), and 10 μg/ml (nkd-dsRNA-10). The relative gene expression levels are shown as mean values ± SEM. All groups had the same sample size ($n = 3$). The calibrator used to normalize the gene expression is indicated by a solid five-pointed star inside the bar. One-way ANOVA was employed to analyze the differences between data, and *post hoc* effects were identified by Tukey HSD tests. Significant differences between groups are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$).

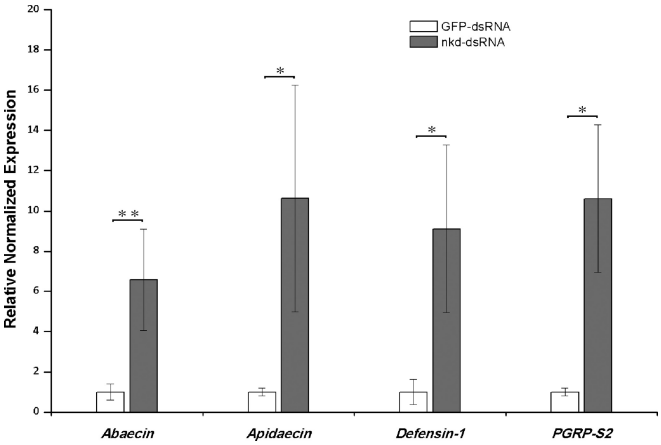

FIG 4 Effects of *nkd* gene silencing on immune gene expression in *Nosema*-infected bees. The relative mRNA levels of immune genes (*x* axis) were compared between control bees fed with 20 μg/ml *GFP* dsRNA (GFP-dsRNA; $n = 6$) and treatment bees fed with 20 μg/ml *nkd* dsRNA (nkd-dsRNA; $n = 5$). Both groups were inoculated with *Nosema* spores before dsRNA feeding. The expression levels are shown as mean values ± SEM. Data were analyzed by independent-sample *t* test. Significant differences between groups are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$).

gene silencing, and the differences compared to the *nkd* expression in controls became significant ($P = 0.018$ for 20 μg/ml and $P = 0.004$ for 40 μg/ml) (Fig. 3).

Silencing of the *nkd* gene upregulates immune gene expression in *N. ceranae*-infected bees. As shown by the results in Fig. 4, the *nkd*-silenced bees expressed significantly larger amounts of mRNA for three antimicrobial peptide (AMP) genes, *Abaecin*, *Apidaecin*, and *Defensin-1*, relative to the expression of these genes in the control bees (for *Abaecin*, *t* test, $t = -3.689$, df = 9, $P = 0.005$; for *Apidaecin*, *t* test, $t = -3.047$, df = 4.982, $P = 0.029$; and for *Defensin-1*, *t* test, $t = -2.855$, df = 9, $P = 0.019$). Moreover, the expression of a peptidoglycan recognition protein (PGRP) gene, *PGRP-S2*, was also significantly upregulated in *nkd*-silenced bees compared with its expression in the control bees (*t* test, $t = -3.183$, df = 5.043, $P = 0.024$) (Fig. 4).

Silencing of *nkd* gene reduces *Nosema* spore levels and extends the life span of *N. ceranae*-infected bees. After feeding with *nkd* dsRNA, the spore load of infected bees was significantly (approximately 50%) lower than that in controls (fed with *GFP* dsRNA) (*t* test, $t = 2.458$, df = 15.485, $P = 0.026$) (Fig. 5), indicating that knockdown of *nkd* gene expression results in a decrease in *Nosema* infection levels. Survival analysis was performed to further examine the effect of silencing the *nkd* gene on the life span of honey bees after *Nosema* infection. The survival distributions for the groups tested were significantly different (log rank test, $\chi^2 = 24.472$, df = 2, $P < 0.001$; and Wilcoxon test, $\chi^2 = 25.020$, df = 2, $P < 0.001$) (Fig. 6). *Nosema* infection indeed induced higher mortality in the infected honey bees (no treatment versus *GFP* dsRNA treatment, Wilcoxon test, $\chi^2 = 25.218$, $P < 0.001$) (Fig. 6); however, silencing of *nkd* significantly reduced the inci-

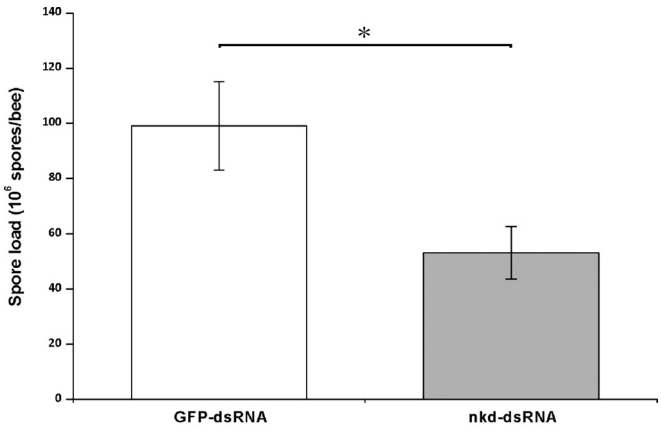

FIG 5 Effect of *nkd* gene silencing on *Nosema* infection levels in adult bees. The *Nosema* infection levels were determined by spore counting. The spore loads of the two groups of bees, the control bees fed with 20 μg/ml *GFP* dsRNA ($n = 11$) and the treatment bees fed with 20 μg/ml *nkd* dsRNA ($n = 8$), were compared. The spore loads are expressed as mean values ± SEM. Significant difference between data was analyzed by independent-sample *t* test and is indicated with an asterisk (*, $P < 0.05$).

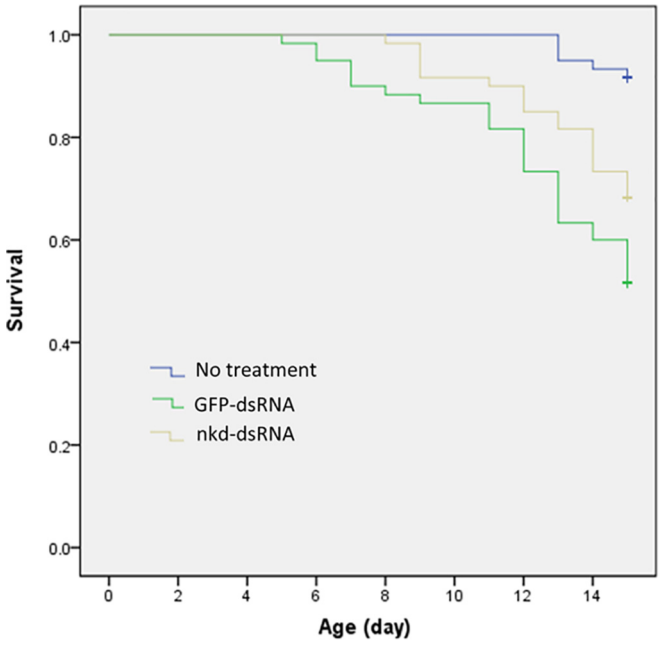

FIG 6 Effect of *nkd* gene silencing on the life span of honey bees infected by *N. ceranae*. Survival curves for bees inoculated with 100,000 *N. ceranae* spores at day 0 (i.e., within 24 h after adult emergence) and fed with 50% (mass/vol) sucrose solution containing 20 μg/ml *nkd* dsRNA (nkd-dsRNA; $n = 60$) or 20 μg/ml *GFP* dsRNA (GFP-dsRNA; $n = 60$) for 15 days and for the bees that did not receive any treatment and were only fed with 50% sucrose solution (no treatment; $n = 60$). Knockdown of *nkd* gene reduced the incidence of death ($P = 0.041$ by Wilcoxon test).

dence of death of adult bees (Fig. 6) (*nkd* dsRNA versus *GFP* dsRNA, Wilcoxon test, $\chi^2 = 4.165$, $P = 0.041$).

DISCUSSION

Significant progress has been made in exploring RNAi as a therapeutic strategy for controlling diseases in honey bees, with much attention focused on pathogen-specific virulence determinants. *Nosema* are obligate intracellular parasites and, therefore, require host cell proteins and pathways to support their replication and many phases of their life cycles (12, 13). In the present study, the identification and characterization of a host-based factor that is required for parasite pathogenesis in hosts allow us to obtain important insights into the host-parasite interactions and to propose a potential drug target against the parasite.

Wnt signaling is an evolutionarily conserved pathway that plays a critical role in embryogenesis and cell proliferation and differentiation (64). Additionally, recent studies found that activation of the Wnt signaling pathway regulates the immune response to certain pathogenic bacterial infections by upregulating the expression of anti-inflammatory factors and downregulating the expression of inflammatory factors (65–68). *nkd* is linked to Wnt signaling and was originally found to act as an inducible antagonist of Wnt signaling during embryonic development in *Drosophila* (52). The results of our study confirmed our hypothesis that *nkd* and Wnt signaling might be also involved in the honey bee responses to *Nosema* infections. Indeed, *Nosema* infection can result in dramatic host transcriptional responses (47–49), and the expression of the *nkd* gene is significantly upregulated during *N. ceranae* infection in honey bees, suggesting that *nkd* and Wnt signaling might be targeted by *N. ceranae* during the infection process to alter the defensive function of hosts.

Knockdown of *nkd* by feeding *Nosema*-infected bees with dsRNA specific to *nkd* led to several remarkable alternations, one of which was the modulation of host immune responses. Previous studies have revealed that *N. ceranae* infection induces immunosuppression in honey bees by downregulating several immune genes, such as the AMP genes *Apidaecin*, *Abaecin*, *Defensin-1*, and *Hymenoptaecin* (69, 70). Our results show that silencing the *nkd* gene in bees infected with *N. ceranae* can reverse immune suppression and enhance the host immune response. The exact mechanism of host immune induction by *nkd* silencing remains unclear. However, silencing of the *nkd* gene ultimately reduces the *Nosema* infection levels and extends the life span of infected adult bees.

Since infection always involves interaction between host and parasite/pathogen, infections can theoretically be controlled by targeting the host of the parasite/pathogen. All previous gene-based efforts to control honey bee parasites or pathogens have targeted these biotic threats directly. Here, by targeting the honey bee *nkd* gene with RNAi, we demonstrate that silencing a honey bee gene can suppress the reproduction of parasites/pathogens and improve the overall health of honey bees. Our results provide a novel host-derived strategy to mitigate honey bee disease. Similar studies have been reported in various species. For example, silencing of the *Cactus* gene, an inhibitor of the Toll pathway, reduces the extent of dengue virus infection in the midgut by fourfold in *Aedes aegypti* mosquitoes (71). Downregulation of *scavenger receptor class B type 1* (*SR-B1*) expression by RNAi dramatically decreases the susceptibility of human hepatoma cells to hepatitis C virus (HCV) infection, resulting in the inhibition of this virus infection (72). These studies together indicate that targeting host factors by RNAi can potentially protect the hosts from infections of parasites/pathogens and promote the overall health of hosts.

RNAi technology has great potential for relieving the impacts of honey bee diseases. Other previous studies (46), combined with our efforts, demonstrate that silencing parasite/host genes by RNAi manipulation is efficient to suppress parasite development and improve honey bee health to some extent in the laboratory. It is likely that the combination of both strategies, meaning targeting both host and parasite genes in the same RNAi manipulation, will lead to better results for controlling *Nosema* infection. As for field application, more experiments are needed to determine the ideal treatment time, the suitable concentration of dsRNA, and other factors. The present study will help direct the application of RNAi to mitigate *N. ceranae* infection in honey bees.

In sum, these studies have identified a host factor required for *Nosema* infection and highlight the potency of host-derived RNAi-based therapeutics to inhibit not only microsporidian parasite infection but also potentially a wide range of pathogens and parasites that cause serious diseases in honey bees.

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