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Apoptosis as a primary defense mechanism in response to viral infection in invasive fire ant *Solenopsis invicta*

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ABSTRACT

Apoptosis is considered an innate defense mechanism of insect hosts at the early stage of pathogen infection. The present study attempts to determine whether apoptosis is involved in defending the fire ant, *Solenopsis invicta* from a natural viral pathogen *Solenopsis invicta* virus 1 (SINV-1). Results of TEM examination and TUNEL assay both revealed the signature of apoptosis in the midgut epithelium of SINV-1-infected fire ant larvae. A time-course study was conducted to monitor changes in the dynamics of SINV-1 viral titers and apoptosis levels in the midgut epithelium of SINV-1-infected larvae. We found that the viral titer significantly decreases as apoptosis level increases, suggesting that the apoptotic epithelium constitutes a barrier against dissemination of SINV-1. These findings serve as the very first empirical evidence for the virus-induced apoptosis in ants and also help explain some previously observed mortality patterns and behavioral alterations associated with SINV-1 in fire ants.

1. Introduction

Dicistroviridae is a viral family of positive-sense, single-strand RNA (ssRNA) viruses that infect arthropod hosts and are usually associated with an epidemic of chronic infections in the hosts, although dicistrovirus infections can vary considerably in virulence and pathogenicity (Bonning and Miller, 2010). Previous studies have demonstrated that dicistroviruses are associated with molting failure, reduced host longevity and fecundity, and even incur significant mortality in host populations (Bailey and Woods, 1977; Bekesi et al., 1999; Golubovsky and Plus, 1982; Williamson et al., 1988). With their host specificity and other traits (Lacey et al., 2015), some dicistroviruses have been considered as potential biological control agents against invasive insect pests such as the red imported fire ant (*Solenopsis invicta*) and Argentine ant (*Linepithema humile*) (Gruber et al., 2017; Valles et al., 2018). As these viruses are currently being evaluated as self-sustaining biological control agents (Valles et al., 2018), knowledge regarding the interactions between them and their host ants represents baseline information to assure the control efficiency of any management framework that may be devised.

Solenopsis invicta virus 1 (SINV-1) is arguably the most well-studied ssRNA virus infecting ants (Valles et al., 2012). SINV-1 is found to mainly replicate at the alimentary canal of larvae in *S. invicta*, and brood death is occasionally found in SINV-1-infected colonies following translocation to the lab from the field (Hashimoto and Valles, 2007; Valles et al., 2004). As late-instar larvae are generally more vulnerable to pathogen infections than other colony members due to the fact that they are the center of nutrient flow in the colony (i.e., they digest solid food particle for the entire colony) (Oi et al., 2005), their innate immune systems must have played a significant role in defending against SINV-1 and possibly other pathogens but remained overlooked.

The programmed cell death pathways (e.g., apoptosis, and necrosis in some cases) are considered the intracellular innate immune mechanisms that restrict the dissemination of pathogens through directly destroying infected cells (Clarke and Clem, 2003; Nainu et al., 2015). In general, necrosis is a passive type of cell death caused by external factors, such as acute viral infection and cell destruction, and ends up with cell lysis, while apoptosis is commonly described as a non-inflammatory response of host against pathogen infection. The microsporidian pathogen of the genus *Nosema* in honeybees is recognized as an excellent model system to study the role of such defensive mechanisms in social insects. For example, Kurze et al. (2015) found that *N. ceranae* is able to manipulate cell apoptosis in *Nosema*-sensitive honeybees but has a negligible effect on tolerant honeybees,

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highlighting the immune function of apoptosis in honeybees when dealing with an intracellular gut pathogen such as microsporidium (Kurze et al., 2015). To our surprise, empirical studies reporting apoptosis as an immune pathway in virus-infected honeybees are lacking, not to mention studies on other groups of social insects such as ants. Although social insect colonies have evolved collective immune defenses against parasites and pathogens (Cremer et al., 2007), we argue that immune responses at the individual level (e.g., apoptosis) may also be important in preventing pathogen epidemics in the colony.

In this study, we therefore hypothesized that apoptosis in the midgut epithelium of the late-instar larvae of *S. invicta* is likely prevalent when challenged with SINV-1 infection. To test this hypothesis, we investigated the apoptotic status of 4th instar larval epithelia challenged by SINV-1 infection. To determine whether apoptosis in the midgut epithelium acts as a barrier to dissemination of SINV-1, we conducted a time-course study to monitor changes in both apoptosis signals and viral titers in the midgut epithelium of SINV-1-infected larvae. Results based on these two experiments are expected to improve our understanding of the interplay between a viral pathogen and its social-insect host, but may also facilitate the development of future control framework on invasive ants.

2. Materials and methods

2.1. Colony collection and viral detection

Colonies of *S. invicta* ($N = 40$) were collected in northern Taiwan, and 10–15 workers were subject to molecular detection for the presence of numerous *S. invicta* viruses (SINV-1, 2 and 3) using species-specific RT-PCR (Hashimoto et al., 2007). SINV-1 and SINV-2 were detected in 60% ($N = 24$) and 25% ($N = 10$) of the collected colonies, respectively. As a result, a total of six colonies were confirmed to be uninfected and thus were maintained under the laboratory conditions for at least one month prior to the experiments.

2.2. Time course of viral replication

We isolated colony fragments, containing 10–20 4th instar larvae and 200 workers, from each of the six uninfected colonies, and maintained the rest of the colonies for sampling of uninfected larvae as control. SINV-1-infected workers were homogenized and ultra-centrifuged by a sucrose density gradient for 2 h at 350,000 rpm at 4 °C (Valles et al., 2004). Subsequently, the viral supernatants were quantified by real-time PCR and stored in −20 °C. Each fragment was then artificially inoculated with SINV-1 by feeding a viral suspension (10^9 genome equivalents/ml) mixed with 10% aqueous solution of honey for 24 h after two days of starvation. One or two larvae (ten in total) were randomly taken from each of the six fragments for detection of viral amplification at four separate time points (1st, 3rd, 5th and 7th day after viral inoculation). Total RNA was extracted from an individual larva using TRIzol reagent (Invitrogen, CA, USA). cDNAs with SINV-1 positive-sense were transcribed with a primer containing the 5' tag sequence GGCCGTCATGGTGGCGAA (Plaskon et al., 2009; Valles et al., 2012). Viral amplification was detected by strand-specific quantitative PCR in an ABI 7500 Real-Time PCR system following the protocol described by Hashimoto et al. (2007). The correlation between inoculation period and viral titer was analyzed with a linear mixed model with the colony as the random effect. The effect of inoculation period was examined by likelihood ratio test by comparing the full model and the model without it as the fixed effect. The models were built by "lme4" package in R (R Development Core Team, 2014) with the function, "lmer".

2.3. Apoptosis detection in the larval midgut

One or two larvae (ten in total) were collected from each of the six artificially infected fragments at four time points and were then subject to TUNEL assay to monitor the apoptosis level, whereas larvae of the same instar also were sampled from the original uninfected colonies as a control group. TUNEL assay is commonly used to detect the DNA fragmentation, a key feature of apoptosis, by staining terminal deoxynucleotidyl transferase (TdT) that catalyzes attachment of modified deoxynucleotides on the DNA strand breaks during the late stage of apoptotic pathway (Darzynkiewicz and Zhao, 2011). Both uninfected and infected larvae were soaked in 20% sucrose for 1 h to avoid tissue damage and were then embedded in Tissue-Tek® O.C.T.™ Compound before being transferred to the cryomolds. The cryo-sections (30 μm) were mounted on the poly-L-lysine-coated glass slides and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature. The slides were processed and stained by TdT according the protocol of an *in situ* Apoptosis Detection Kit (TaKaRa, Japan). The apoptotic signals labeled with fluorescence-dUTP were monitored by fluorescence microscopy with a 20 × fluorescent objective. We focused on the larval midgut for detecting the signal of apoptosis because replication of SINV-1 has been reported to center in this particular part of the alimentary canal (Valles, 2012). We measured the TdT-positive area in the midgut tissue in each tissue section using ImageJ (NIH, imagej.nih.gov), and estimated apoptosis rate by dividing the TdT-positive area by the total area of midgut (apoptosis rate = TdT-positive area/total area of midgut). The effect of time elapsed since inoculation on apoptotic status was analyzed using the generalized linear mixed model with binomial errors and colony as random effects. The significance of the effect was examined by the likelihood ratio test by comparing the full model and the model without it as the fixed effect. The models were built by "lme4" package with the function, "glmer".

2.4. Ultrastructural examination

To understand the morphological alteration(s) associated with SINV-1 in the midgut epithelium, we collected 4th instar larvae from uninfected colonies and infected fragments of the same colonies on the 7th day after viral inoculation. The fire ant larvae were dissected and fixed overnight with 1% glutaraldehyde in 0.05-M cacodylate buffer (pH = 7.0), and secondarily fixed with 1% 0.05 M OsO_4 solution in cacodylate buffer for 1 h. The fixed tissues were then dehydrated through serial acetone. The larvae were embedded in Spurr resin (Spurr, 1969), and tissues were identified in 1-μm-thick sections using Toluidine blue O stains (TBO). Ultra-thin (100 nm) sections were stained with uranyl acetate and lead citrate and visualized under transmission electron microscopy (Hitachi H-7650, TC5 Bio-image Tools, NTU).

3. Results

3.1. Dynamics of viral replication and apoptosis signals

We characterized host-pathogen dynamics by examining changes in viral replication at four time points after infection. Results indicated a trend of viral amplification reduction with the time elapsed since inoculation; a significant negative correlation was found between the two parameters (p-value < 0.001) (Fig. 1a, Table 1). As detected by TUNEL assay, only a few apoptotic cells were found in the midgut of the uninfected larvae and infected larvae during the early viral inoculation (the 1st and 3rd day). Apoptotic cells, however, were abundant in the midgut epithelium of the infected larvae on the 5th day and reached a peak on the 7th day after inoculation during our experimental period (Fig. 1b and c). The delayed increase in apoptotic level may simply reflect the nature of TUNEL assay as DNA fragmentation is a late-stage event during the apoptotic progression (Collins et al., 1997; Wadskog et al., 2004). The intensity, as expressed by apoptosis rate, was positively correlated with the time elapsed since initial inoculation (p-

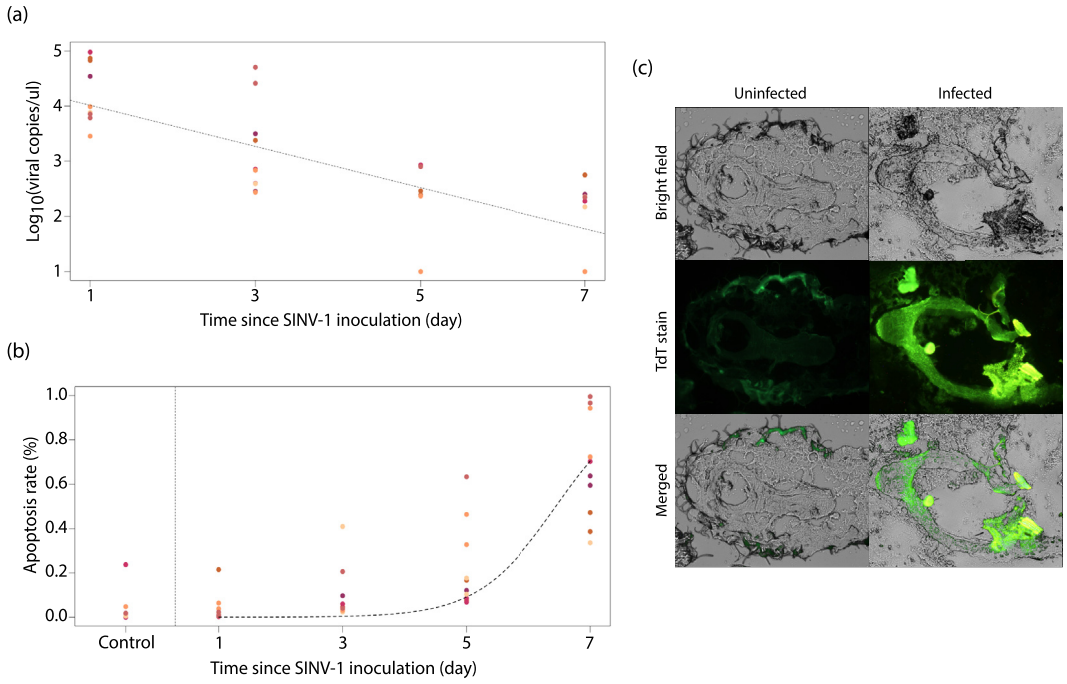

Fig. 1. A negative correlation between viral titer and apoptotic level during the first 7 days after artificial SINV-1 infection. a) viral titers across four time points; b) apoptosis rates across four time points; c) the proportions of TdT-positive tissue in the midgut epithelium by TUNEL assay in uninfected and infected larvae (original magnification: 20 ×). Note that a similarly low apoptosis level is observed among all uninfected larvae, and hence we only present the apoptosis level of an individual larva in (c).

Table 1
The significances of "viral titer" and "apoptotic level" after viral inoculation.

Source	β_0	β_1	*d.f.*	X^2	P
Viral titer	3.387	−0.373	1	37.127	< 0.001
Apoptotic level	−10.224	1.585	1	21.215	< 0.001

value < 0.001) (Fig. 1b, Table 1).

3.2. Impacts of SINV-1 infection on the epithelial cell morphology

The microscopy examination showed that the midgut epithelium of uninfected larvae remains functionally active as evidenced by abundant lipid droplets and cell organelles such as mitochondria and endoplasmic reticulum (Fig. 2a–d). Furthermore, the microvilli-covered apical membrane contained bundles of filaments, suggesting maintenance of the functions and properties of the midgut epithelium (Fig. 2a). Unlike uninfected larvae, numerous apoptotic cells were found in midgut epithelium of infected larvae (Fig. 2e). The ultrastructure of the midgut epithelium in the infected larvae was characterized by chromatin condensation and reduction of the number of the microvilli; in addition, quite a few cytoplasmic vacuoles were present at the base, suggesting that the cells had undergone a series of degradation processes (Fig. 2a and b). Numerous midgut epithelial cells in SINV-1-infected larvae were found to have swollen cytoplasm, nuclear fragmentation and condensed chromatin (Fig. 2e and f). Furthermore, reduced numbers of organelles and less blank space in some epithelial cells were observed after infection (Fig. 2e and f). We found plenty of cytoplasmic vacuoles rather than organelles in the infected larvae (Fig. 2f). Moreover, the structural disorganization and dilated intercristal space suggested mitochondria were in the early stages of degeneration (Fig. 2g). The formation of apoptotic bodies in a midgut epithelial cell was observed in the late stages of apoptosis (Fig. 2h). On the contrary, few epithelial cells, if any, were observed to be undergoing apoptosis in the midgut epithelium of uninfected larvae. Hence, the presence of apoptotic cells in the midgut epithelium was confirmed to be a response to the challenge by SINV-1.

4. Discussion

While viruses are considered potential biological control agents against invasive fire ants (Valles et al., 2018), studies towards a better understanding of the complex interactions between microbe and host ant are much needed to assure control efficiency. This study demonstrated that SINV-1 infection induces apoptosis in the midgut epithelium of fire ants and that the viral replication of SINV-1 decreases as the number of apoptotic cells increases. These data are consistent with previously observed tissue tropisms for SINV-1 (Hashimoto and Valles, 2007) and other insect-infecting positive-sense ssRNA viruses (Valles, 2012). These findings also suggest that apoptosis of midgut epithelium cells in fire ant larvae likely serves as a barrier to prevent SINV-1 dissemination, which in turn may explain the low virulence of SINV-1 in fire ants. However, it remains unclear how a high SINV-1 titer might be retained in field colonies of fire ants, as has been observed in some cases (Hashimoto and Valles, 2007). One possibility is that apoptosis is only effective in the containment of viral dissemination when the viral titer is below a certain threshold; indeed, apoptosis is considered a threshold-dependent response (Fragkoudis et al., 2009). In this study, we fed the experimental colonies with a fixed viral dose only at the beginning of the experiment, with only a 24-hr inoculation period; hence, the titer may well have been sub-threshold.

While our results showed that an apoptotic midgut epithelium may function as a potentially effective defensive mechanism against SINV-1, significant brood mortality has been observed in SINV-1-infected colonies, particularly when colonies are challenged with certain stressful conditions (e.g., colony translocation) (Valles et al., 2004). Many arthropod-infecting positive-sense ssRNA viruses share a similar chronic, asymptomatic infection nature but cause overt symptoms under certain circumstances including rapid viral replication under environmental stress triggers (Chen and Siede, 2007; de Miranda and Genersch, 2010),

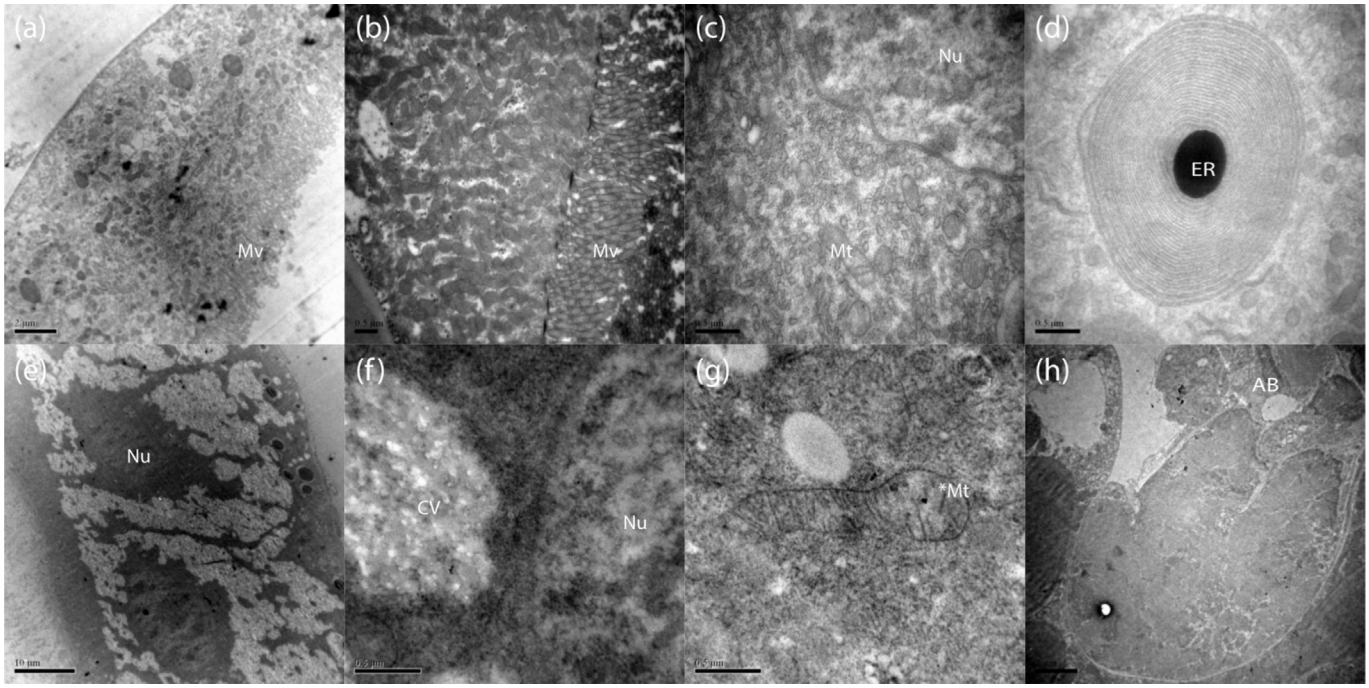

Fig. 2. Ultrastructure features of the larval midgut epithelial cells collected from uninfected colonies [a-d] and infected colonies [e-h]: (a) epithelial cells filled with organelles; (b) microvilli are abundant in the epithelial cells; (c) numerous mitochondria observed in a healthy epithelial cell; (d) normal structure of the endoplasmic reticulum; (e) nuclear condensation; (f) reduction of organelles and formation of cytoplasmic vacuoles; (g) structural disorganization and dilated intracristal space of mitochondria; (h) the formation of apoptotic bodies. [Note: AB, apoptotic bodies; CV, cytoplasmic vacuoles; ER, endoplasmic reticulum. Mt, mitochondria; Mv, microvilli; Nu, nucleus].

and SINV-1 seems to be no exception (Valles, 2012). This possibility is supported by multiple recent studies in which widespread apoptosis in response to pathogen challenge resulted in increasing mortality of various insect hosts due to overwhelming immunopathology (Wang et al., 2012). Studies examining the interactions of viral titer, environmental stress, apoptosis level and colony mortality are needed in the future.

Recent evidence indicates that SINV-1 infection alters the food preference of fire ants towards carbohydrate-rich foods compared to uninfected congeners (Hsu et al., 2018; Valles et al., 2013, 2014), which is believed to prefer a high-protein, high-lipid diet (Stein et al., 1990; Vander Meer et al., 1995). Late-instar larvae in fire ants are the primary colony members that digest solid food and redistribute the food through trophallaxis in liquid form to nestmates (Cassill and Tschinkel, 1999), and it is likely that an increased level of epithelial apoptosis as result of defending against SINV-1 infection may impair the digestive capabilities of these larvae (Hausmann, 2010; Schafer et al., 2013; Sonakowska et al., 2016), driving workers to forage only liquid foods such as honey solution in response to the larval incompetence. Furthermore, larval conditions in a colony play a critical role in regulating the ontogeny of workers' forging behaviors (Ulrich et al., 2016). This may also explain why SINV-1-infected fire ants forage much less, another key reported behavioral change induced by SINV-1 infection (Hsu et al., 2018). Hence, the reduced foraging performance of workers may simply reflect larval status (e.g., loss of appetite) associated with widespread SINV-1-induced apoptosis in the intestinal epithelium. The results of TEM examination appear to support this possibility given the fact that the morphology of the midgut epithelium in infected larvae is significantly altered with apparent reduction of organelles and nuclear deformation, which likely reduces the functions of food processing (e.g., secretion of digestive enzymes) and thus digestion competence in SINV-1-infected larvae (Huang et al., 2015).

Accumulating evidence in other species has shown that pathogen-induced apoptosis may potentially incur fitness costs (Medzhitov et al., 2012; Sonakowska et al., 2016), leading researchers to suggest disease tolerance as an alternative, less costly defensive mechanism in coping with infection through anorexia (Povey et al., 2014) or dietary changes (e.g., compensatory feeding) (Abbott, 2014; Medzhitov et al., 2012; Shikano and Cory, 2016). SINV-1 in fire ants seems to be an excellent model system for such studies, as similar illness behaviors including reduced foraging activities (analogical to anorexia) and shifts in macronutrient preference have been reported in SINV-1-infected fire ants (Hsu et al., 2018). Furthermore, the host-virus interactions during early (acute) and late (chronic) infection phases may involve different immune cascades, host immune responses and gene expression profiles (Belov et al., 2003; Buenz and Howe, 2006; Croft et al., 2017). Thus, further studies on SINV-1 and fire ants would facilitate our understanding of early-late switch temporal regulations associated with a focal virus, and provide useful knowledge for integrating viruses into current control schemes for fire ants.

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