Antiviral Role of Apoptosis-Dependent Phagocytosis of Virus-Infected Cells in *Drosophila*

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Dissertation

Antiviral Role of Apoptosis-Dependent Phagocytosis of Virus-Infected Cells in *Drosophila*

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SUMMARY

To ensure host survival, virus-infected cells are targeted for elimination by host immune mechanisms. While vertebrates successfully accommodate such task to their sophisticated immune system, the mechanism how invertebrates, being only equipped with a simple innate immunity, accomplish this vital responsibility remains unknown. Our lab previously reported that influenza virus-infected cells are engulfed by macrophages in a manner dependent on apoptosis leading to the mitigation of viral threat in mice. Considering that phagocytes are the sole immune cells present in most invertebrates, including insects, I here examined if such mechanism, may also play an antiviral role in *Drosophila*.

I established *in vitro* and *in vivo* infection model systems using *Drosophila* C virus. Infection of *Drosophila* S2, an embryonic cell-derived cell line, was characterized by massive production of progenitor virus accompanied by an elevated level of cells with apoptosis-like properties. Such cells were phagocytosed by l(2)mbn, a larval phagocyte-derived cell line, in a manner inhibitable by either a caspase inhibitor, phosphatidylserine-containing liposomes, or by RNA interference-based knockdown of engulfment receptors in phagocytes. Furthermore, the pathogenic effect of *Drosophila* C virus *in vivo* was increased when flies were pre-treated with latex beads or manipulated to ectopically express a phosphatidylserine-binding protein to inhibit phagocytosis. Taken together, these results suggested that apoptosis-dependent phagocytic removal of virus-infected cells exists as a part of innate immunity and is evolutionarily conserved from flies to mice.

INTRODUCTION

Viral infection is one of the most significant maladies giving a life-threatening pressure to all living organisms (1, 2). For the affected species to survive, successful adaptation to such biological pressure is, therefore, indispensable. One of means available to achieve this purpose is the active engagement of various host defense mechanisms against the incoming viral threat. As viruses are required to enter cells of host organisms due to a lack of machinery to produce proteins, there should be two chances for the host to protect themselves against successful virus invasion, at the entrance and inside the cells. Host strategies to fight against viral attack is represented by the immune system that recognizes pathogens, alerts the body of invasion, evokes and amplifies biological reactions, kills and eliminates virus, and cures damaged tissues (3). Failure to perform such tasks may lead to the impairment in cellular homeostasis, development of infectious disease, and, to the worst, lethality to the infected host (4).

In response to foreign viral particles, vertebrates, including mammals, employ an extensive array of cellular defense mechanisms, starting from the production of interferon either by a classical Toll-like receptor-mediated pathway (5, 6) or a recently defined pathway involving mitochondrial antiviral-signaling protein (7) and cyclic GMP-AMP synthase-stimulator of interferon genes (8, 9), which is categorized into an built-in or innate arms of the immune system, to a more sophisticated and specific reactions mediated by the antibody in an adaptive part of defense mechanism (10). In addition to these, a self-consumption process termed autophagy as well as dedicated RNA interference-pathway targeting viral genomes have been added to a list of major innate antiviral arsenals (11-13).

Similar protection systems have also been described in evolutionarily lower organisms such as invertebrates. However, unlike vertebrates, invertebrates including insects are only equipped with an innate part of the immunity system (14, 15), leaving them with fewer options to resist infection compared to higher organisms. Nevertheless, mechanisms that are based on RNA

interference (16-19) and autophagy (20-23) have been proposed to be efficient in dealing with a wide range of viruses in insects. By recognizing the characteristics of viral entities, host organisms may restrict viral behavior, attachment on the surface of host cells, replication in host cells, and escape from host cells for further invasion. However, in the epic struggle between host and virus, viruses also have evolved their strategies for evading immune responses and achieving flawless hijack of host cells to produce daughter virions (24-26).

In addition to virus entities, virus-infected cells can be targeted for elimination by host immune system (2, 3). In vertebrates such as mammals, this is achieved through the actions of natural killer (27, 28) and cytotoxic CD8-positive T (29, 30) cells to recognize viral components that have been processed and expressed at the surface of infected cells. In addition to such mechanisms, another procedure by which infected cells are recognized independent of viral components: cells infected with virus are induced to undergo apoptosis and eliminated by a mechanism of phagocytic clearance that generally targets apoptotic cells. This was first shown with cells infected with influenza virus that are recognized and engulfed by macrophages and neutrophils (31-33). However, whether or not this mechanism is available in invertebrates remains to be known.

As are vertebrates including humans, invertebrates, including insects, are suffered from infection with a broad range of viruses (34-36). Although well-defined antiviral cellular effectors of vertebrates, such as natural killer cells and cytotoxic CD8-positive T cells, are absent in invertebrate animals, they have managed to survive and existed on earth. Therefore, invertebrates should possess a robust mechanism to deal with virus-infected cells during viral invasion. I decided to challenge this issue, elucidation of a cellular mechanism responsible for the elimination of virus-infected cells in invertebrates, using fruit fly *Drosophila melanogaster* as host for viral infection with the hypothesis that virus-infected cells are removed by apoptosis-dependent phagocytosis.

MATERIALS AND METHODS

Fly stocks and cell culture

The *Drosophila* embryonic cell-derived cell line S2 was used as host cells for virus infection and target cells in an assay for phagocytosis *in vitro*. S2 cells were cultured at 25 °C with Schneider's Drosophila medium (Life Technologies Japan, Tokyo, Japan) containing 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. l(2)mbn, another cell line established from larval hemocytes of a tumorous *Drosophila* mutant, was maintained in a similar fashion to S2 cells and used as the phagocytes in an assay for phagocytosis after treatment with the molting hormone 20-hydroxyecdysone (Sigma-Aldrich Japan, Tokyo, Japan) at 1 μM for 48 h. All fly lines, listed in Table 1, were raised with standard laboratory cornmeal-agar medium at 25 °C. w^{1118}, a common white-eye laboratory stock of *Drosophila* (provided by Bloomington Drosophila Stock Center, Indiana University, Bloomington, IN, USA), was used as a general control for the *in vivo* infection experiments. $betaInt\text{-}nu^{2}$ (37) and $drpr^{\Delta 5}$ clone 15 were used as mutant flies lacking Draper and integrin βv, respectively. The original $drpr^{\Delta 5}$ line (38) was backcrossed with w^{1118} to obtain a stable fly line, which was later named as 'clone 15'. *UAS-MFGE8* and *UAS-MFGE8-ΔC2* were 'UAS lines' for the expression of milk fat globule epidermal growth factor 8 (MFG-E8) and MFG-E8 lacking C2 domain (MFGE8-ΔC2) (39). *UAS-CD8::PARP::Venus* is another UAS line for the expression of human poly(ADP-ribose) polymerase (PARP) fused to a portion of murine CD8 and the fluorescence protein Venus as a membrane-bound form (CD8-PARP-Venus) (40). *da-Gal4* is a 'GAL4 driver' for the ubiquitous expression of GAL4 and mated with the above-described UAS lines for the expression of the trans-genes. *pxn-Gal4 UAS-GFP* (41) is a fly line where hemocytes are labelled with green fluorescence protein (GFP).

Table 1. Fly lines used in this study

No.	Fly line	Description
1	*w*^{1118}	control fly line
2	*drpr*$^{\Delta 5}$ clone 15	mutant fly line lacking Draper
3	*betaInt-nu*^{2}	mutant fly line lacking integrin βν
4	*betaInt-nu*^{2}*; drpr*$^{\Delta 5}$	mutant fly line lacking both Draper and integrin βν
5	*UAS-MFGE8*	UAS line for MFG-E8 expression
6	*UAS-MFGE8-ΔC2*	UAS line for MFGE8-ΔC2 expression
7	*UAS-CD8::PARP::Venus*	UAS line for CD8-PARP-Venus expression
8	*da-Gal4*	GAL4 driver for ubiquitous expression of GAL4
9	*pxn-Gal4 UAS-GFP*	fly line with GFP-labelled hemocytes

Preparation, titration, and UV-inactivation of *Drosophila* C virus

Drosophila C virus (DCV), natural pathogen for *Drosophila*, was used to infect culture cell lines and adult flies. To prepare a stock of DCV, semi-confluent S2 cells were incubated with an aliquot of original DCV stock (a gift from Takayuki Kuraishi of Tohoku University) at 25 °C, and the culture media were collected at day 4. A 50% tissue culture-infective dose ($TCID_{50}$) of the harvested culture media was determined by the Reed-Muench titration approach (42). In brief, S2 cells (2.5×10^4) were seeded in wells of a 96-well culture dish with 150-µl medium and incubated overnight. On the next day, they were inoculated with the harvested DCV-containing culture medium at 10-fold serial dilutions (50 µl/well), 8 wells for each dilution, and further incubated at 25 °C for 7 days. All wells were assessed for the presence of cell debris and the loss of cell confluency, indicative of DCV infection, and a virus titer was determined according to an established procedure (43). The culture medium gave a DCV concentration of 1.6×10^9 $TCID_{50}$/ml and was kept frozen at -80 °C as a stock until use. UV-inactivation of DCV was carried out by exposing the original virus stock to 15W lamp emitting 253.7-nm light at a distance of 10 cm for 5 min at room temperature, and this was repeated for 3 times.

Virus infection and determination of virus titer

In the *in vitro* experiments, S2 cells were treated with either DCV (40 $TCID_{50}$) or Schneider's Drosophila medium alone (mock infected), and maintained at 25 °C until further analysis. In the *in vivo* experiments, adult flies were infected with DCV according to an established procedure (44) with slight modification. Male flies 3~6 days after eclosion were anesthetized with CO_2 and abdominally injected with DCV (8,000~80,000 $TCID_{50}$ in 50 nl) using a N_2 gas-operated microinjector (IM300; Narishige, Tokyo, Japan). As a negative control, flies were injected with PBS. All flies were subsequently maintained in a 25 °C incubator (15-20 flies per vial, 3 vials for each experiment). Flies that died within 2 h were excluded from the analysis, considering that they were injured with a needle. To determine the growth of DCV after infection, media of S2 cell cultures, lysates of S2 cells, and lysates of whole flies were subjected to an assay for virus titer. For preparing S2 cell lysates, 10^7 pelleted cells were lyzed with 100 µl of a buffer consisting of 50 mM Tris-HCl (pH 7.5), 0.1% (v/v) Nonidet P-40, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 0.15 M NaCl, 5 mM $MgCl_2$, and 1% (v/v) protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) (*Drosophila* lysis buffer), centrifuged at 14,000 rpm for 10 min, and supernatants were collected. For the preparation of fly lysates, five live flies were chosen randomly and homogenized with 100 µl of *Drosophila* lysis buffer using a plastic pestle, centrifuged at 14,000 rpm for 10 min, and supernatants were collected as test samples. Prior to use, samples were diluted based on the expected virus concentration, which ranged from 10^{-2} to 10^{-13}.

Western blotting

Lysates of culture cell lines and whole flies were subjected to a Western blotting analysis according to established procedures. Primary and secondary antibodies used are listed in Table 2.

Table 2. List of antibodies used in this study

No.	Product	Types of antibodies	Description
1.	anti-drICE	Primary	Rabbit-origin, polyclonal, affinity-purified
2.	anti-PARP	Primary	Rabbit-origin, polyclonal, affinity-purified
3.	anti-DIAP1	Primary	Rabbit-origin, antiserum
4.	anti-Draper	Primary	Rat-origin, antiserum
5.	anti-integrin βν	Primary	Rat-origin, antiserum
6.	Anti-Croquemort	Primary	Rat-origin, antiserum
7.	anti-DmCaBP1	Primary	Rat-origin, antiserum
8.	anti-DCV	Primary	1. Chicken-origin, polyclonal, affinity-purified 2. Rabbit-origin, polyclonal, affinity-purified
9.	HRP-conjugated anti-chicken IgY	Secondary	Rabbit-origin, polyclonal, affinity purified
10.	HRP-conjugated anti-rabbit IgG	Secondary	Donkey-origin, polyclonal, affinity purified
11.	AP-conjugated anti-rat IgG	Secondary	Goat-origin, polyclonal, affinity purified

Anti-drICE (#13085) and anti-PARP (#9542), which were raised by immunizing rabbits with a synthetic peptide corresponding to residues Asp230 of drICE and a synthetic peptide corresponding to the caspase cleavage site of human PARP, respectively, were purchased from Cell Signaling Technology Japan, Tokyo, Japan. Anti-*Drosophila* inhibitor of apoptosis protein 1 (DIAP1) was raised in rabbits after immunization with a recombinant DIAP1 protein (45) and provided by Masayuki Miura of the University of Tokyo. Anti-Draper, anti-integrin βν, and anti-DmCaBP1 were rat anti-sera obtained after immunization with recombinant proteins containing the intracellular portions (amino acid positions 881-1200) of Draper (46), the intracellular regions (amino acid positions 753-799) of integrin βν (47), and full length of DmCaBP1 (48), respectively. I used two differently prepared anti-DCV antibodies: anti-DCV

chicken antibody (44) was kindly provided by Sara Cherry (University of Pennsylvania); and anti-DCV capsid polyprotein antibody (ab92954) was purchased from Abcam (Cambridge, UK). To locate the primary antibodies, horse radish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (GE Healthcare Bio-Sciences KK, Tokyo, Japan), HRP-conjugated anti-chicken IgY antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and alkaline phosphatase (AP)-conjugated anti-rat IgG antibody (KPL, Gaithersburg, MD, USA) were employed as the secondary antibodies, and Western Lightning (PerkinElmer Japan Co., Ltd., Yokohama, Kanagawa, Japan) and Immune-Star system (Bio-Rad Laboratories Co., Ltd., Tokyo, Japan) were used as substrates for luminescence reactions with HRP and AP, respectively.

Immunochemical detection of DCV

Mock-infected and DCV-infected S2 cells were washed twice with PBS and subsequently treated with 2% (w/v) paraformaldehyde, 0.1% (w/v) glutaraldehyde, and 0.05% Triton X-100, and then with methanol for fixation. These cells were moderately washed with PBS containing 0.1% Triton X-100 (PBST), treated with PBST containing 5% (v/v) whole swine serum for blocking, and incubated with PBST containing anti-DCV antibody at 4 °C overnight. Samples were then washed with PBS, incubated with PBS containing appropriate HRP-labelled secondary antibodies at room temperature for 1 h, and were washed with PBS. They were subjected to an enzymatic reaction of HRP with 3,3'-diaminobenzidine tetrahydrochloride (DAB) as a substrate and examined by microscopy. In some experiments, samples were further stained with hematoxylin to locate the nucleus.

Phagocytosis assay

To examine phagocytosis *in vitro*, S2 cells (2×10^6) mock-infected or infected with DCV were mixed with 20-hydroxyecdysone-treated l(2)mbn cells (2×10^5) in triplicate wells of 24-well culture plates with 1 ml of Schneider's Drosophila medium supplemented with 1% fetal

bovine serum at 25 °C for 2 h. The cells were immunostained with anti-DCV antibody and examined under a microscope for the occurrence of phagocytosis. The ratio of l(2)mbn cells that had engulfed DCV-positive S2 cells as well as the number of target cells engulfed by 100 l(2)mbn cells were determined. To examine phagocytosis *in vivo*, hemocytes contained in hemolymph collected from adult flies were subjected to a cytochemical analysis according to published procedures (49) with modifications (personal communication, Róbert Márkus of the University of Nottingham). Briefly, adult males of *pxn-Gal4 UAS-GFP* mock-infected or infected with DCV were anesthetized with CO_2, and a small slit was made at the last abdominal region using a forceps. Then, PBS was inoculated into the thorax using a glass capillary and discharged onto an aminosilane-coated glass slide. More than 90% of cells collected from hemolymph in this way were positive for GFP (see Fig. 16A and 16A') and thus considered to be hemocytes. These cells were subjected to hematoxylin staining as well as immunostaining with anti-DCV antibody. Cells containing homatoxylin-stained two nuclei and positive for the capsid protein of DCV were considered as adult hemocytes that had engulfed DCV-infected cells.

Other materials and methods

The extents of viability and chromatin condensation of DCV-infected S2 cells were assessed by dye exclusion test with trypan blue and staining with Hoechst 33342, respectively, according to established procedures. For the examination of DNA fragmentation, a TUNEL assay was conducted, and the resulting signals were visualized by HRP-cleavage of DAB (47) (ApopTag Peroxidase In Situ Apoptosis Detection Kit; Merck Millipore, Darmstadt, Germany). To inhibit caspase-mediated apoptosis, the synthetic peptide benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) (Peptide Institute, Inc., Osaka, Japan) was included in medium of S2 cell cultures at 20 μM 1 h prior to DCV infection. Expression of Draper and integrin βν in l(2)mbn cells was inhibited by RNA interference by treatment with double-stranded RNA, as described previously (46). To inhibit phosphatidylserine-mediated

phagocytosis *in vitro*, liposomes consisting of phosphatidylcholine and phosphatidylserine at a molar ratio of 7:3 or phosphatidylcholine only, prepared as described previously (50), were included in the reaction of phagocytosis. For the inhibition of phagocytosis in flies, two approaches were adopted: one, adult flies were abdominally injected with 2 μM-diameter latex beads (Life Technologies Japan) into the abdomen of adult flies 6 h prior to DCV infection (49); two, phosphatidylserine-binding MFG-E8 was forcedly expressed in adult flies harnessing the use of GAL4-UAS system (39).

Data processing and statistical analysis

Unless otherwise stated, data obtained from three independent experiments were subjected to an appropriate statistical analysis and expressed as the mean ± standard deviation. In some cases, for example Western blotting, a representative of at least two independent experiments that yielded similar results was shown. Number of replication in each experiment is specified in the corresponding figure legends. In an assay for fly survival, a single-vial experiment was first conducted at least three times to confirm reproducibility, and a 3-vial experiment was then carried out as a final experiment. Statistical analyses were performed by log-rank test (Kaplan-Meier method) for the data obtained in an assay for fly survival or two-tailed Student *t*-test for all other data. *p* values are indicated in figures or figure legends, and any *p* values of less than 0.05 were considered significant.

RESULTS

Establishment of virus infection platform in *Drosophila* S2 cells

Our laboratory previously reported that mammalian cells undergo apoptosis upon infection with influenza virus and subsequently eliminated by host macrophages by apoptosis-dependent phagocytosis (32, 33, 51). Due to the fact that phagocytosis is an ancient process conserved within metazoan species (52, 53), I hypothesized that a similar phagocytosis-mediated antiviral reaction might also available in other organisms, including insects.

To examine this, I started to establish an *in vitro* infection system using S2 cells, an embryonic cell-derived *Drosophila* cell line, and *Drosophila* C virus (DCV), a natural pathogenic virus of *Drosophila*. S2 cell cultures were incubated in the absence or presence of DCV for three days and examined, in a time course manner, under the microscope to investigate morphological changes occurring in the host cells, if any, during virus infection. From microscopic observation, it is revealed that DCV-infected S2 cells had a tendency to shrink to become debris-like small particles (Fig. 1). Such a characteristic is typical of the cytopathic effect (CPE) of viral infection.

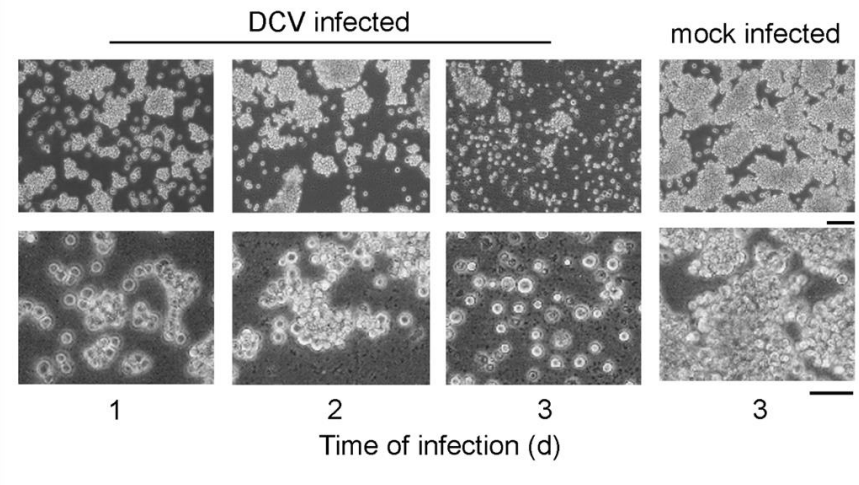


Fig. 1 Morphological changes of DCV-infected cells.
S2 cells were subjected to microscopic analysis at the indicated time points after infection with DCV. The panels at the bottom are higher-magnified views of the squared area in the top panels. Data from one of two independent experiments that yielded similar results are presented. Scale bars, 100 μm (*top*) and 50 μm (*bottom*).

CPE is often correlated with viral growth. To assess this, I carried out two independent time-course experiments to determine the expression level of DCV capsid proteins and the dynamics of virus concentration in infected cell cultures over time. S2 cells were incubated with or without DCV for five days and subjected to two subsequent experiments: Western blotting using anti-DCV capsid protein antibody and viral titer examination by the $TCID_{50}$ method.

The intensity of DCV capsid proteins, visualized as two signals corresponding to proteins with molecular masses of 38 and 29 kDa in Western blotting using anti-DCV antibody (Fig. 2), as reported previously (44), continued to increase up to three days post infection and was maintained until the end of experiment. In addition, the release of biologically active DCV in the culture medium was elevated in a time-dependent manner (Fig. 3). Both data indicate that S2 cells are permissive for infection with DCV.

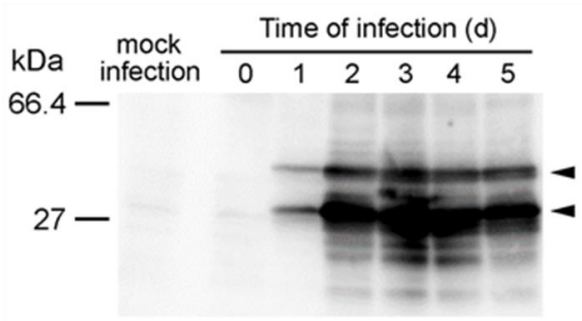


Fig. 2 Expression of DCV capsid proteins during infection.
S2 cells were lysed at the indicated time points and analyzed by Western blotting with anti-DCV antibody. A portion of the blotted membrane after a chemiluminescence reaction is shown. The arrowheads denote positive signals. Data from one of two independent experiments that yielded similar results are presented.

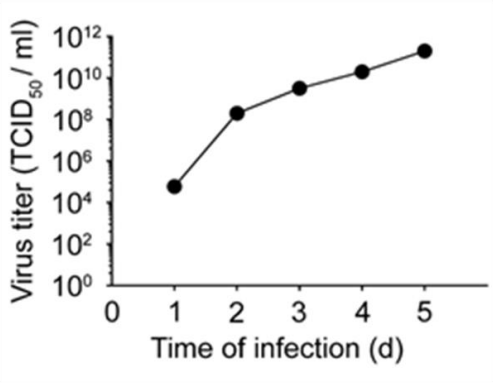


Fig. 3 Propagation of DCV during a course of infection.
Culture medium was collected at the indicated time points and analyzed for the virus titer. Representative data of two independent experiments that gave similar results are shown.

Occurrence of apoptotic cell death in *Drosophila* S2 cells in response to DCV infection

Cells may undergo apoptosis when exposed to life-threatening stimuli, and the observation of cell debris in S2 cell cultures upon infection with DCV suggests the occurrence of this type of cell death (54, 55). To examine this possibility, DCV-infected cells were analysed for biochemical changes often evident in apoptosing cells, including a decrease in the activity of excluding a membrane-permeable dye, the occurrence of chromatin condensation, the activation of caspases, and the fragmentation of nuclear DNA (46).

A dye exclusion test was carried out using trypan blue, and I found that the number of S2 cells remaining stained with this dye increased upon infection with DCV (left panel in Fig. 4).

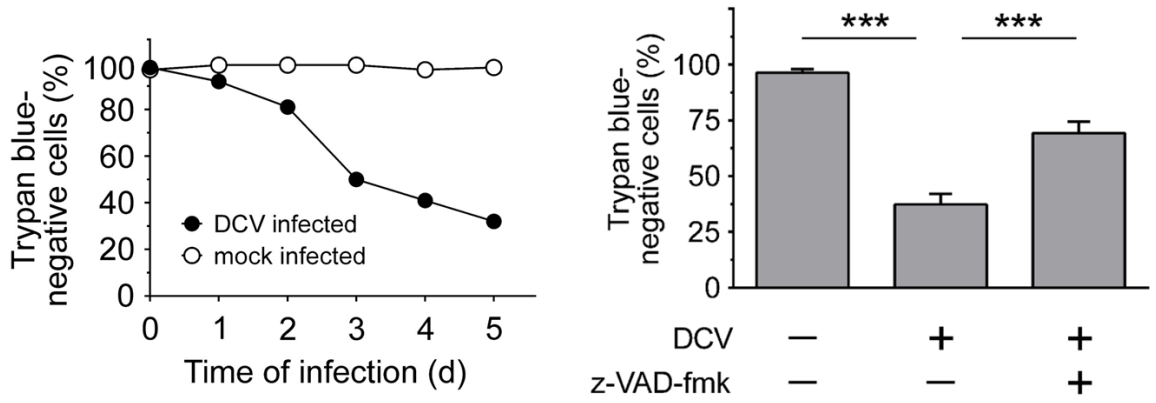


Fig. 4 Caspase-dependent decrease of dye exclusion in S2 cells upon DCV infection. (**left**) S2 cells collected at the indicated time points were subjected to a dye exclusion test with trypan blue. Data representative of four independent experiments that yielded similar results is shown. (**right**) S2 cells were infected with DCV for 3 days in the presence or absence of z-VAD-fmk and analyzed for viability. Mean ± SD of the data obtained from three independent experiments are shown. ***$p < 0.001$.

This indicated that DCV-infected cells lose activity of excluding trypan blue. The occurrence of chromatin condensation was assessed by staining S2 cells with the DNA-binding fluorescence dye Hoechst 33342. An observation by fluorescence microscopy revealed that the ratio of S2

cells containing intensely stained nuclei was raised in a time-dependent manner after DCV infection (left panel in Fig. 5). Either change was reversed, though partly, when S2 cells were infected with DCV in the presence of z-VAD-fmk, an inhibitor of caspases (right panels in Figs. 4 and 5). These results suggested that DCV infection induces caspase-dependent apoptosis in S2 cells.

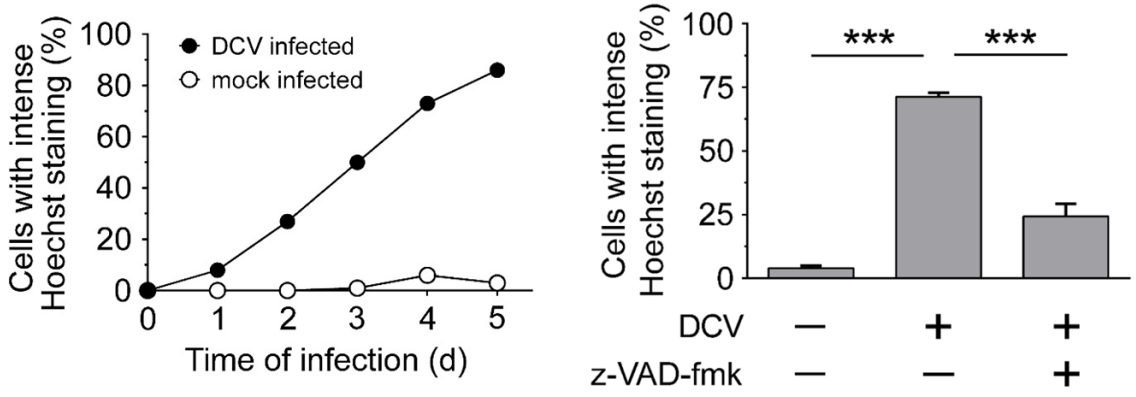


Fig. 5. Occurrence of chromatin condensation in S2 cells after DCV infection
(**left**) S2 cells were collected at the indicated time points of infection, stained with Hoechst 33342, and examined under a fluorescence microscope. Data are representative of four independent experiments that yielded similar results. (**right**) S2 cells were infected with DCV for 3 days in the presence or absence of z-VAD-fmk and similarly analyzed. Mean ±standard deviations of the data obtained from three independent experiments are shown. *** $p < 0.001$.

To further confirm the induction of apoptosis in S2 cells by DCV infection, I examined another biochemical indication of apoptosis, cleavage of chromosomal DNA. I employed an assay called terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) and applied this method to the analysis of DCV-infected cells. Some portion of S2 cells became positive for TUNEL after infection with DCV (left panel in Fig. 6), and such cells increased in a time-dependent manner (right panel in Fig. 6). These results indicated the fragmentation of chromosomal DNA in S2 cells infected with DCV and allowed us to draw the conclusion that DCV infection induces typical caspase-mediated apoptosis in S2 cells.

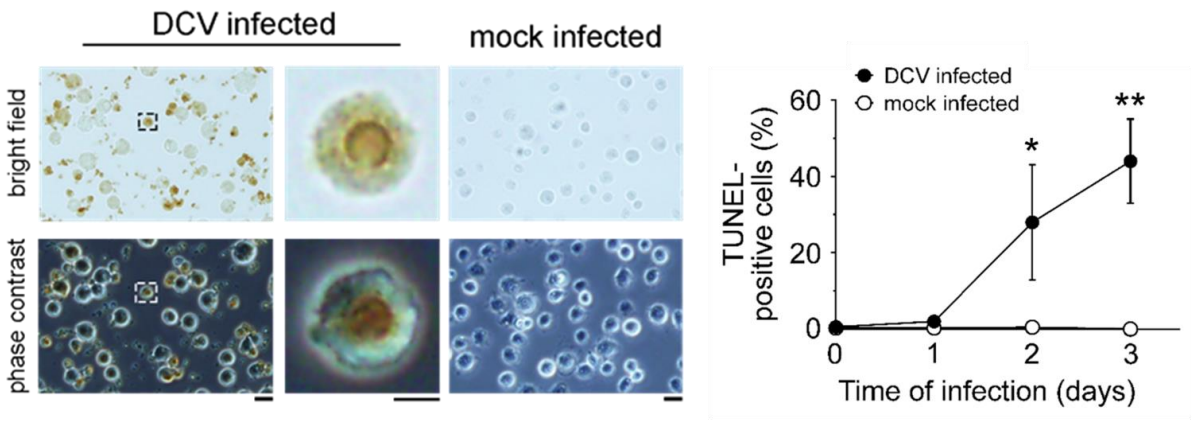


Fig. 6. DNA fragmentation in S2 cells after infection with DCV
(**left**) S2 cells infected or mock-infected with DCV for 3 days were analyzed by TUNEL assay. TUNEL-positive signals are seen in brown. The same microscopic fields are shown as bright field (*top*) and phase-contrast (*bottom*) views. Middle panels are higher-magnified views of the cells squared in the left panels. The data from one of three independent experiments that yielded similar results are presented. Scale bars, 10 μm (*left and right*) and 5 μm (*middle*). (**right**) DCV- or mock-infected S2 cells collected at the indicated time points of infection were subjected to TUNEL assay. The ratio of TUNEL-positive cells is shown as mean ± standard deviations of the data from three independent experiments. * $p < 0.05$, ** $p < 0.01$.

Caspases are classified into two types, initiators and effectors (56). In *Drosophila*, Dronc is the only initiator caspase while *Drosophila* interleukin-1 converting enzyme (drICE) and Dcp-1 are known as effector caspases (57, 58). Dronc exists in cells as an active form, but its activity is bound and repressed by an inhibitor protein called *Drosophila* inhibitor of apoptosis 1 (DIAP1) (57, 58). Therefore, the onset of apoptosis in *Drosophila* cells is a step of inactivation or degradation of DIAP1 that enables Dronc to cleave and activate effector caspases (57). To gain cue for understating a mechanism of DCV-induced apoptosis, I determined the levels of drICE and DIAP1 by Western blotting in lysates of S2 cells infected or mock-infected with DCV (Fig. 7). The data from analyses with anti-DCV and anti-DmCaBP1 showed successful infection with DCV and constant loading of proteins in lanes, respectively. I found the appearance of a smaller protein bound by anti-drICE in the lysates of DCV-infected S2 cells, suggesting the cleavage and activation of this effector caspase. In addition, a signal corresponding to DIAP1 decreased upon viral infection. These results suggested that DCV infection caused a decrease in the level of

DIAP1 leading to the activation of Dronc and subsequent cleavage of drICE. Induction of apoptosis after viral infection in *Drosophila* cells was previously reported with Flock House virus, one of the simplest non-enveloped virus with a genome of single-stranded RNA (59). This preceding study describes global inhibition of host protein synthesis upon viral infection. However, this is not the case for cells infected with DCV: the level of DmCaBP1, a Ca^{2+}-binding endoplasmic reticulum protein, did not significantly change before and after infection. This suggested that a mechanism underlying virus-induced decrease of DIAP1 varies among viral species.

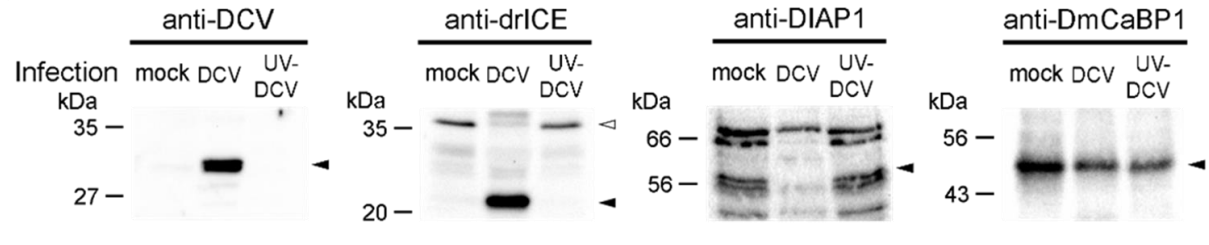


Fig. 7. Cleavage of drICE and decrease of DIAP1 in S2 cells after infection with DCV
S2 cells were infected or mock-infected with DCV for 3 days, lysed, and subjected to Western blotting with the indicated antibodies. Anti-DCV used here is anti-DCV capsid polyprotein antibody. DCV that had been treated with UV (UV-DCV) was also used to infect S2 cells. The open and closed arrowheads in the panel with anti-drICE antibody point to uncleaved and cleaved drICE, respectively. DmCaBP1 (12) was analyzed to confirm that a similar amount of proteins was loaded in each lane. The positions of molecular mass markers are indicated on the left in each panel. Representative data of three independent experiments that yielded similar results are shown.

During viral infection, robust expression of viral proteins often drives the host cell to undergo apoptosis (60). However, some viral species have been shown to induce apoptosis only by attachment on the surface of host cells, in the absence of virus replication (61, 62). To further define the characteristics of apoptosis induced by DCV, I next asked if replication-compromised DCV causes apoptosis in S2 cells after infection. To this end, S2 cells were infected with DCV that had been irradiated with UV. There observed a significant decrease in the levels of capsid protein production (Fig. 7) and viral replication (left panel in Fig. 8), indicating successful

inactivation of DCV by UV. Under such a condition, all biochemical changes evident in S2 cells infected with intact DCV were diminished (Fig. 7 and right panel in Fig. 8). This suggested that induction of apoptosis in S2 cells requires the productive infection of DCV.

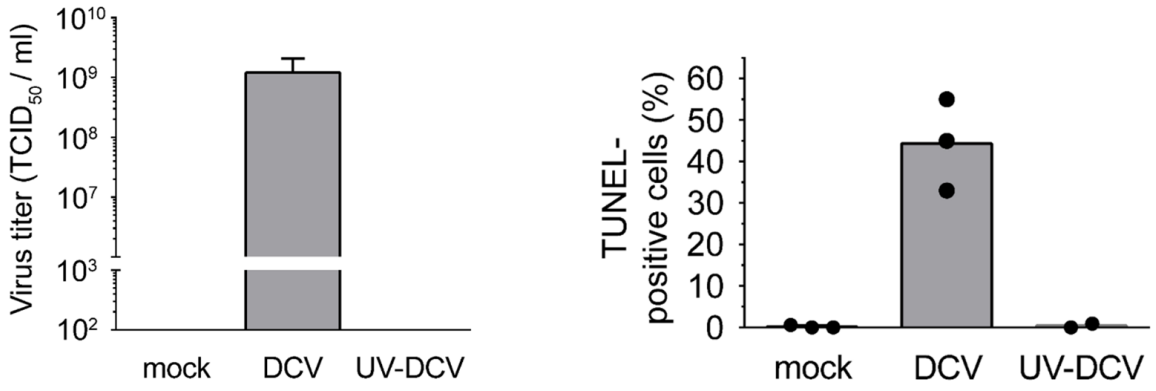


Fig. 8. Infection of S2 cells with UV-treated DCV
(**left**) S2 cells were infected with DCV that had been exposed (*UV-DCV*) or not exposed to UV for 3 days. The culture medium was collected and analyzed for the virus titer. Mean ± standard deviations of the data obtained from three independent experiments are shown. Note that virus titers for the samples '*mock*' and '*UV-DCV*' are less than 100 $TCID_{50}$ per ml. (**right**) S2 cells similarly infected with virus were subjected TUNEL assay. Data from three (*mock* and *DCV*) and two (*UV-DCV*) independent experiments with average values are shown. Note that the values for the samples '*mock*' and '*UV-DCV*' are near 0%.

Phagocytosis of DCV-infected cells and restriction of virus growth by *Drosophila* phagocytes

In general, apoptotic cells are subjected to a homeostatic disposal process carried out by phagocytes (63-65). I thus examined whether apoptotic, DCV-infected S2 cells are susceptible for phagocytosis by *Drosophila* phagocytes. To explore this possibility, I took an advantage of using l(2)mbn cells, a cell line established from *Drosophila* larval hemocytes, that had been successfully used as phagocytes against cells undergoing apoptosis (46). When incubated in the presence of 20-hydroxyecdysone, l(2)mbn cells acquires a flattened macrophage-like shape and becomes active in phagocytosis (46).

S2 cells, infected or mock-infected with DCV, were mixed with hormone-treated l(2)mbn cells and subjected to immunostaining using anti-DCV antibody followed by counterstaining with hematoxylin. Macrophage-like l(2)mbn cells (cells marked '*a*' in Fig. 9) were easily distinguishable from spherical S2 cells (cells marked '*b*' in Fig. 9). Uninfected S2 cells were stained blue with hematoxylin while they became brownish once infected with DCV after a reaction with DAB as a substrate to locate a secondary antibody (cells marked '*c*' in Fig. 9). Some l(2)mbn cells contained brown-stained particles besides their own nuclei (cells marked '*d*' in Fig. 9). I considered such cells as those that had engulfed virus-infected S2 cells.

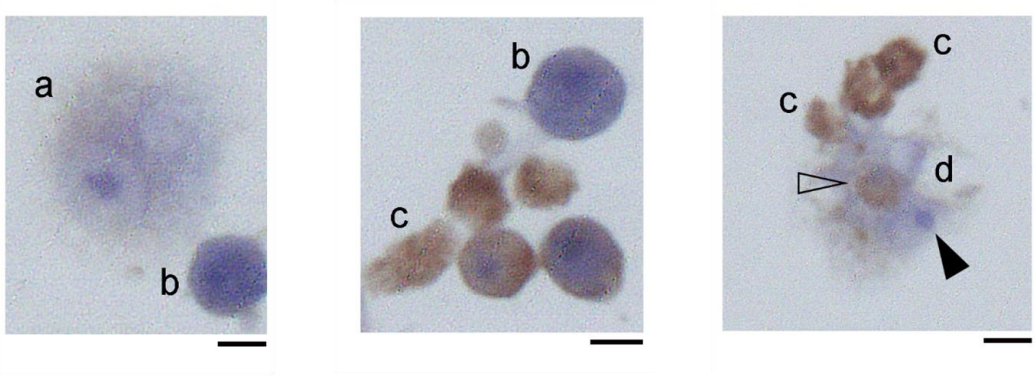


Fig. 9. Microscopic observation of DCV-infected S2 cells and hormone-treated l(2)mbn cells
DCV-infected S2 cells were mixed with 20-hydroxyecdysone-treated l(2)mbn cells and subjected to immunocytochemistry with anti-DCV antibody followed by counterstaining with hematoxylin. Cells '*a*', '*b*', and '*c*', are l(2)mbn cells, uninfected S2 cells, and DCV-infected S2 cells, respectively. Cell '*d*' is an l(2)mbn cell that has engulfed DCV-infected S2 cells. The open and closed arrowheads point to a DCV-positive S2 cell contained in an l(2)mbn cell and the nucleus of a l(2)mbn cell, respectively. Scale bars, 10 μm.

Phagocytosis level was numerically analyzed in two criteria: the ratio of phagocytes that had accomplished phagocytosis (left panel in Fig. 10) and the number of target cells engulfed by a given number of phagocytes (right panel in Fig. 10). Either value increased as incubation of mixed cultures was prolonged. I thus concluded that S2 cells become susceptible to phagocytosis by hormone-treated l(2)mbn cells upon infection with DCV.

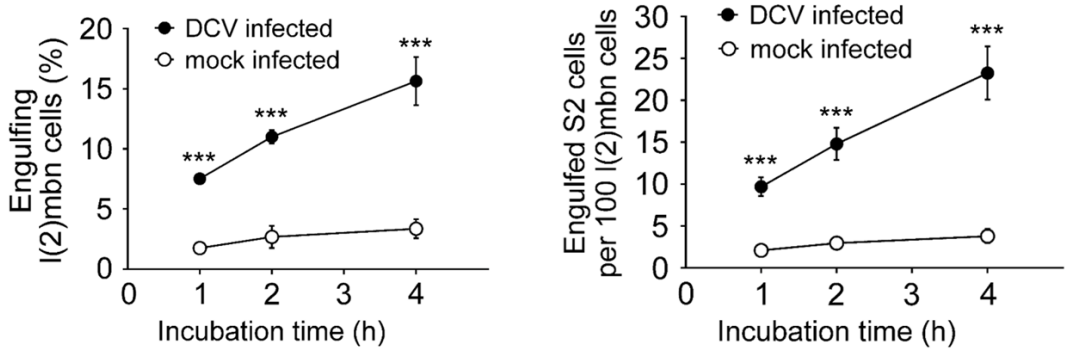


Fig. 10. Time-course analysis of phagocytosis
DCV-infected S2 cells were co-incubated with ecdysone-treated l(2)mbn cells for the indicated periods and analyzed for the ratio of l(2)mbn cells that had accomplished phagocytosis (**left**) and the number of S2 cells that had been engulfed by 100 l(2)mbn cells (**right**). Mean ± standard deviations of the data obtained from three independent experiments are presented. *** $p < 0.001$.

Phagocytosis of virus-infected cells could lead to two opposing manifestations: inhibition of virus growth (51) or dissemination of infection (66). To assess the consequences of phagocytosis in this *in vitro* model system, S2 cells infected with DCV were divided into two groups, one added with hormone-treated l(2)mbn cells and the other none treated. The cultures were further maintained until virus titer was determined in the culture media. I found that the level of virus released into the culture medium was significantly lower in the presence of phagocytes (Fig. 11), suggesting that virus particles, together with

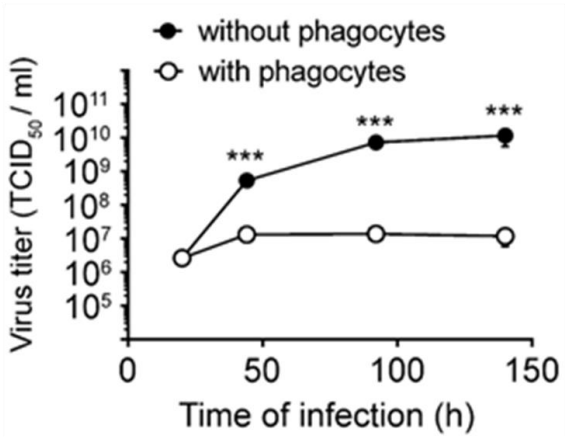


Fig. 11. Virus propagation in the presence of phagocytes
S2 cells infected with DCV for 18 h were further maintained in the presence and absence of ecdysone-treated l(2)mbn cells, and the amount of virus existing in the culture medium was determined at the indicated time points. Mean ± standard deviations of the data obtained from three independent experiments are presented. *** $p < 0.001$.

infected host cells, are digested in l(2)mbn cells after engulfment. Taken together, the data obtained so far indicate that phagocytosis of DCV-infected cells leads to the reduction of virus propagation and thus inhibition of dissemination of infection, at least as analyzed *in vitro*.

Apoptosis-dependent phagocytosis of DCV-infected cells

I next examined if phagocytosis of DCV-infected cells depends on apoptosis occurring in the target cells. For this purpose, S2 cells were infected with DCV in the presence or absence of z-VAD-fmk and used as target cells in an assay for phagocytosis. I found that inclusion of the caspase inhibitor reduced the susceptibility of DCV-infected S2 cells to phagocytosis by hormone-treated l(2)mbn cells (Fig. 12). This indicated that apoptosis is required for DCV-infected S2 cells to be effectively phagocytosed *in vitro*.

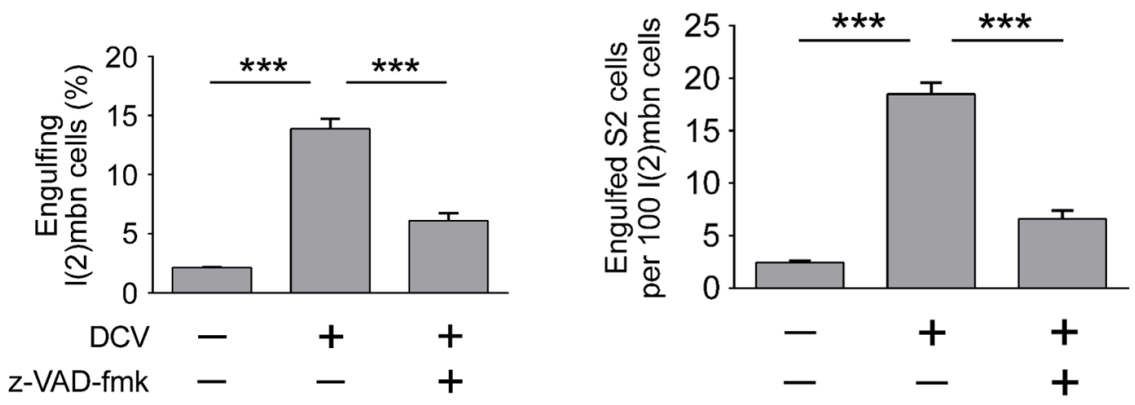


Fig. 12. Phagocytosis of DCV-infected S2 cells in the presence of caspase inhibitor
S2 cells were infected with DCV for 3 days in the presence or absence of the caspase inhibitor z-VAD-fmk and used as target cells in an assay for phagocytosis by 20-hydroxyecdysone-treated l(2)mbn cells. The ratio of l(2)mbn cells that had accomplished phagocytosis (**left**) and the number of S2 cells that had been engulfed by 100 l(2)mbn cells (**right**) were determined. Mean ± standard deviations of the data obtained from three independent experiments are presented. *** $p < 0.001$.

Phagocytic clearance of cells undergoing apoptosis is an evolutionarily conserved event from nematodes to humans (64, 67, 68). Genetic studies have revealed the presence of partly overlapping two signalling pathways for the induction of engulfment in phagocytes (68). In *Drosophila*, the membrane proteins Draper (46) and integrin αPS3-βν (47, 69) serve as receptors responsible for the recognition of apoptotic cells and the activation of signalling pathways. I thus wondered if those receptors also play a role in the phagocytosis of DCV-infected cells. To test this, RNA interference was applied to l(2)mbn cells to reduce the expression of these receptors. When l(2)mbn cells were incubated with double-stranded RNA possessing sequences corresponding to parts of the mRNA of Draper and integrin βν, the level of the receptors was significantly lowered as assessed by Western blotting of whole-cell lysates (upper panel in Fig. 13). When such l(2)mbn cells were tested in an assay for phagocytosis with DCV-infected S2 cells as target cells, I found that RNA interference of either Draper or integrin βν reduced the phagocytic activity of phagocytes by about 60% (lower panels in Fig. 13). Furthermore, there observed further reduction in the level of phagocytosis when two receptors were simultaneously knocked down. These results indicated that Draper and integrin βν were independently involved in the recognition and engulfment of DCV-infected cells by hormone-treated l(2)mbn cells. Collectively, phagocytosis of DCV-infected S2 cells by l(2)mbn cells involves a canonical mechanism underlying the clearance of apoptotic cells in *Drosophila*.

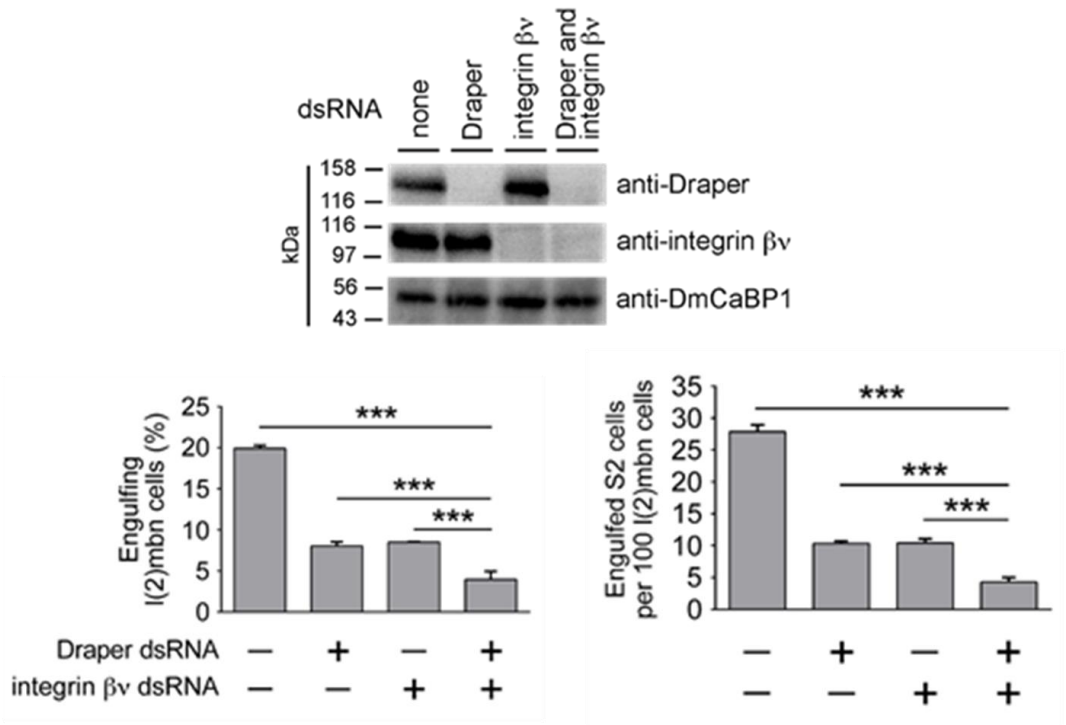


Fig. 13. Involvement of Draper and integrin βν in phagocytosis of DCV-infected cells (**top**) Hormone-treated l(2)mbn cells were incubated for 3 days in the presence of double-stranded RNA (dsRNA) possessing sequences corresponding to the mRNA of Draper and integrin βν, and lysed. The lysates were then subjected to Western blotting with antibodies specific for these proteins. Level of DmCaBP1 was also determined as an internal control. The positions of molecular mass markers are indicated at the left of each panel. Data are representative of three independent experiments that yielded similar results. (**bottom**) dsRNA-treated l(2)mbn cells were tested in an assay for phagocytosis with S2 cells infected with DCV for 3 days as targets. Mean ± standard deviations of the data obtained from three independent experiments are presented. *** $p < 0.001$

The fact that knockdown of both Draper and integrin βν did not completely cancelled the phagocytic activity of l(2)mbn cells suggests the presence of another engulfment receptor. A membrane protein named Croquemort has been reported to act as an engulfment receptor for the phagocytosis of apoptotic cells by *Drosophila* hemocytes (70, 71). I thus asked if this receptor is also involved in the phagocytosis of DCV-infected S2 cells by l(2)mbn cells. Hormone-treated l(2)mbn cells were similarly subjected to RNA interference, and the reduction in the level of Croquemort was confirmed (upper panels in Fig. 14). I found that knockdown of this protein did not influence the phagocytic activity of l(2)mbn cells (lower panels in Fig. 14). The data

indicated that Croquemort does not participate in the phagocytosis of DCV-infected S2 cells by l(2)mbn cells under conditions employed in this study.

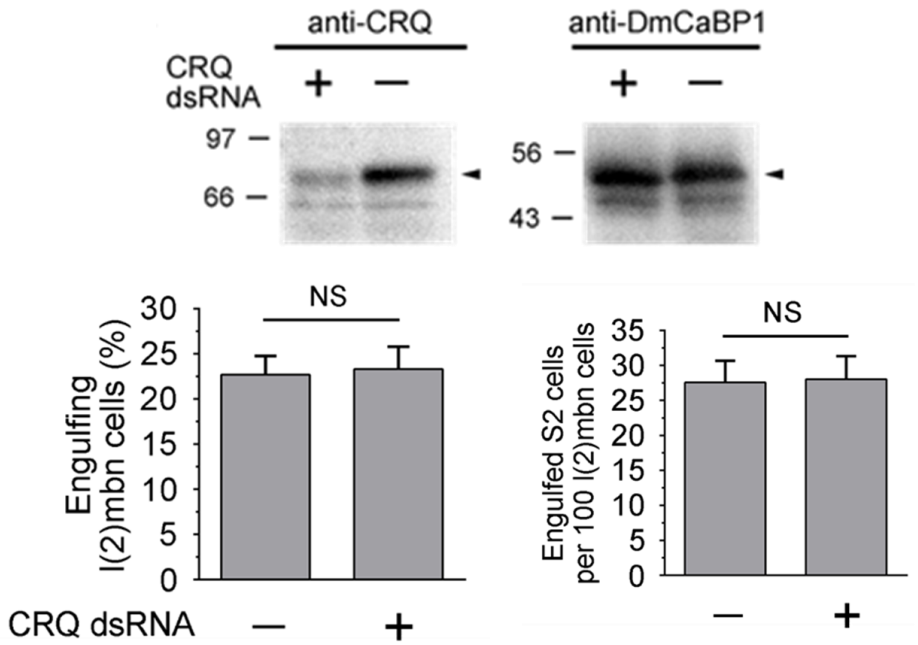


Fig. 14. No participation of Croquemort in phagocytosis of DCV-infected S2 cells by l(2)mbn cells

(**top**) Hormone-treated l(2)mbn cells were incubated for 3 days in the presence of double-stranded RNA (dsRNA) possessing a sequence corresponding to the mRNA of Croquemort (CRQ) and lysed. The lysates were then subjected to Western blotting. Level of DmCaBP1 was determined as an internal control. The positions of molecular mass markers are indicated at the left of each panel. Data are representative of three independent experiments that yielded similar results. (**bottom**) dsRNA-treated l(2)mbn cells were tested in an assay for phagocytosis with S2 cells infected with DCV for 3 days as targets. Mean ± standard deviations of the data obtained from three independent experiments are presented. NS, difference not significant.

Apoptosis and phagocytosis of DCV-infected cells in *Drosophila* upon infection with DCV

To confirm whether the *in vitro* findings are manifestable in living animals, I next examined the induction of apoptosis and the phagocytosis of virus-infected cells in *Drosophila* after infection with DCV.

The cleavage of PARP, a known target for effector caspases, in flies was determined as indication of apoptosis. Adult flies that expressed human PARP fused to the transmembrane domain of murine CD8 at the N-terminal and Venus, an improved version of yellow florescence protein, at the C-terminal (CD8-PARP-Venus) (40, 72) were abdominally injected with DCV. Lysates of whole flies were then analysed by Western blotting to determine the propagation of DCV as well as the cleavage of CD8-PARP-Venus (Fig. 15). Upon infection, viral capsid protein became detectable, and this indicated a successful infection of adult flies with DCV. The analysis with anti-PARP antibody showed the presence of intact and cleaved CD8-PARP-Venus in flies before DCV infection. This is probably due to apoptosis ongoing in tissues and organs of *Drosophila* during normal development. I found that cleaved CD8-PARP-Venus increased after infection with DCV, indicating an elevated level of apoptosis. However, this was not the case when UV-inactivated DCV was used to infect flies. These results suggested that caspase-mediated apoptosis is induced in adult flies upon infection with DCV in a manner dependent on virus replication.

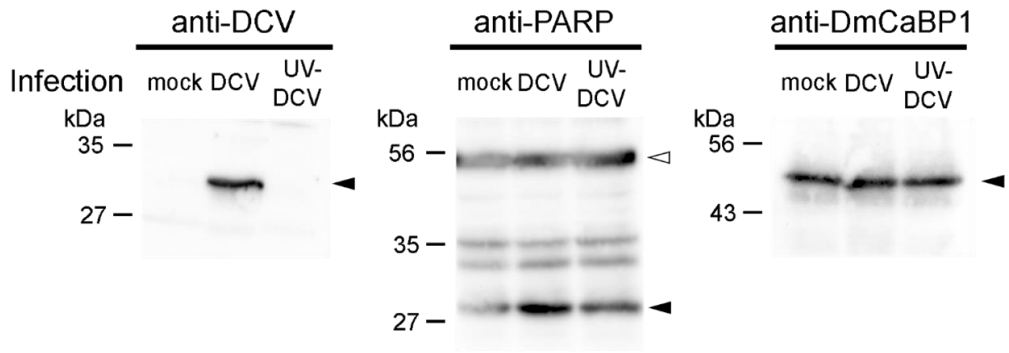


Fig. 15. Caspase activation in flies after infection with DCV
Adult flies systemically expressing CD8-PARP-Venus were infected with DCV for 2 days and lysed. The resulting lysates were subjected to Western blotting using the indicated antibodies. The open and closed arrowheads in the middle panel denote intact and cleaved CD8-PARP-Venus, respectively. Representative data of three independent experiments that yielded similar results are shown.

I next asked if DCV-infected cells are phagocytosed in flies. For this purpose, I decided to analyze cells present in the hemolymph of adult flies for the presence of hemocytes that have engulfed DCV-infected cells. There are three types of hemocytes in adult *Drosophila*, namely, plasmatocytes, lamellocytes, and crystal cells (14, 52, 73). Among them, plasmatocytes are hemocytes responsible for the phagocytosis of invading microbes and apoptotic cells (73, 74). To analyze plasmatocytes, I used a genetically manipulate fly line that expresses GFP specifically in this type of hemocytes. When cells collected from adult hemolymph were examined under a fluorescence microscope, greater than 90% of the cells were positive for GFP expression (Figs. 16A and 16A'), indicating most cells obtained in this way are plasmatocytes, which are thereafter called hemocytes. Staining with hematoxylin revealed that those hemocytes possessed a flattened shape resembling 20-hydroxyecdysone-treated l(2)mbn cells (Fig. 16B). When hemocytes

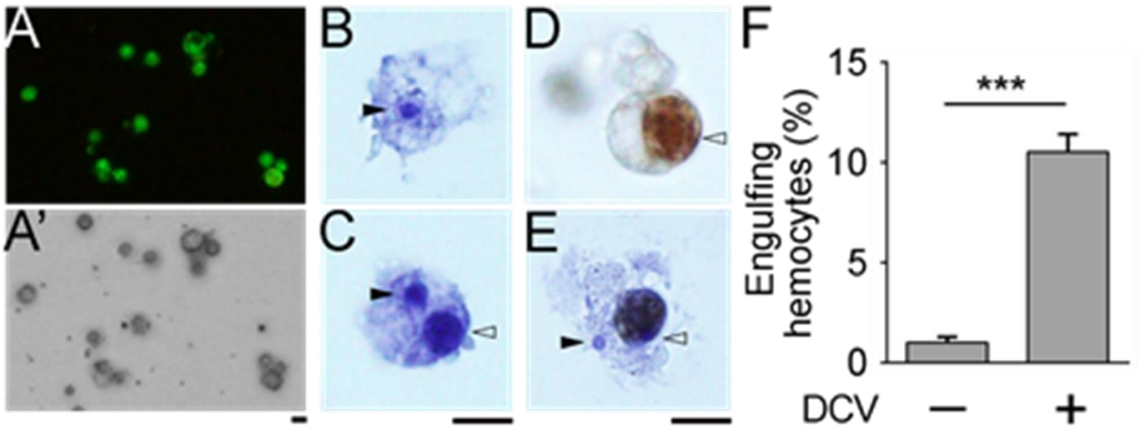


Fig. 16. Phagocytosis of DCV-infected cells in adult flies
Adult flies (*pxn-Gal4 UAS-GFP*) expressing GFP in hemocytes were infected (**C**, **D**, **E**, **F**) or not infected (**A**, **A'**, **B**, **F**) with DCV for 20-32 h. Cells contained in fly hemolymph were examined by fluorescence microscopy, and fluorescence (**A**) and phase-contrast (**A'**) views of the same microscopic field are shown. The cells were subjected to staining with hematoxylin (**B**, **C**) as well as immunocytochemistry with anti-DCV capsid polyprotein antibody (**D**), and both staining and immunocytochemistry (**E**). The open and closed arrowheads point to nuclei of DCV-infected cells and hemocytes, respectively. Scale bars, 10 μm. The ratio of hemocytes containing multiple nuclei and positive for viral capsid protein was determined and shown as mean ± standard deviations of the data from three independent experiments (**F**).

collected from flies infected with DCV were examined after hematoxylin staining, I found that a portion of cells contained two or more nuclei of different sizes (Fig. 16C). Further analysis of hemocytes adopting immunostaining with anti-DCV antibody revealed that one of nucleus-like particles was bound by the antibody (Figs. 16D and 16E). Hemocytes with two or more nuclei and positive for DCV were considered to have engulfed DCV-infected cells, and the number of such hemocytes significantly increased in adult flies upon infection with DCV (Fig. 16F). All these data indicated the occurrence of phagocytosis of DCV-infected cells in adult flies after viral infection.

Antiviral role for phagocytosis of DCV-infected cells in *Drosophila*

Cellular homeostasis is maintained by a constant removal of apoptotic cells by phagocytes (75). In the context of viral infection, similar mechanism has also been shown to provide antiviral protection in mammal (33, 51). In the *in vitro* studies described above, the presence of phagocytes, thus the occurrence of phagocytosis restricted the growth of DCV in S2 cells. I next examined if this is also true *in vivo*. For this purpose, an *in vivo* system for the systemic infection of *Drosophila* with DCV was first established. When adult flies were abdominally injected with increasing amounts of DCV, the survival rate of the infected flies was inversely correlated with the dose of virus infected (Fig. 17), indicating the establishment of pathogenic infection of adult flies with DCV.

Several procedures have been reported to inhibit phagocytosis in *Drosophila* (76-79). I chose a relatively simple method, injection of undigestible latex beads into the hemocoel of flies. In this procedure, injected beads once taken up by hemocytes are expected to inhibit their subsequent phagocytic activity. Adult flies were injected with latex beads at the abdomen prior to the infection with DCV and analysed for their survival. I found that pre-injection of beads led to earlier death of flies after DCV infection (Fig. 18). This is not due to a toxic effect of latex beads

because flies with beads injected maintained near 90% survival when left uninfected. The data suggested an important role for hemocyte phagocytosis in the protection of *Drosophila* against pathogenic infection with DCV.

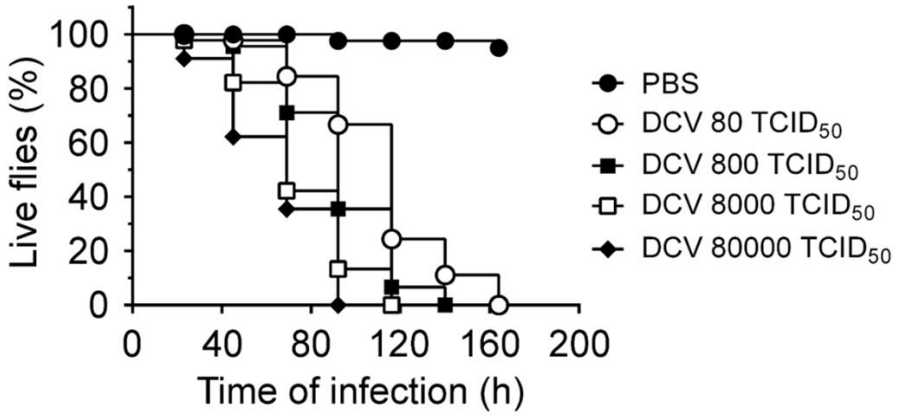


Fig. 17. Fly death after infection with DCV
Adult w^{1118} flies were abdominally injected with the indicated amounts of DCV, and the ratio of live flies was determined at the indicated time points. Average values of the data from a 3-vial experiment are presented. The data were analyzed by log-rank test: *** $p < 0.001$ for 80 $TCID_{50}$ vs 800 $TCID_{50}$ and for 800 $TCID_{50}$ vs 8000 $TCID_{50}$, * $p < 0.05$ for 8000 $TCID_{50}$ vs 80000 $TCID_{50}$.

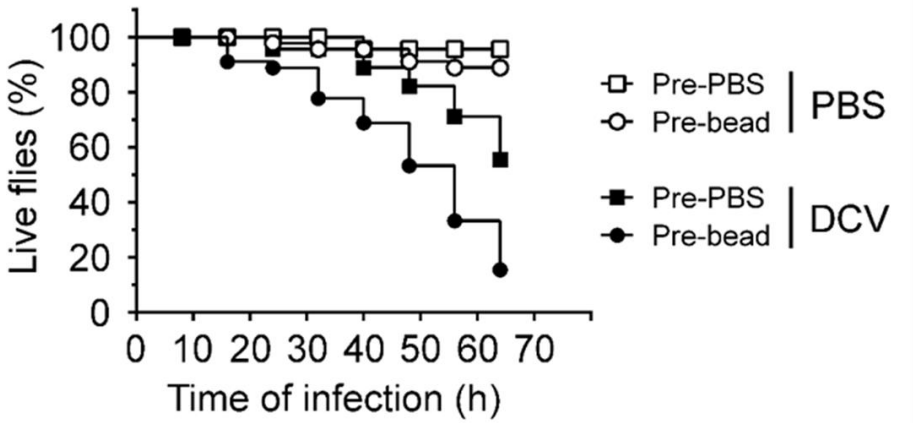


Fig. 18. Early death of DCV-infected flies by pre-injection of latex beads
Adult w^{1118} flies, which had been injected with latex beads (*Pre-bead*) or PBS (*Pre-PBS*) for 6 h, were abdominally infected with DCV, and the ratio of live flies was determined at the indicated time points. Average values of the data from a 3-vial experiment are presented. $p < 0.001$ for Pre-PBS vs Pre-bead (with DCV infection), analyzed by log-rank test.

I next examined if loss of engulfment receptors worsens the pathogenic infection with DCV. To test this, adult flies having a null mutation in the genes coding for Draper and integrin βν were abdominally injected with DCV and subjected to an assay for survival (Fig. 19). Flies lacking either Draper or integrin βν died at an almost similar rate, much earlier than flies that normally expressed both receptors. A loss of both receptors made flies more severely succumb to viral infection. These results are well in line with those from the *in vitro* experiments and indicated the requirement for Draper and integrin βν in anti-viral actions in *Drosophila*.

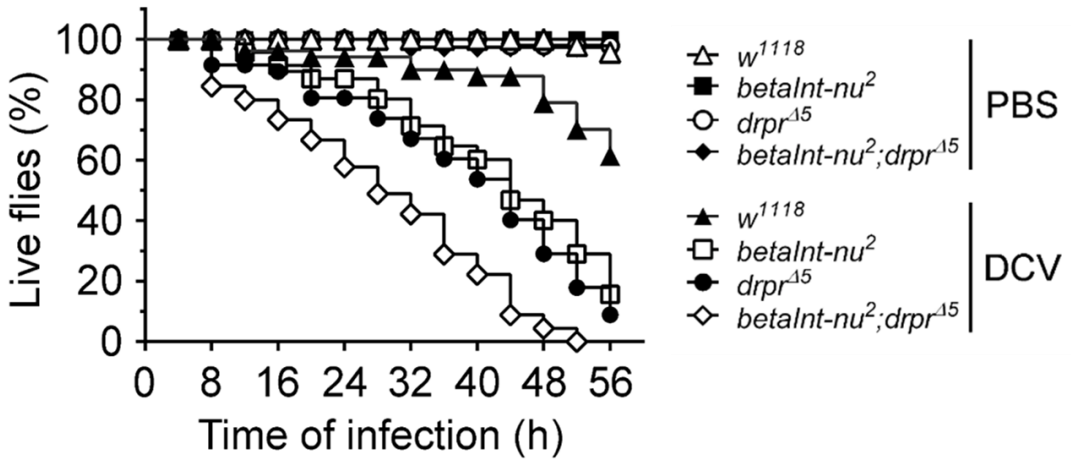


Fig. 19. Early death of engulfment receptor-deficient flies upon infection with DCV
Adult flies of the indicated lines were infected with DCV, and the rate of fly death was determined at the indicated time points. Average values of the data from a 3-vial experiment are presented. *betaInt-nu*^{2}, a null-mutation allele of the gene coding for integrin βν; *drpr*$^{\Delta 5}$, a null-mutation allele of the gene coding for Draper. *p* values (for flies infected with DCV) analyzed by log-rank test: *** $p < 0.0001$ for w^{1118} vs *betaInt-nu*^{2}, for w^{1118} vs *drpr*$^{\Delta 5}$, for w^{1118} vs *betaInt-nu*^{2}*;drpr*$^{\Delta 5}$, for *betaInt-nu*^{2} vs *betaInt-nu*^{2}*;drpr*$^{\Delta 5}$, and for *drpr*$^{\Delta 5}$ vs *betaInt-nu*^{2}*;drpr*$^{\Delta 5}$. Difference was not significant for *betaInt-nu*^{2} vs *drpr*$^{\Delta 5}$.

Early death of engulfment receptor-lacking flies after DCV infection is most likely due to a lowered level of phagocytosis of virus-infected cells. If so, those flies should be burdened with greater virus load. To determine the level of virus in flies, DCV-infected flies were lysed and subjected to an assay for virus titer (Fig. 20). I found that the mount of virus in control flies steadily increased during a course of infection. Under such conditions, flies lacking either Draper or integrin βν gave an almost similar level of virus titre, significantly higher than that from

control flies. Furthermore, the highest titer was obtained when flies with a null mutation on both Draper- and integrin βν-encoding genes. Taken together, it is most likely that phagocytic elimination of DCV-infected cells plays an important role in antiviral immunity in *Drosophila*.

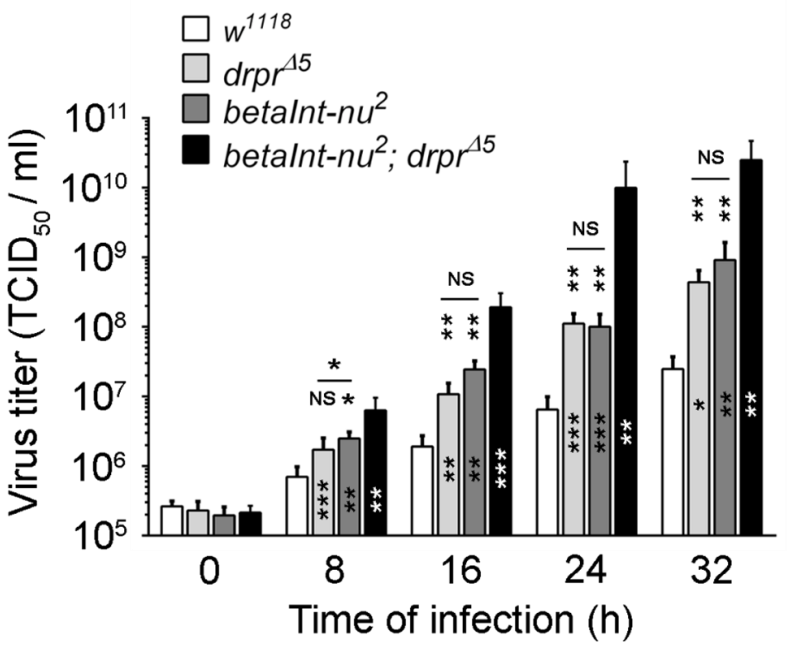


Fig. 20. Enhanced virus propagation in flies lacking engulfment receptors
The indicated lines of flies were infected with DCV, collected at the indicated time points, and lyzed. The resulting lysates were analyzed for virus titer. Mean ± standard deviations of the data obtained from three independent experiments are presented. *p* values: those indicated in the bars are after comparison with the data with w^{1118} flies, and those shown above the bars are after comparison with the data of *betaInt-nu*^{2}*; drpr*$^{\Delta 5}$ flies. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. NS, difference not significant.

Involvement of phosphatidylserine in phagocytosis of DCV-infected cells

Successful phagocytosis of apoptotic cells is achieved in a manner dependent on a ligand, or eat-me signal, on the surface of target cells and its recognition by an engulfment receptor on phagocytic cells (64, 68). Phosphatidylserine, one of glycerophospholipids, is the best-characterized ligand involved in the phagocytic clearance of apoptotic cells in varieties of cell types and animal species (80-83). This phospholipid is almost exclusively present in the inner

layer of plasma membrane but relocated to the outer layer when cells are induced to undergo apoptosis (84). A mechanism for the trans-bilayer movement of phosphatidylserine in the process of apoptosis has been well studied (85). Phagocytes possess several types of receptors that bind to phosphatidylserine exposed at the surface of apoptotic cells (86, 87). In *Drosophila*, Draper serves as a phosphatidylserine-recognizing receptor in the phagocytosis of apoptotic cells (39).

I examined if DCV-infected cells are phagocytosed in a phosphatidylserine-mediated manner. To test this *in vitro*, an assay for phagocytosis was conducted in the presence of liposomes, which consisted of phosphatidylcholine alone or a combination of phosphatidylcholine and phosphatidylserine. This strategy has successfully demonstrated phosphatidylserine-mediated phagocytosis *in vitro* (88). The inclusion of phosphatidylcholine -only liposome did not alter the level of phagocytosis while phosphatidylserine-containing liposomes significantly inhibited phagocytosis (Fig. 21). These results suggested the involvement of phosphatidylserine in the recognition of DCV-infected cells by l(2)mbn cells.

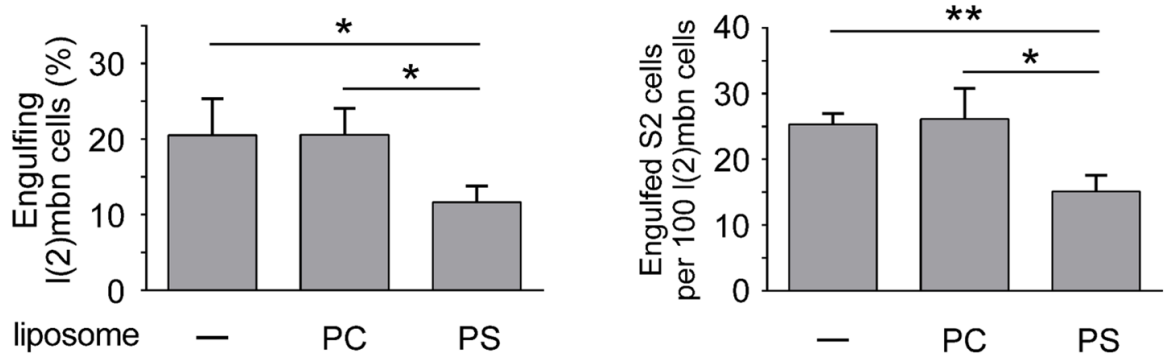


Fig. 21. Inhibition of phagocytosis of DCV-infected cells by phosphatidylserine-containing liposome

S2 cells infected with DCV for 3 days were subjected to an assay for phagocytosis with ecdysone-treated l(2)mbn cells as phagocytes in the presence and absence of liposome (1 mM). Mean ± standard deviations of the data obtained from three independent experiments are presented. PC, liposome composed of phosphatidylcholine alone; PS, liposome consisting of a mixture of phosphatidylcholine and phosphatidylserine (7:3). * $p < 0.05$ and ** $p < 0.01$.

I next tested this *in vivo*. To do so, a fly line that systemically expressed MFG-E8, a human protein that binds to phosphatidylserine, was used anticipating its inhibitory action against phosphatidylserine-mediated phagocytosis (39). Another fly line was also employed, which expressed MFGE8-ΔC2, a mutant form of MFG-E8 lacking a domain responsible for phosphatidylserine-binding activity, and used as a negative control. Adult flies of these lines together with several control fly lines were abdominally injected with DCV and subjected to an assay for fly survival. I found that flies expressing MFG-E8 died after viral infection earlier than control flies including that expressing MFGE8-ΔC2 (Fig. 22). When the level of virus existing in those flies was determined, it was well correlated with the rate of fly death (Fig. 23). Taken together, it is conclude that the phagocytosis of DCV-infected cells is accomplished at least partly through recognition of phosphatidylserine exposed at the surface of DCV-infected, apoptotic cells by hemocytes of adult *Drosophila*.

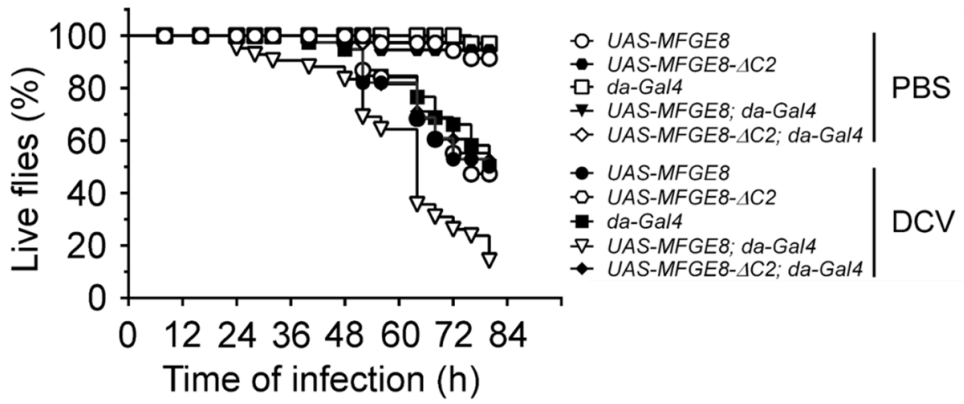


Fig. 22. Early death of MFG-E8-expressing flies after DCV infection
Adult flies of the indicated lines were abdominally injected with DCV or PBS as control, and the ratio of live flies was determined at the indicated time points. Average values of the data from a 3-vial experiment are presented. *p* values (for flies infected with DCV) analyzed by log-rank test: *** $p < 0.001$ for *UAS-MFGE8* vs *UAS-MFGE8;da-Gal4*, for *da-Gal4* vs *UAS-MFGE8;da-Gal4*, and for *UAS-MFGE8;da-Gal4* vs *UAS-MFGE8-ΔC2;da-Gal4*. Difference is not significant for *UAS-MFGE8-ΔC2* vs *UAS-MFGE8-ΔC2;da-Gal4* and for *da-Gal4* vs *UAS-MFGE8-ΔC2;da-Gal4*.

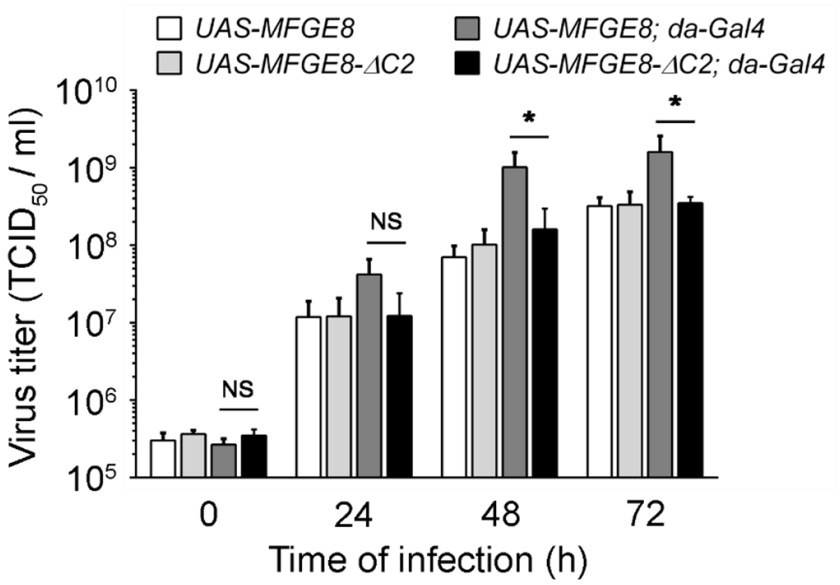
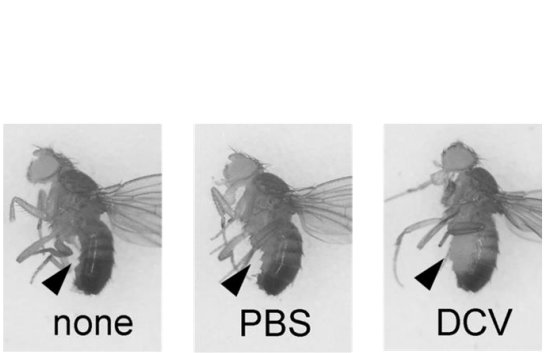


Fig. 23. Enhanced virus propagation in flies expressing MFG-E8
Flies containing the indicated trans-genes were injected with DCV. Lysates of whole flies were analyzed for the virus titer at the indicated time points. Mean ± standard deviations of the data obtained from three independent experiments are presented. * $p < 0.05$; NS, difference not significant.

Acceleration of pathological damage in DCV-infected *Drosophila* lacking engulfment receptors

Several independent research groups reported the occurrence of pathogenic state-related events upon virus infection in *Drosophila* (89-91). Two of such events, abdominal swelling and crop enlargement, are evident in DCV-infected flies (89, 90). In the studies described above, lack of engulfment receptors accelerated the death of flies after infection with DCV. I thus examined if such flies are accompanied by the two morphological changes. To test this, adult flies of control and a mutant line lacking both Draper and integrin βν were infected with DCV and subjected to a morphological analysis for the sizes of abdomen and crop (Figs. 24 and 25). I first confirmed the occurrence of abdominal swelling and crop enlargement upon viral infection in control flies. Loss of engulfment receptors, and thus impairment of virus elimination, enhanced both events.

Taken together, these data suggested that the phagocytosis of virus-infected cells comprehensively contributes to the mitigation of pathological damage caused by dissemination of DCV infection.



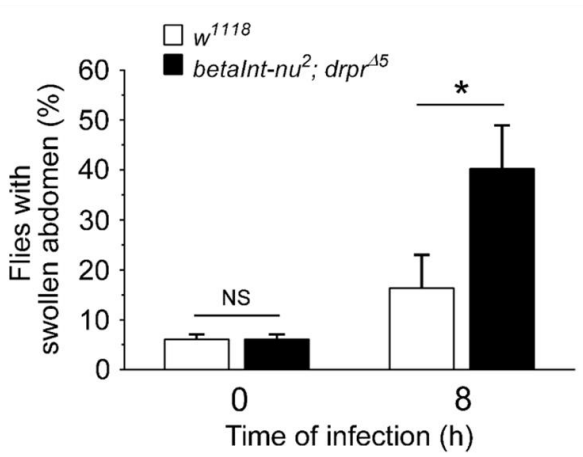
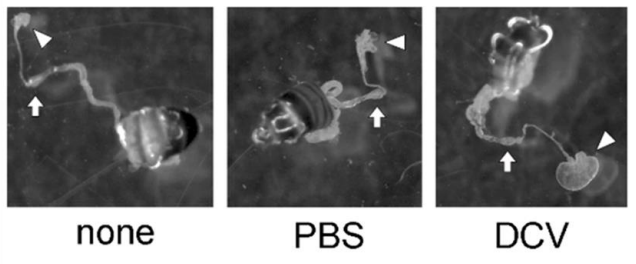


Fig. 24. Enhanced abdominal swelling upon DCV infection in flies lacking engulfment receptors
Control (w^{1118}) and Draper- and integrin βv-lacking (*betaInt-nu*^{2}*; drpr*$^{\Delta 5}$) flies were abdominally injected with DCV for 8 h. They were photographed (**A**) and examined for the ratio of flies with swollen abdomen (**B**, n=20~25). The arrowheads point to the abdomen (**A**), and mean ± standard deviations of the data obtained from three independent experiments are presented (**B**). * $p < 0.05$; NS, difference not significant.



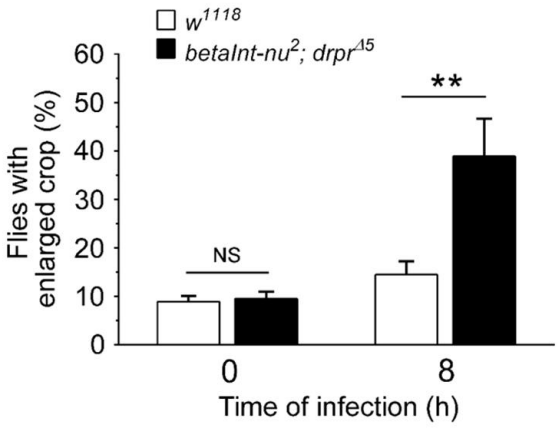


Fig. 25. Enhanced crop enlargement upon DCV infection in flies lacking engulfment receptors
Control (w^{1118}) and Draper- and integrin βv-lacking (*betaInt-nu*^{2}*; drpr*$^{\Delta 5}$) flies were abdominally injected with DCV for 8 h. The flies were dissected, and the digestive tract was isolated and photographed (**A**). The arrowheads and arrows point to the crop and cardia, respectively. The ratio of flies with enlarged crop (**B**, n=20~25) is shown as mean ± standard deviations of the data obtained from three independent experiments. ** $p < 0.01$; NS, difference not significant.

DISCUSSION

Living organisms are continuously threatened by viral infection all year round. To cope with this, metazoan hosts are equipped with a number of antiviral strategies to detect and eliminate virus entities as well as virus-infected cells. To date, research in the area of insect antiviral immunity has provided adequate evidences to explain how host immune systems are able to restrain virus replication through destruction of viral entity or parts of virion including viral genome by autophagy, RNA interference, and other intrinsic reactions (21, 35). In this study, I aimed to clarify if another antiviral mechanism, which has been known in mammals, i.e., elimination of virus-infected cells by apoptosis-dependent phagocytosis (33, 51), is also evident in insects.

Through a series of *in vitro* and *in vivo* experiments, I successfully confirmed the presence of a cellular immune reaction involving phagocytosis to remove virus-infected, apoptotic cells in insects that are equipped with only innate immunity. This should be added to a list of antiviral mechanisms that have been known in *Drosophila*: mechanisms relying on RNA interference (16, 19), Toll-Dorsal pathway (90, 92), JAK-STAT pathway (93), or phagocytosis of virus itself (94, 95). More importantly, this finding indicates that apoptosis-dependent phagocytosis of virus-infected cells is an anti-viral mechanism evolutionarily conserved from insects to mammals, and also that this mechanism is categorized into an innate immune response.

Mechanism and consequences of phagocytosis of DCV-infected cells in *Drosophila*

The phagocytosis of DCV-infected cells observed in both *in vitro* and *in vivo* assays was mediated at least in part by Draper and integrin βν, engulfment receptors that were previously

identified in the phagocytosis of apoptotic cells. In fact, DCV-infected target cells are required to undergo apoptosis for gaining their susceptibility to phagocytosis by *Drosophila* hemocytes, similar to influenza virus-infected cells in mammals (32, 33). Although this issue needs to be demonstrated *in vivo*, it is most probable that the phagocytosis of DCV-infected cells depends on the induction of apoptosis in the target cells. I thus conclude that phagocytosis of virus-infected cells occurs in *Drosophila* by a canonical mechanism for the clearance of apoptotic cells (67, 68), which also plays an important role during animal development (64, 68, 75).

The data obtained in an assay for phagocytosis *in vitro* showed that about 20% of phagocytosis remained when phagocytes lacking both Draper and integrin βν were used to target DCV-infected S2 cells. This suggested the existence of another mechanism for phagocytosis, most likely the involvement of another engulfment receptor other than Draper (39, 46) and integrin αPS3-βν (47, 69). Croquemort, the first to be reported as an engulfment receptor of *Drosophila* (70, 71), is not the one because RNA interference-knock down of this protein in phagocytes did not influence the level of phagocytosis. Further studies are required to solve this issue. The membrane phospholipid phosphatidylserine, the best-characterized markers for phagocytosis or eat-me signal (80-82), seems to be involved in the phagocytosis of DCV-infected cells both *in vitro* and *in vivo*. This phospholipid is likely to be recognized by Draper (39), but probably not by integrin αPS3-βν. It is thus necessary to identify another ligand bound by integrin αPS3-βν.

I showed in both *in vitro* and *in vivo* experiments that the phagocytosis of DCV-infected cells contribute to restrict viral propagation, probably through degradation of virus together with infected cells in phagocytes. In mammals, engulfment of virus-infected cells by dendritic cells and macrophages leads to the presentation of viral antigens to T cells (96). However, this secondary effect in unlikely in *Drosophila* that is not equipped with adaptive immunity. Another consequence of the phagocytosis of apoptotic cells could be a change of gene expression in

phagocytes. Mammalian macrophages were shown to express genes coding for proteins that act to terminate inflammation after phagocytosing apoptotic neutrophils (97). The occurrence of inflammation in *Drosophila* is not clear, but alteration of gene expression pattern in hemocytes that have engulfed DCV-infected cells is possible. If so, the phagocytosis of virus-infected cells could play a role more than inhibiting infectious diseases in *Drosophila*. Further investigation from a point of view different from previous ones is necessary to gain an answer to this question.

Mechanism of apoptosis induced by infection with DCV

In DCV-infected S2 cells, apoptosis was inhibited, though partially, by the pan-caspase inhibitor z-VAD-fmk, and partial digestion, thus the activation of the effector caspase drICE was evident. In addition, an increase of cleaved PARP, a known target protein for effector caspases, was observed in *Drosophila* after infection with DCV. These results strongly suggested the occurrence of caspase-mediated apoptosis in DCV-infected *Drosophila* cells.

In *Drosophila*, the initiator caspase Dronc resides in cells in its active form. The activity of Dronc is maintained at a dormant state by the endogenous caspase inhibitor DIAP1 that forms a complex with Dronc (57). Dronc becomes active when DIAP1 is degraded. The degradation of DIAP1 is induced by a mechanism dependent (57) or independent (98) of the pro-apoptotic proteins Reaper, Hid, and Grim. Liberated, active Dronc cleaves and activates the effector caspases drICE and Dcp-1 (57, 58) so that apoptotic changes occur. Therefore, a decrease in the level of DIAP1 is an indication of the onset of apoptosis in *Drosophila* cells. Settles and Friesen (59) previously claimed that a decreased level of DIAP1 is solely responsible for the induction of apoptosis in a *Drosophila* culture cell line upon infection with Flock house virus. A similar mechanism could be responsible for apoptosis in DCV-infected S2 cells because the level of DIAP1 was severely decreased after viral infection. It is thus probable that DCV infection leads

to the inhibition of expression of a DIAP1-encoding gene or the degradation of DIAP1. If the latter is the case, induced degradation should be specific to DIAP1 because the amount of DmCaBP1 analysed as an internal control did not significantly change before and after viral infection. This issue must be solved to gain a complete view of the induction of apoptosis in DCV-infected cells.

Growth of virus and death of flies

Although *Drosophila* is equipped with various mechanisms to fight against viral infection including one discovered in this study, they eventually died after infection with DCV. There are several questions to be asked regarding physiological and pathological events occurring in DCV-infected flies. First, a mechanism how daughter virus is produced and released from an originally infected cell, especially amid the apoptosis, needs to be known. Although the egress of newly assembled enveloped virus has been subject of a major spotlight (99, 100), mechanism to explain the release of non-enveloped virus, including DCV, from infected cells has just started to receive its scientific interest (101). It has been widely suggested that non-enveloped virus is released via the lysis of virus-producing cells (1, 2). However, recent findings argued that some non-enveloped viruses escape from infected cells by a non-lytic mechanism (102-104). Nevertheless, both views have been largely supported by the data derived from experiments with culture cell lines where phagocytes were not present. An *in vivo* experimental system described in this study is suitable for a study to elucidate mechanisms underlying the growth and release of a non-enveloped virus under conditions where apoptosis and phagocytosis are actively being pursued.

Data obtained in this study indicate that the size of viral burden is closely related to the mortality of flies. However, it remains to be determined how flies succumb to DCV infection. DCV infects a variety of tissues and organs in *Drosophila*, including the fat body, periovarial

sheath, trachea, muscle, and digestive tract (44, 89, 90, 105, 106). A recent report argued that alteration in a variety of physiological and metabolic phenomena is related to the death of DCV-infected flies (91). I also noticed that DCV-infected adult flies showed retarded migration, but it is unclear whether this phenomenon has something to do with fly mortality. Another report reasoned that a defect in the uptake of nutrients from digestive tract is a direct cause for fly death after the intestine is infected with DCV (89). It is necessary to clarify how viral infection causes lethal damage in host organisms in order to develop an effective remedy to prevent and cure viral diseases. The model system I adopted in this study using DCV and *Drosophila* is suitable for such research because the availability of robust genetics and a huge number of specimens should help to identify the genes involved that should explain the underlying mechanisms.

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