AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology

Analysis of the Contribution of Hemocytes and Autophagy to *Drosophila* Antiviral Immunity

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ABSTRACT

Antiviral immunity in the model organism *Drosophila melanogaster* involves the broadly active intrinsic mechanism of RNA interference (RNAi) and virus-specific inducible responses. Here, using a panel of six viruses, we investigated the role of hemocytes and autophagy in the control of viral infections. Injection of latex beads to saturate phagocytosis, or genetic depletion of hemocytes, resulted in decreased survival and increased viral titers following infection with Cricket paralysis virus (CrPV), Flock House virus (FHV), and vesicular stomatitis virus (VSV) but had no impact on Drosophila C virus (DCV), Sindbis virus (SINV), and Invertebrate iridescent virus 6 (IIV6) infection. In the cases of CrPV and FHV, apoptosis was induced in infected cells, which were phagocytosed by hemocytes. In contrast, VSV did not trigger any significant apoptosis but we confirmed that the autophagy gene *Atg7* was required for full virus resistance, suggesting that hemocytes use autophagy to recognize the virus. However, this recognition does not depend on the Toll-7 receptor. Autophagy had no impact on DCV, CrPV, SINV, or IIV6 infection and was required for replication of the sixth virus, FHV. Even in the case of VSV, the increases in titers were modest in *Atg7* mutant flies, suggesting that autophagy does not play a major role in antiviral immunity in *Drosophila*. Altogether, our results indicate that, while autophagy plays a minor role, phagocytosis contributes to virus-specific immune responses in insects.

IMPORTANCE

Phagocytosis and autophagy are two cellular processes that involve lysosomal degradation and participate in *Drosophila* immunity. Using a panel of RNA and DNA viruses, we have addressed the contribution of phagocytosis and autophagy in the control of viral infections in this model organism. We show that, while autophagy plays a minor role, phagocytosis contributes to virus-specific immune responses in *Drosophila*. This work brings to the front a novel facet of antiviral host defense in insects, which may have relevance in the control of virus transmission by vector insects or in the resistance of beneficial insects to viral pathogens.

Experiments in the model organism *Drosophila melanogaster* have shown that RNA interference (RNAi) plays a major role in antiviral immunity in insects: (i) flies with mutations for the three key genes of the small interfering RNA (siRNA) pathway, *Dicer-2*, *Argonaute 2*, and *r2d2*, show increased sensitivity to infection by RNA and DNA viruses (1–6); (ii) Dicer-2-dependent 21-nucleotide siRNAs of viral origin accumulate in virus-infected flies (1, 3, 4, 7–9); (iii) several insect viruses express viral suppressors of RNAi (5, 10–12). The importance of this pathway in the control of viral infections has been confirmed in other insects, in particular, the vector mosquito genera *Aedes* and *Culex*, which transmit important human pathogens such as dengue virus, West Nile virus, and other arthropod-borne viruses (13–18).

Inducible responses also contribute to the antiviral host defense in *Drosophila*, although they remain poorly characterized and involve virus-specific mechanisms (reviewed in references 19 and 20). We previously reported that a number of genes are induced following viral infection via the Jak/STAT pathway (21). Accordingly, flies with mutations for the Jak kinase Hopscotch are susceptible to infection by Drosophila C virus (DCV) and Cricket paralysis virus (CrPV), two members of the *Dicistroviridae* family, although they are as resistant as wild-type controls to other viruses (e.g., the alphavirus Sindbis virus [SINV] or the rhabdovirus vesicular stomatitis virus [VSV]) (3). Virus-induced autophagy and apoptosis have also been associated with antiviral immunity in *Drosophila* (22–24) and other insects (reviewed in reference 25).

Insects also mount cellular responses to fight infections mediated by blood cells called hemocytes. In *Drosophila*, macrophage-like plasmatocytes and two other nonphagocytic cells, the crystal cells and lamellocytes, have been described (26, 27). Plasmatocytes form the majority of differentiated blood cells (90 to 95% of hemocytes in *Drosophila* larvae). Commonly referred to as macrophages, they can engulf and degrade dead cells, debris, and invading pathogens (28, 29). Crystal cells (5% of larval hemocytes in *Drosophila*) are round cells with a 10- to 12-μm diameter and

Received 10 February 2016 **Accepted** 16 March 2016

Accepted manuscript posted online 23 March 2016

Citation Lamiable O, Arnold J, de Faria IJDS, Olmo RP, Bergami F, Meignin C, Hoffmann JA, Marques JT, Imler J-L. 2016. Analysis of the contribution of hemocytes and autophagy to *Drosophila* antiviral immunity. J Virol 90:5415–5426. doi:10.1128/JVI.00238-16.

Editor: R. M. Sandri-Goldin

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JVI.00238-16.

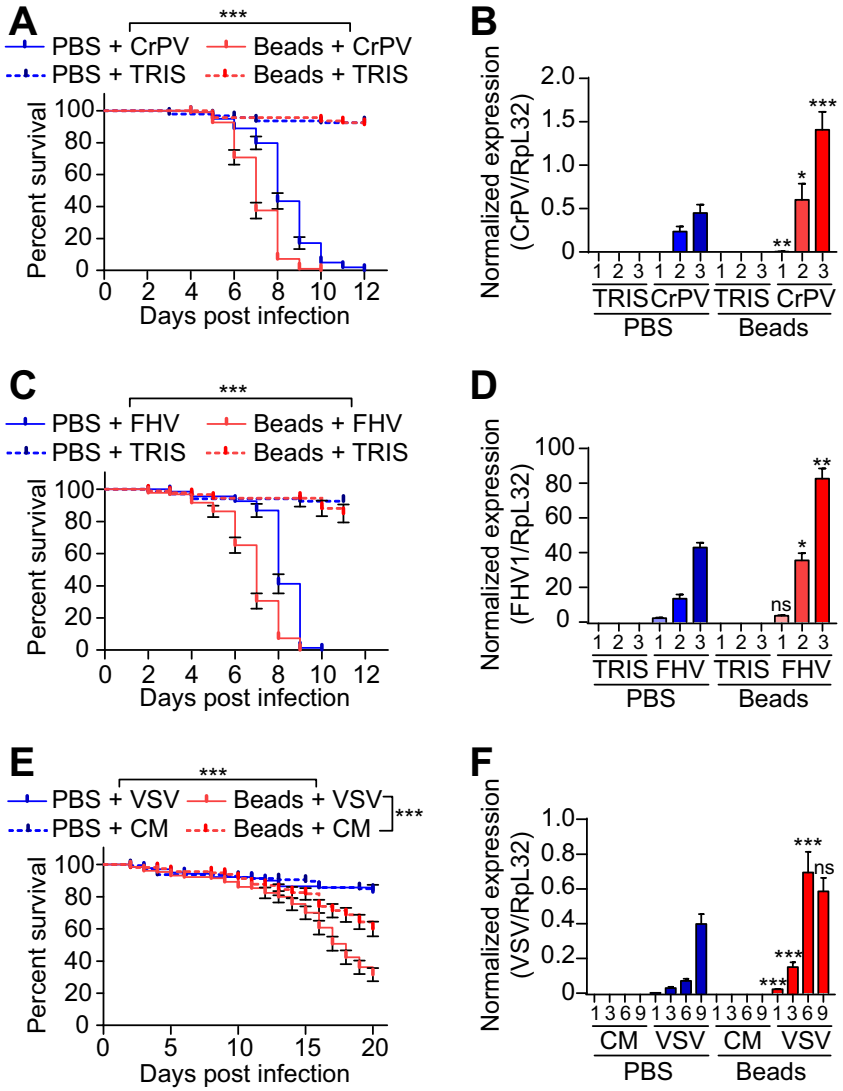

FIG 1 Inhibition of phagocytosis affects resistance to CrPV, FHV, and VSV. (A, C, and E) Survival of Canton-S wild-type flies injected with latex beads or PBS 1 day before challenge with CrPV (A), FHV (C), or VSV (E). (B, D, and F) Quantitative RT-PCR analysis of the accumulation of viral RNA at the indicated days postinfection in Canton-S flies injected with latex beads or PBS 1 day before challenge with CrPV (B), FHV (D), or VSV (F). Tris or conditioned medium (CM) was used as a control. Data represent the means ± standard errors (A, C, and E) or SD (B, D, and F) of 3 independent experiments, each containing three groups of 10 (A, C, and E) or 6 (B, D, and F) flies. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (log rank [A, C, and E] or unpaired *t* test [B, D, and F]).

characteristic paracrystalline cytoplasmic inclusions. They play a key role in host defense through melanization and they participate in wound healing. Lamellocytes are large (15 to 40 μm in diameter), flat adherent cells which encapsulate and neutralize objects too large to be phagocytosed by plasmatocytes (30–32). Of note, the question of the involvement of hemocytes in antiviral immunity has not been a focus of interest so far for *Drosophila*.

Here, we performed a comparative analysis of the contribution of cellular immunity and autophagy to antiviral host defense, using a panel of 6 different viruses. We show that hemocytes participate in antiviral host defense against CrPV, Flock House virus (FHV), and VSV, but not against the three other viruses tested. FHV- and CrPV-infected cells undergo apoptosis and can be cleared by hemocytes. In addition, we confirmed that autophagy participates in the host defense against VSV infection, although its contribution is modest compared to that of RNAi. However, *Drosophila* strains with mutations of the essential autophagy gene *Atg7* are more resistant to FHV infection, indicating that autophagy has a pro- rather than antiviral function in this context. Our results indicate that blood cells and autophagy display virus-specific functions in *Drosophila* and are not general antiviral pathways, in contrast to RNAi.

MATERIALS AND METHODS

***Drosophila* strains.** The fly stocks were raised on standard cornmeal agar medium at 25°C. *Canton-S*, w^{1118}, y^1 w^1, *Df(2R)BSC22/SM6a* (stock number 7441), *w**; *P{UAS-mCherry.NLS}3* (stock 38424), *Df(2R)BSC45*, w^{+mC}*/SM6a* (stock 7441), UAS-Mito::GFP (stock 8443), and *actin*-GAL4 (stock 25374) genotype flies were obtained from the Bloomington Fly Stock Center (Bloomington, IN). $Atg7^{d14}$*/Cyo-GFP*, $Atg7^{d77}$*/Cyo-GFP*,

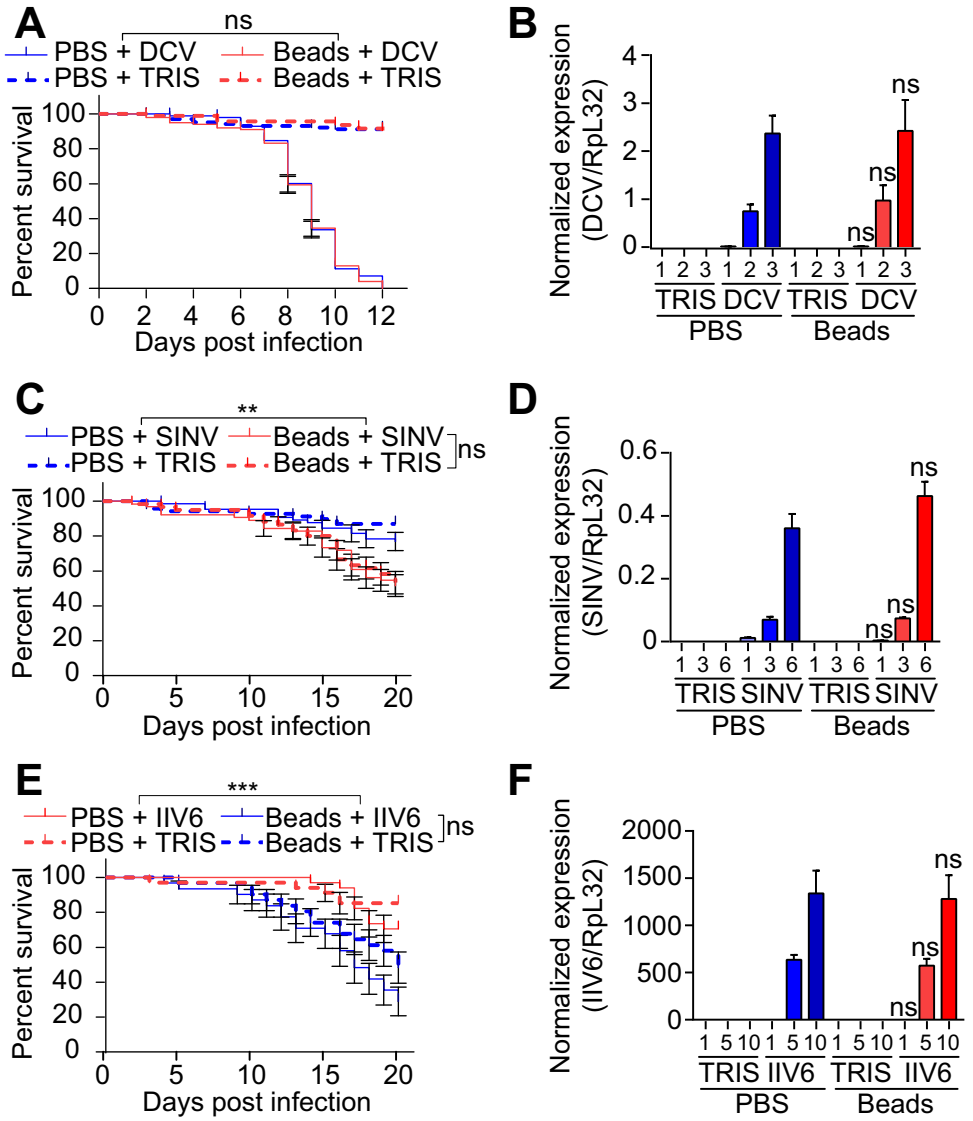

FIG 2 Phagocytosis is not required during DCV, SINV, and IIV6 infection. (A, C, and E) Survival of Canton-S wild-type flies injected with latex beads or PBS 1 day before challenge with DCV (A), SINV (C), or IIV6 (E). (B, D, and F) Quantitative RT-PCR analysis of the accumulation of viral RNA at the indicated days postinfection in Canton-S flies injected with latex beads or PBS 1 day before challenge with DCV (B), SINV (D), or IIV6 (F). Tris was used as a control. Data represent the means ± standard errors (A, C, and E) or SD (B, D, and F) of 3 independent experiments, each containing three groups of 10 (A, C, and E) or 6 (B, D, and F) flies. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (log rank [A, C, and E] or unpaired *t* test [B, D, and F]).

CG5335^{d30}*/Cyo-GFP* (33), *Dcr-2*^{L811fsX} (3), *UAS-bax/CyO-actin-GFP*, *hml*(Δ)-GAL4, *UAS-eGFP* (34), *Toll-7*$^{g1-1}$ (35), and *Toll-7*^{p8} and *Toll-7*^{p114} (36) stocks have been described previously. A genomic rescue of the *Toll-7* gene was established with the fosmid FlyFos 030116 (http://transgeneome.mpi-cbg.de) inserted at the landing site attP40 (3L), and the transgenic chromosome was associated with the deficiency *Df(2R)BSC22*, which uncovers the *Toll-7* locus. For the rescue experiments, *Toll-7*$^{g1-1}$ mutants were crossed with the *Df(2R)BSC22–gToll-7* rescue line. All fly lines were tested and cleared of any *Wolbachia* spp. infection.

Phagocyte ablation experiments. Surfactant-free, red, 0.3-μm-diameter carboxylate modified latex beads (Interfacial Dynamics Corp.) were washed and resuspended at a 4× concentration in 1× phosphate-buffered saline (PBS) (corresponding to 5 to 10% solids). Flies were injected with 69 nl of this solution 24 h prior to virus infection.

Infections. Adult flies (half males and half females) 4 to 6 days old were used in infection experiments. VSV and VSV with a green fluorescent protein inserted (VSV-GFP) were grown and titers were determined on Vero cells. Supernatants of infected cells were centrifuged at 1,000 × *g* to pellet cell debris. The resulting virus suspensions were used to infect flies. A supernatant from uninfected cells was used as a control. For all other viruses, stocks were prepared, titers were determined as described previously (3), and the stocks were resuspended in 10 mM Tris-HCl (pH 7.5). Infections were done by intrathoracic injection (Nanoject II apparatus; Drummond Scientific) of 4.6 nl of a viral suspension (500 PFU/fly for DCV and FHV, 5 PFU/fly for CrPV, 2,500 PFU/fly for SINV, 5,000 PFU/fly for Invertebrate iridescent virus 6 [IIV6], and 10,000 PFU/fly for VSV and VSV-GFP). The size of the inoculum was chosen to take into account the kinetics of replication and colonization of *Drosophila* by the different viruses (3). Injection of the same volume of 10 mM Tris-HCl (pH 7.5) or mock-infected Vero cell culture supernatant for VSV and VSV-GFP experiments was used for controls. Infected flies were incubated at 25°C and monitored daily for survival or frozen for RNA or DNA isolation at the indicated time points.

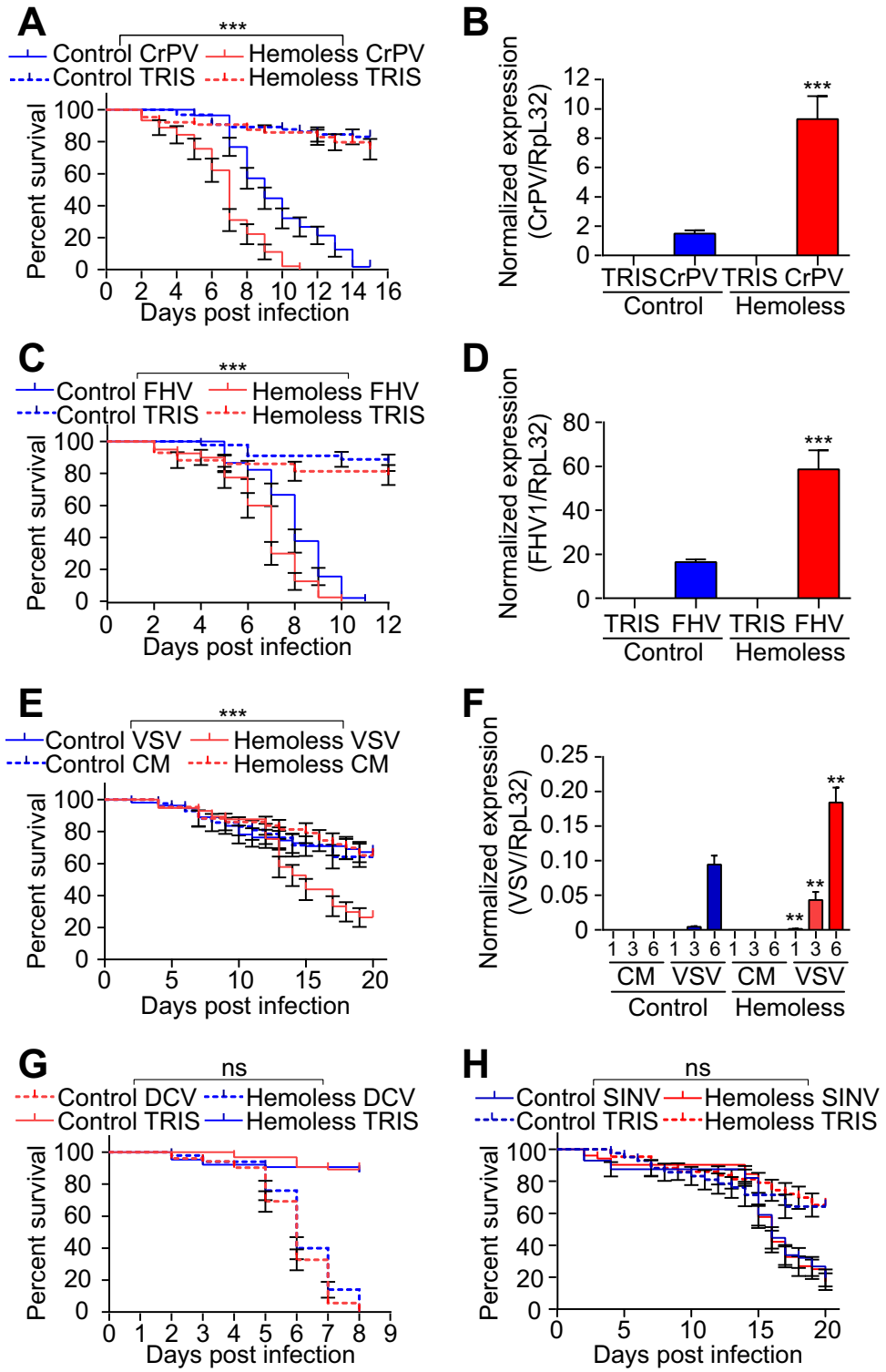

FIG 3 Hemocyte-depleted flies are susceptible to some, but not all, viral infections. (A, C, E, G, and H) Survival of hemoless [*hml*(Δ)-GAL4, *UAS-eGFP*, or *UAS-Bax*] and control flies [*hml*(Δ)-GAL4, *UAS-eGFP*,+] after CrPV (A), FHV (C), VSV (E), DCV (G), or SINV (H) infection. (B, D, and F) Quantitative RT-PCR analysis of the accumulation of viral RNA in hemoless and control flies 3 days postinfection with CrPV (B) or FHV (D) and at the indicated days postinfection following VSV infection (F). Tris or conditioned medium (CM) was used as the injection control. Data represent the means ± standard errors (A, C, E, G, and H) or SD (B, D, and F) of 3 independent experiments, each containing three groups of 10 (A, C, E, G, and H) or 6 (B, D, and F) flies. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (log rank [A, C, and E] or unpaired *t* test [B, D, and F]).

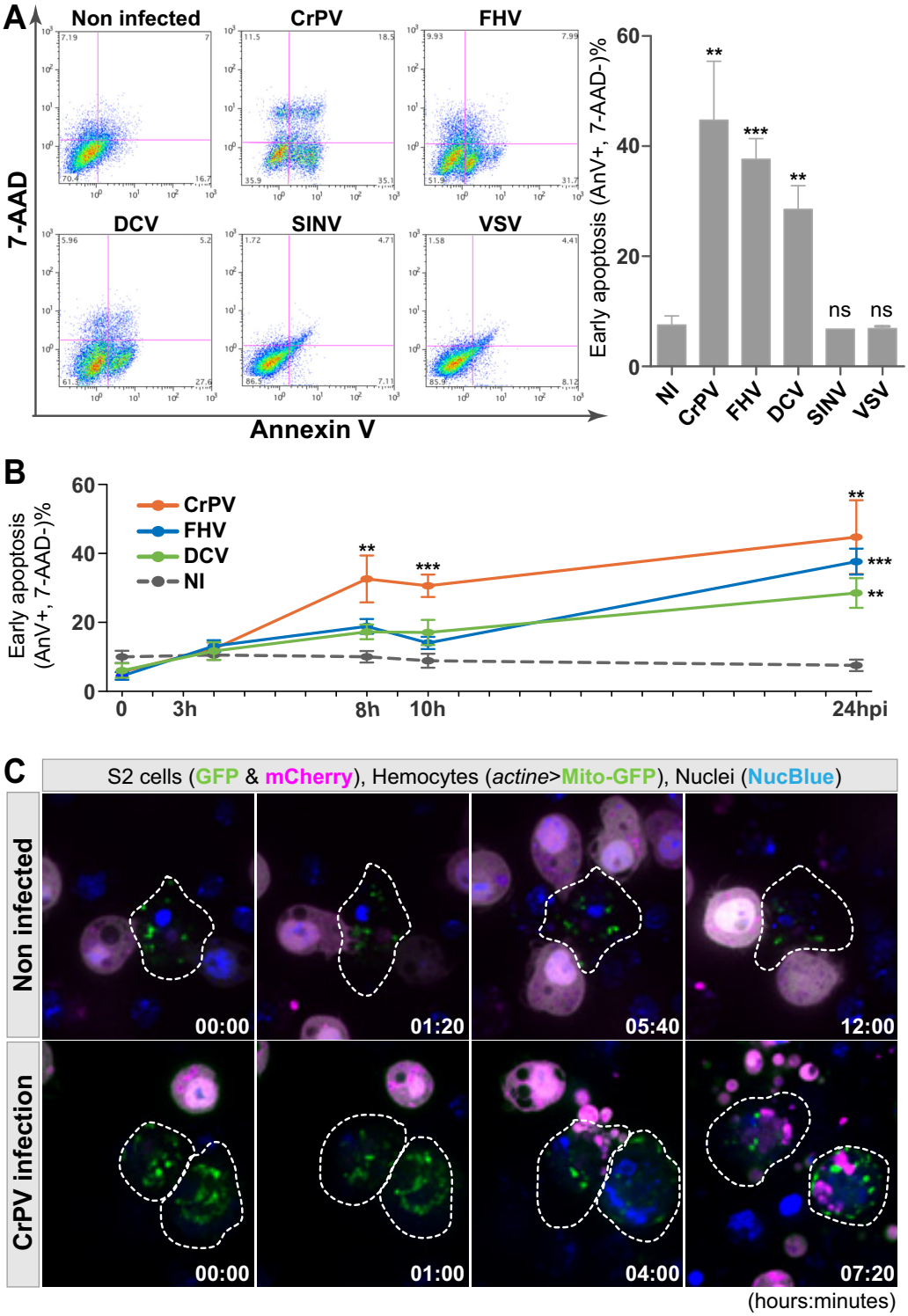

FIG 4 Apoptosis-dependent phagocytosis of CrPV-infected cells. (A) Annexin-V/7-AAD double staining to quantify viable S2 cells after infection by the indicated viruses to quantify viable cells (Annexin-V$^-$, 7-AAD$^-$), early apoptotic cells (Annexin-V$^+$, 7-AAD$^-$), and late apoptotic/necrotic cells (AnnexinV$^+$, 7-AAD$^+$) 24 h postinfection. The right panel represents the percentage of early apoptotic cells (Annexin-V$^+$, 7-AAD$^-$) after 24 h of infection with different viruses. (B) Kinetics of appearance of early apoptotic cells (Annexin-V$^+$, 7-AAD$^-$) during infection of S2 cells with CrPV, FHV, or DCV. Measurements were made at 0, 3, 8, 10, and 24 h postinfection. NI, noninfected. For panels A and B, graphs represent means ± standard errors of the means. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ (Student's *t* test). (C) Phagocytosis of apoptotic cells after CrPV infection. Hemocytes (*actine*>mito-GFP cells), surrounded by the dotted lines, were incubated with mock- or CrPV-infected S2 cells (cytoplasmic GFP and mCherry markers) for 12 h. NucBlue was used to stain DNA in live cells. One picture was taken every 20 min. The corresponding movies can be found in the supplemental material.

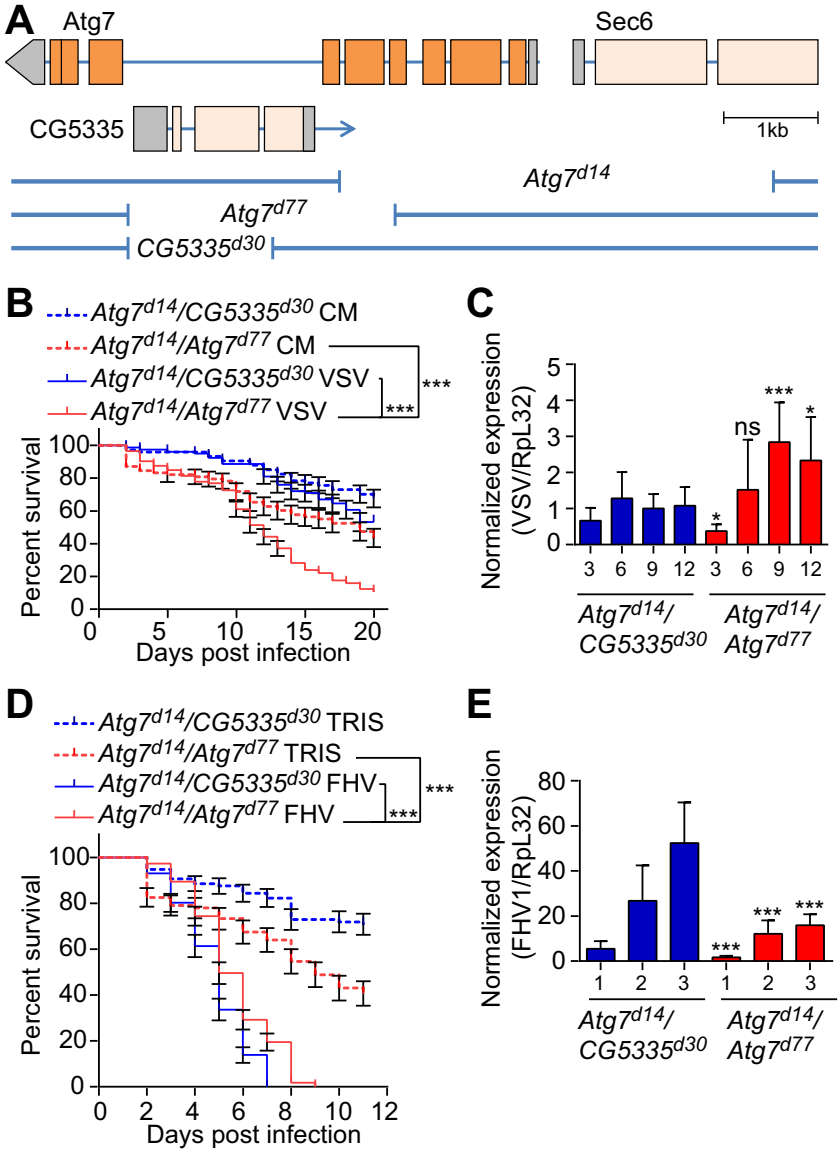

FIG 5 *Atg7* can have pro- or antiviral activity in *Drosophila*. (A) Schematic representation of the *Atg7* locus and the deletion mutants used. Exons are indicated by rectangles with the coding region highlighted in orange. Deletions are indicated by the gaps in the blue lines. (B and D) Survival of *Atg7* mutant ($Atg7^{d14}/Atg7^{d77}$) and control ($Atg7^{d14}/CG5335^{d30}$) flies after VSV (B) or FHV (D) infection. Tris or conditioned medium (CM) was used as an injection control. (C and E) Quantitative RT-PCR analysis of the accumulation of viral RNA at the indicated day postinfection in control ($Atg7^{d14}/CG5335^{d30}$) and *Atg7* mutant ($Atg7^{d14}/Atg7^{d77}$) flies injected with VSV (C) or FHV (E). Data represent the means ± standard errors (B and D) or SD (C and E) of three independent experiments, each containing three groups of 10 (B and D) or 6 (C and E) flies. ns, not significant; *, $P < 0.05$; ***, $P < 0.001$ (log rank [B and D] or unpaired *t* test [C and E]).

Quantitative RT-PCR. Analysis of RNA expression or viral DNA was performed by real-time quantitative reverse transcription-PCR (RT-PCR) as previously described (3). Primers used for quantitative PCR (qPCR) were as follows: RP49 (forward, 5′-GACGCTTCAAGGGACAGTATCTG-3′; reverse, 5′-AAACGCGGTTCTGCATGAG-3′), DCV (forward, 5′-TCATCGGTATGCACATTGCT-3′; reverse, 5′-CGCATAACCATGCTCTTCTG-3′), CrPV (forward, 5′-GCTGAAACGTTCAACGCATA-3′; reverse, 5′-CCACTTGCTCCATTTGGTTT-3′), FHV RNA1 (forward, 5′-TTTAGAAGCACATGCGTCCAG-3′; reverse, 5′-CGCTCACTTTCTTCGGGTTA-3′), VSV (forward, 5′-CATGATCCTGCTCTTCGTCA-3′; reverse, 5′-TGCAAGCCCGGTATCTTATC-3′), SINV (forward, 5′-CAAATGTGCCACAGATACCG-3′; reverse, 5′-ATACCCTGCCCTTTCAACAA-3′), Toll-7 (forward, 5′-GGGCGAGAATCAAATTCGTA-3′; reverse, 5′-CAGACCAGTCAGCTGGTGAA-3′), IIV6 (forward, 5′-TTGTTAGGAATTGGAACTGGAA-3′; reverse, 5′-GCCCTAGATGCTGCTTGTTC-3′).

Flow cytometry. Cell death was assessed by Annexin-V–fluorescein isothiocyanate/7-aminoactinomycin D (7-AAD) double staining (catalog numbers 559925 and 556419; BD Biosciences) after infection at a multiplicity of infection (MOI) of 10 during 1 h at 4°C. After acquisition by a Gallios flow cytometry apparatus (Beckman Coulter), data were analyzed with FlowJo software (Tree Star) or imaged with a cell observer (spinning disk; Zeiss, Oberkochen, Germany) with adapted settings.

Live imaging. Lab-Tek II chambered coverglasses (catalog number 155382; Thermo Scientific Nunc) were coated with a Cell-Tak solution (catalog number 354240; Corning) diluted in ultrapure water (1 to 5 μg/

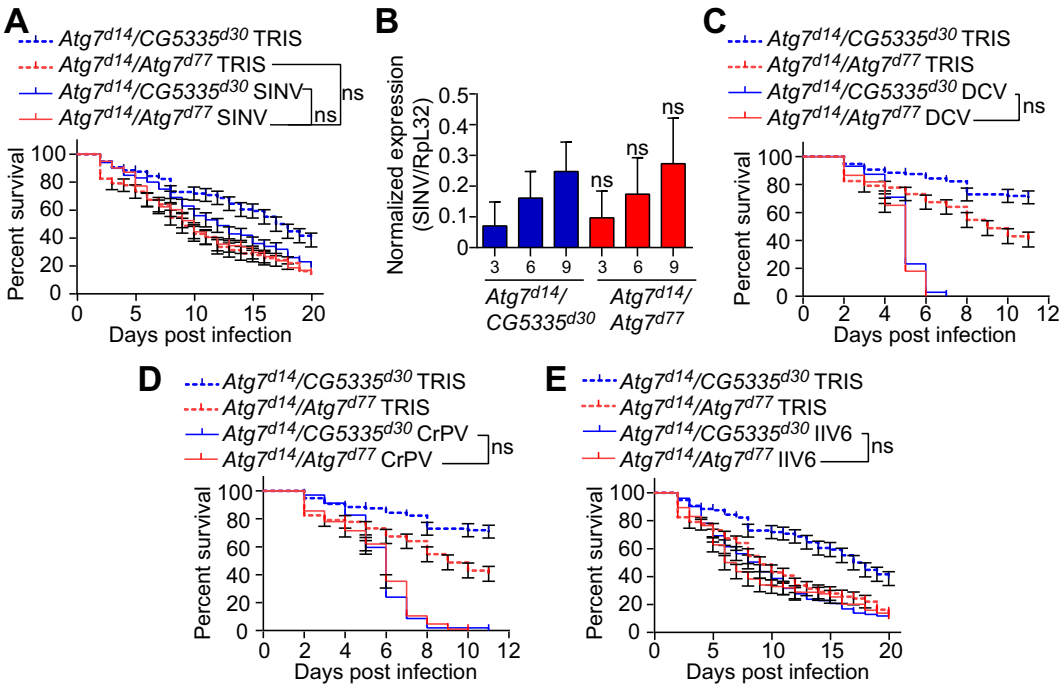

FIG 6 *Atg7* mutant flies show resistance similar to controls after infection by SINV, DCV, CrPV, or IIV6. (A, C, D, and E) Survival of *Atg7* mutant ($Atg7^{d14}/Atg7^{d77}$) and control ($Atg7^{d14}/CG5335^{d30}$) flies after SINV (A), DCV (C), CrPV (D), or IIV6 (E) infection. Tris injection was used as a control. (B) Quantitative RT-PCR analysis of the accumulation of viral RNA at the indicated day postinfection in control ($Atg7^{d14}/CG5335^{d30}$) and *Atg7* mutant ($Atg7^{d14}/Atg7^{d77}$) flies injected with SINV. Data represent means ± standard errors (A, C, D, and E) or SD (B) of 3 independent experiments, each containing three groups of 10 (A, C, D, and E) or 6 (B) flies. ns, not significant (log rank [A, C, D, and E] or unpaired *t* test [B]).

cm^2). A total of 200,000 S2 cells were added per chamber during 30 min at 25°C. Cells were incubated with different viruses at an MOI of 10 during 1 h at 4°C. NucBlue was used for live DNA staining (catalog number R37605; Molecular Probes). Cells were then observed using adapted settings on a cell observer (spinning disk; Zeiss, Oberkochen, Germany).

Cocultured hemocytes/infected S2 cells. A stable cell line derived from plasmatocyte-like S2 cells was established for the expression of cytoplasmic GFP and mCherry, using the construct Flag-mCherry-T2A-GFP-T2A-neo (catalog number 32426; Addgene) and G418 at a concentration of 2 mg/ml. Hemocytes were collected from *actin*-GAL4>UAS-Mito-GFP adult flies and added to Lab-Tek II chambered coverglasses with Schneider medium for 1 h at 25°C. Aliquots (100 μl) of infected S2 cells (at a concentration of 10^6 cells/ml) were added to each well. Cells were imaged for 12 h, with 1 picture every 20 min, under a confocal microscope (spinning disk cell observer; Zeiss, Oberkochen, Germany) using adapted settings.

Statistical analysis. An unpaired two-tailed Student *t* test was used for statistical analysis of data within GraphPad Prism (GraphPad Software). Survival curves were plotted and analyzed by log-rank analysis (Kaplan-Meier method) using the Prism program (GraphPad Software). *P* values less than 0.05 were considered statistically significant.

RESULTS

Virus-specific role of plasmatocytes in antiviral host defense. Injection of latex beads into wild-type flies blocks phagocytosis and provides a convenient way to address the contribution of plasmatocytes in host defense (37, 38). As observed previously (39), flies injected with latex beads showed decreased survival upon infection with the dicistrovirus CrPV (Fig. 1A). In addition, bead injection led to a significant increase in the CrPV titer, suggesting that phagocytes are important to control viral replication (Fig. 1B). Resistance of wild-type flies to the nodavirus FHV and the rhabdovirus VSV was also significantly reduced when phagocytosis was impaired (Fig. 1C to F). In contrast, injection of latex beads did not affect survival or virus load following challenge with the alphavirus SINV, the iridovirus IIV6, or DCV, which belongs to the same family as CrPV (Fig. 2). Similar results were obtained using transgenic flies genetically depleted of hemocytes, referred to as hemoless (34). Hemoless flies were more sensitive to infection by CrPV, FHV, and VSV than were the wild-type controls [*hml*(Δ)-GAL4, *UAS-eGFP*/+] (Fig. 3). In agreement with results using latex beads, no differences were observed for the other viruses. These findings uncover the involvement of hemocytes in the control of viral infections, thus revealing a novel arm of the antiviral defense in insects. Significantly, however, they also showed that this host defense reaction is limited to some viral species.

FHV and CrPV, but not VSV, induce apoptosis of infected cells. The requirement for phagocytosis in antiviral immunity raises the question of the recognition of virus-infected cells by plasmatocytes. FHV was one of the viruses impacted by hemocytes and is known to induce apoptosis (23, 40), raising the possibility that plasmatocytes are involved in the clearance of infected apoptotic cells. One hallmark of apoptosis is the early surface exposure of phosphatidylserine, which can readily be detected by Annexin-V staining. As expected, *Drosophila* S2 cells (a hemocyte-like cell line) infected with FHV had a significant increase in Annexin-V staining 24 h postinfection (Fig. 4A). CrPV-infected cells, and to a lesser extent DCV-infected cells, also showed increased Annexin-V staining compared to controls, suggesting that *Dicistroviridae* trigger apoptosis as well, although we did not detect a contribution of hemocytes in the control of DCV (Fig. 4A) (see Discussion). Notably, CrPV-infected cells showed a significant in-

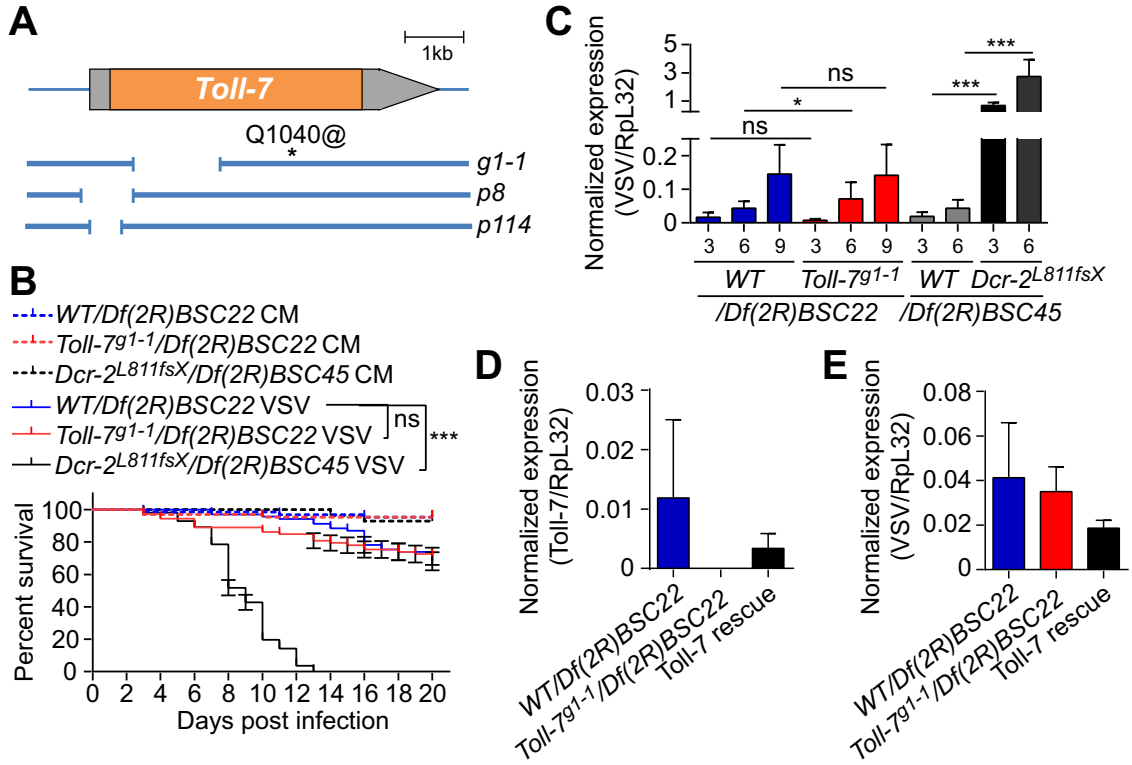

FIG 7 *Toll7*$^{g1-1}$ mutant flies are not sensitive to VSV infection. (A) Schematic representation of the *Toll-7* gene and the deletion mutants evaluated. The coding region is highlighted in orange, and deletions are indicated by the gaps in the blue lines. For the *Toll-7*$^{g1-1}$ allele, a stop codon replaces a glutamine at position 1040. (B) Survival of *Toll-7*$^{g1-1}$ null mutant and control flies after VSV infection. Flies carrying a null allele of *Toll-7* or controls from the same genetic background (*yw*; *Toll-7*$^{g1-1}$ and y^1 w^1) were crossed with a line containing a deficiency covering the *Toll-7* gene [*Df(2R)BSC22*] and hemizygous flies from the progeny were injected with conditioned medium (CM) or VSV. *Dcr-2* null mutant (*Dcr-2*^{L811fsX}) and WT flies crossed with the deficient *Df(2R)BSC45* flies were used as positive controls. (C) Quantitative RT-PCR analysis of the accumulation of VSV RNA on the indicated day postinfection in control flies [*WT/Df(2R)BSC22* and WT/*Df(2R)BSC45*], *Toll-7* null mutant flies [*Toll-7*$^{g1-1}$*/Df(2R)BSC22*], and *Dcr-2* mutant flies [*Dcr-2*^{L811fsX}*/Df(2R)BSC45*]. (D and E) Quantitative RT-PCR analysis of *Toll-7* mRNA expression (D) and VSV viral RNA accumulation (E) in control [*WT/Df(2R)BSC22*], *Toll-7* null mutant [*Toll-7*$^{g1-1}$*/Df(2R)BSC22*], and *Toll-7* rescue [*Toll-7*$^{g1-1}$*/Df(2R)BSC22*; *+/gToll-7*] flies at 6 dpi. Data represent means ± standard errors (B) or SD (C, D, and E) of three independent experiments, each containing three groups of 10 (B) or 6 (C, D, and E) flies. ns, not significant; *, $P < 0.05$; ***, $P < 0.001$ (log rank [B] or unpaired *t* test [C, D, and E]).

crease in Annexin-V as early as 8 h postinfection (Fig. 4B). *Ex vivo*, plasmatocytes isolated from adult flies engulfed apoptotic bodies derived from CrPV-infected cells, supporting the hypothesis that plasmatocytes clear apoptotic cells and prevent the release of infectious particles from dead or dying cells (Fig. 4C; see also Movies S1 and S2 in the supplemental material). Intriguingly, we did not detect Annexin-V staining or cell death of VSV-infected S2 cells (Fig. 4A), despite the importance of phagocytosis and plasmatocytes in the control of this virus *in vivo*. This suggests that blood cells participate in the control of VSV by a mechanism different from clearance of apoptotic cells.

Autophagy has opposite effects on VSV and FHV in *Drosophila*. Plasmatocytes could be involved in the direct recognition of viruses. In mammals, VSV infection promotes autophagy in dendritic cells, leading to recognition of viral RNA by Toll-like receptor 7 (TLR-7) upon fusion of autophagosomes with lysosomes (41). Of note, autophagy and a Toll receptor, Toll-7, were reported to participate in the control of VSV infection in *Drosophila* (22, 42). To address the global role of autophagy in antiviral defenses in flies, we used transheterozygous adult flies carrying two null alleles of the *Atg7* gene (*Atg7*$^{d14/d77}$). These flies are viable but exhibit a strong impairment of the autophagy pathway and succumb rapidly to starvation (data not shown) (33). We compared these flies to controls carrying one of the null alleles in *trans*, with a shorter deletion affecting the gene *CG53335* but not the coding sequence of *Atg7* (*Atg7*^{d14}*/CG5335*^{d30}) (Fig. 5A). Of note, the three different deletion mutants are in the same genetic background (33). In agreement with their shorter life span and reported susceptibility to oxidative stress, *Atg7* mutant flies (*Atg7*$^{d14/d77}$) were more sensitive to an injection of buffer than were control flies (*Atg7*^{d14}*/CG5335*^{d30}) (Fig. 5B and D). In addition, *Atg7* mutants exhibited reduced survival upon VSV infection (Fig. 5B), as previously described (22). A significant but modest increase (almost 3-fold) in the VSV viral titer was observed in *Atg7* mutant flies at late time points of infection that coincided with a decrease in survival (9 and 12 days postinfection [dpi]) (Fig. 5C). In contrast, we did not observe significant differences in the resistance of *Atg7* mutants versus control flies to infection with SINV, DCV, CrPV, or IIV6 (Fig. 6). Surprisingly, *Atg7* mutant flies showed increased survival and decreased viral loads compared to controls infected with FHV, suggesting that autophagy plays a proviral role in this context (Fig. 5D and E). Altogether, these data indicate that autophagy is not a broad antiviral mechanism in *Drosophila* and can impact viral replication negatively or positively, depending on the virus.

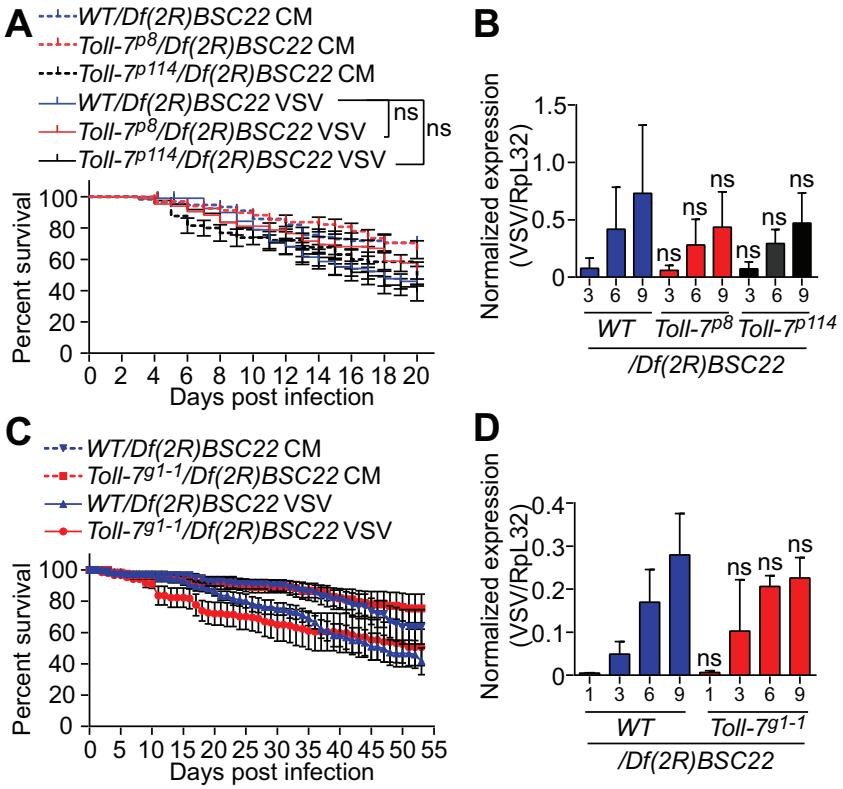

FIG 8 Toll-7 is not required to control VSV infection. (A and C) Survival of *Toll-7* mutant and control flies after VSV (A) or VSV-GFP (C) infection. Flies carrying a null allele of *Toll-7* or controls from the same genetic background (w^{1118}; *Toll-7*^{p8} or *Toll-7*^{p114} and w^{1118} [A] and *yw*; *Toll-7*$^{g1-1}$ and y^1 w^1 [C] were crossed with a line containing a deficiency covering the *Toll-7* gene [*Df(2R)BSC22*] and hemizygous flies from the progeny were injected with conditioned medium (CM) or VSV (A) or VSV-GFP (C). (B and D) Quantitative RT-PCR analysis of the accumulation of VSV RNA in control and the indicated *Toll-7* mutant flies injected with VSV (B) or VSV-GFP (D) at the indicated day postinfection. Data represent means ± standard errors (A and C) or SD (B and D) of three independent experiments, each containing three groups of 10 (A and C), 6 (B), or 5 (D) individual flies. ns, not significant (log rank [A] or unpaired *t* test [B and D]).

Toll-7 and resistance to VSV infection. The VSV-specific antiviral role of autophagy led us to further investigate the previously described mechanisms. The Toll-7 receptor has been proposed to function as a direct VSV sensor to activate autophagy and control infection (42). However, we observed that *Toll-7*-deficient flies carrying a null allele over a deficiency that covers the *Toll-7* locus [*Toll-7*$^{g1-1}$/*Df(2R)BSC22*] (35) (Fig. 7A) survived VSV infection, similar to control flies carrying one wild-type allele of *Toll-7* [WT/*Df(2R)BSC22*]. For comparison to *Toll-7* mutants, we used *Dcr-2* mutant flies [*Dcr-2*^{L811fsX}/*Df(2R)BSC45*], which showed significantly increased VSV replication and rapidly succumbed to infection (Fig. 7B and C). VSV RNA levels remained similar in *Toll-7* hemizygote mutants and in control flies, despite a 1.6-fold significant increase observed at only one time point. However, this mild phenotype was not reproducible when we rescued mutant flies with a transgene carrying a wild-type copy of *Toll-7* (Fig. 7D and E). In addition, flies carrying two other null alleles of *Toll-7* (p8 and p114) (36) also resisted VSV infection, similar to control flies (Fig. 8A and B). We noted that the original study showed increased sensitivity of *Toll-7* mutants to a recombinant VSV-GFP virus, whereas we used a wild-type VSV (Indiana strain); this could help explain the differences we observed. However, we obtained similar results using a recombinant VSV-GFP in a separate set of experiments (Fig. 8C and D). We conclude that Toll-7 does not participate in the autophagy and hemocyte-mediated host defense against VSV infection.

DISCUSSION

We investigated the involvement of hemocytes and autophagy in the resistance to a panel of six viruses representative of different families of RNA and DNA viruses. Our data revealed that (i) unlike RNAi, which acts via a broad antiviral pathway, blood cells play a critical role only in defense against certain viruses, such as CrPV, FHV, and VSV; (ii) CrPV and FHV induce apoptosis of infected cells that likely act in concert with hemocytes to control these viruses; (iii) autophagy contributes to the containment of VSV infection together with hemocytes, but it does not seem to be important for the other viruses tested.

How can hemocytes sense infected cells and control viral infections in flies? FHV, CrPV, and VSV belong to different virus families and do not share obvious features that would explain why hemocytes are required to control them. However, FHV and CrPV trigger surface exposure of phosphatidylserine, suggesting that plasmatocytes clear apoptotic cells containing infectious particles. Recently, Nakanishi and colleagues reported a role for hemocytes in the control of DCV infection. They demonstrated that DCV infection triggers activation of effector caspases, phosphatidylserine exposure, and efferocytosis of the dying cells that is me-

diated by the receptors integrin β_ν and Draper (24). Curiously, in our hands blood cells were not required to control DCV infection *in vivo*. This virus induced exposure of phosphatidylserine and cell death in tissue culture S2 cells, but at lower rates than FHV and CrPV. Of note, DCV is less virulent than the two other viruses, possibly because its suppressor of RNAi is less potent (43). This could account for a threshold difference for the induction of apoptosis. In this regard, the difference between the two studies may have been due to the high dose of DCV used by Nainu et al. (up to 80,000 50% tissue culture infective doses [$TCID_{50}$] per fly, versus a dose of ~800 $TCID_{50}$ in our study).

Autophagy may be used to combat infection by intracellular pathogens, such as viruses, by isolating them from the cytosol through an isolation membrane and targeting them to lysosomes, where they can be degraded. The role of autophagy in the control of viral infection is complex, since many RNA viruses hijack the autophagy machinery to generate the network of intracellular membranes that will nest their replication sites (44). The complexity of the relationship between viruses and autophagy is well illustrated by the case of measles virus, in which a first wave of autophagy, triggered during viral entry, is antiviral, whereas a second wave of autophagy is proviral (45, 46). Our data indicate that only two of the six viruses tested are affected by mutation of the essential autophagy gene *Atg7*. The first is FHV, for which autophagy is proviral, as shown by the reduced viral titer and increased survival of *Atg7* mutant flies. Interestingly, FHV replicates on the outer membrane of mitochondria, and an important and well-characterized role of autophagy is the regulation of the turnover of mitochondria (mitophagy) (47). We propose that efficient removal of damaged mitochondria, which may not fully support the activity of the viral polymerase, contributes to the success of FHV replication. The second virus affected by autophagy is VSV, for which we observed an increase in replication by *Atg7* mutants, as previously reported (22). This increase is, however, modest (3-fold increase), especially compared to that in RNAi-deficient flies (>50-fold increase). Therefore, we conclude that autophagy does not represent a major pathway of antiviral defense in *Drosophila*.

A previous study reported the provocative finding that one of the nine Toll receptors encoded in the *Drosophila* genome, Toll-7, senses VSV infection and triggers antiviral autophagy (42). However, our genetic studies using three different well-characterized alleles of *Toll-7* do not support this initial observation. Although the sizes of the families of Toll receptors in *Drosophila* and mammals are similar, with about 10 members, phylogenetic analysis has clearly indicated that Toll receptors evolved independently in different animal phyla (48, 49). In *Drosophila*, only Toll itself has so far been shown to participate in the induction of an immune response in flies, although Toll-8 acts as a negative regulator of antimicrobial defenses in the respiratory tract (50–53). *Drosophila* Toll receptors are highly expressed during embryogenesis, pointing to developmental functions (49, 54). Indeed, a subset of Toll receptors, including Toll-7, function as neurotrophin receptors and are activated by members of the Spaetzle family in the developing nervous system (36, 55). *Drosophila* Toll receptors were also recently shown to function as adhesion molecules during elongation of the antero-posterior axis of the embryo (56). In contrast, experimental evidence that members of the Toll family other than Toll itself participate in the activation of innate immunity in *Drosophila* is still lacking.

ACKNOWLEDGMENTS

We thank François Leulier for *Hml*(Δ)-gal4, *uas-Bax* flies, Thomas P. Neufeld for $Atg7^{d14}$, $Atg7^{d77}$ and $CG5335^{d30}$ mutant flies, Tony Ip for $Toll7^{g1-1}$ mutant flies, and Alicia Hidalgo for $Toll7^{p8}$ and $Toll7^{p114}$ mutant flies. Expert technical assistance from Estelle Santiago and Stefanie Pietsch is gratefully acknowledged.

O.L., C.M., J.T.M., and J.-L.I. designed the experiments; O.L., J.A., I.J.D.S.D.F., and R.P.O. performed the experiments; O.L., J.A., I.J.D.S.D.F., R.P.O., F.B., C.M., J.A.H., J.T.M., and J.-L.I. analyzed the data; and O.L., J.A.H., J.T.M., and J.-L.I. wrote the manuscript.

This work was supported by CNRS, the University of Strasbourg Institute for Advanced Study, and grants from the NIH (PO1 AI070167) and ANR (ANR-11-ISV3-002 and ANR-13-BSV3-0009) and Investissements d'Avenir programs (NetRNA ANR-10-LABX-36 and I2MC ANR-11-EQPX-0022). I.J.D.S.D.F. and R.P.O. were supported by fellowships from CNPq and CAPES. J.T.M. was supported by grants from CAPES, CNPq, and FAPEMIG.

FUNDING INFORMATION

This work, including the efforts of Jules A. Hoffmann and Jean-Luc Imler, was funded by HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) (PO1 AI070167). This work, including the efforts of Joao T. Marques, was funded by MCTI | Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). This work, including the efforts of Jean-Luc Imler, was funded by Agence Nationale de la Recherche (ANR) (ANR-11-ISV3-002 ANR-13-BSV3-0009). This work, including the efforts of Joao T. Marques, was funded by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). This work, including the efforts of Joao T. Marques, was funded by Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG).

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