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Parkin-mediated responses against infection and wound involve TSPO-VDAC complex in *Drosophila*

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ABSTRACT

Parkin, an E3 ubuquitin ligase associated with Parkinson's disease (PD), has recently been implicated in mediating innate immunity. However, molecular details regarding parkin-mediated immune response remain to be elucidated. Here, we identified mitochondrial TSPO-VDAC complex to genetically interact with parkin in mediating responses against infection and wound in *Drosophila*. The loss-of-function mutation in *parkin* results in defective immune response against bacterial infection. Additionally, *parkin* mutant larvae showed hypersensitivity against wound regardless of bacterial infection. Interestingly, the combinatorial trans-heterozygotic mutations in *parkin* and *TSPO*, or *parkin* and *VDAC* showed similar lethal tendency with *parkin* homozygous mutants. Furthermore, knockdown of *TSPO* alone also resulted in defective responses to infection and wound analogously to *parkin* mutants. Taken together, we propose that parkin cooperates with TSPO-VDAC complex to mediate responses against infection and wound.

1. Introduction

Parkin is a cytosolic, multifaceted E3 ubiquitin ligase that mediates proteasomal degradation of substrate proteins through various forms of ubiquitination [1–3]. Mutations in *PARK2* that codes for the protein parkin in humans remarkably cause mitochondrial dysfunction and give rise to a subset of autosomal recessive juvenile Parkinson's disease (AR-JP) [4,5]. Indeed, parkin translocates to the mitochondria upon mitochondrial stress to minimize toxicity, and its mutation abolishes this protective function [6–8].

Interestingly, *PARK2* was recently revealed as a susceptibility gene for infections by intracellular pathogens, thereby implicating parkin in innate immunity [9,10]. Previous findings showed parkin to mediate the downstream pathway of NF-kB (nuclear factor-kB) signaling response after pathogenic infection in macrophages and Schwann cells [11]. Additionally, the E3 ubiquitin ligase activity of parkin was shown to be involved in autophagic removal of intracellular pathogens in both mice and flies [12]. Nevertheless, detailed mechanisms and interaction partners of parkin in mediating the innate immune response still remain to be elucidated.

Recently identified role of parkin in innate immunity accentuates the mitochondria as imperative modulator of innate immunity [13]. Mitochondria have been linked to a wide variety of innate immune pathways including antiviral signaling, cellular damage response, and antibacterial signaling [14]. Indeed, accumulating pieces of evidences implicate other mitochondrial proteins in innate immunity as well [15]. One such mitochondrial protein is the 18 kDa translocator protein (TSPO), to which various functions have been ascribed besides its involvement in immunity [16,17]. Notably, TSPO has recently been shown to be involved in parkin-mediated autophagy in cooperation with VDAC1, indicating close association between these three molecules [18]. However, the possibility that these three proteins interact to mediate innate immunity has not hitherto been explored.

In this study, we utilized the *Drosophila* immune system that is well-conserved to mammals [19] to study the molecular mechanisms behind parkin-mediated responses against bacterial infection. Through *Drosophila* genetics, we identified mitochondrial TSPO-VDAC complex to mediate responses against infection and wound. Importantly, we show for the first time that mitochondrial TSPO-VDAC complex genetically interact with parkin to mediate responses against septic injury.

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2. Materials and methods

2.1. Fly strains

The *w1118* strain (Bloomington stock center, stock 6326) was used as the wild-type control. *TSPO* mutant (*P{EPgy2}Tspo*^{EY00814}, stock 15479), *porin* mutant (*PBac{WH}porin*^{f03616}, stock 18676) and *TSPO* deficient strain (*Df(2L)BSC107*, stock 8673) were also obtained from Bloomington stock center. *TSPO* RNAi (v106470/kk) was obtained from VDRC. *Tubulin*-gal4 strain was gifted by JK Chung (Seoul National University, Seoul, Korea). UAS-*park*^{C2} and *park*^{25} lines were gifted by Leo J. Pallanck (University of Washington, Seattle, USA).

2.2. Prediction of transmembrane domain of dTSPO

Amino acid sequences were analyzed with Genedoc and transmembrane domain prediction program (Expasy, TMpred).

2.3. Infection and injury experiment

Wandering stage third instar larvae were first soaked in 1X PBS (phosphate buffered saline). To induce septic injury, a needle dipped in concentrated bacteria was used to infect larvae two times, once in the dorsal middle and another in the dorsal bottom portion of the larvae. Immediately, larvae were incubated in 1 mL of *E. coli* solution (*E. coli* incubated in LB solution for 12 h) for 30 s and washed in 1 mL of 1X PBS for 20 s. To induce wound without bacterial infection, we used the same method as above, except *E. coli* was replaced with 1X PBS in all steps. *w1118* and *tubulin*-gal4/+ strains were used as control groups.

2.4. Analysis of survival rate

To determine survival rate after infection, 20 larvae were placed in each vial with standard cornmeal agar medium after infection and were incubated at 25 °C and approximately 60% humidity. The number of dead larvae was counted every 2 h after infection until 6 h.

2.5. Single colony isolation of remaining bacteria inside the body after infection

Infected larvae at 6 h after infection were homogenized in 200 μl 1X PBS, and serial dilutions were plated onto non-antibiotics solid LB media. After 16 h culture, pictures of isolated colonies were compared between the experimental groups.

2.6. RNA extraction, cDNA synthesis, reverse transcription-PCR (RT-PCR) and quantitative PCR (qPCR)

For qRT-PCR or RT-PCR analysis, the infected live animals (2 h, 4 h, 6 h after infection) or non-infected live animals were collected. Total RNA was extracted from third instar larvae using the easy-Blue™ system (Intron, Korea). 3 mg of total RNA was reverse transcribed using GoScript™ Reverse Transcription System (Promega, USA). For RT-PCR and qPCR analysis, we used following primers: *TSPO* (forward 5′-ATG GCT GAT CGT CCG TGT GCT GG-3'; reverse 5′-GAT CAG GGG CAG TTT GGC CGC CTC-3′), *Diptericin* (forward 5′-ACC GCA GTA CCC ACT CAA TC-3'; reverse 5′-CCC AAG TGC TGT CCA TAT CC-3′) and *rp49* (forward 5′-GCT TCA AGA TGA CCA TCC GCC C-3'; reverse 5′-GGT GCG CTT GTT CGA TCC GTA AC-3′). *rp49* was used as a reference gene and an internal control. Graphs in the figure are representative of at least three independent experiments. qPCR was performed using CFX^{96} Real Time system (BioRad, USA).

3. Results

3.1. Parkin loss-of-function mutation induces defects in immune defense and wound repair

To test whether parkin is involved in the immune response after bacterial infection, we compared the lethality curves between the *w1118* (wildtype control) and *parkin*$^{-/-}$ 3rd instar larvae after septic injury. Septic injury was caused by pricking *Drosophila* larvae using a thin needle dipped into concentrated bacteria. This method of infection was preferred over others (e.g. feeding bacteria) because it best reflected wound infection in humans [20]. Within 6 h after infection, we observed significantly less surviving larvae in the *parkin* mutants compared to the controls (Fig. 1A). Since the larvae were infected using a needle, we examined whether the injury caused by needle during the infection was an important contributor to lethality. To test this, we performed a mock-infection using PBS instead of bacteria. Interestingly, wound infliction resulted in increased lethality in *parkin* mutants compared to the controls (Fig. 1B). Then, we decided to examine more directly the possible changes in the level of immune defense response of *parkin* mutants by measuring remaining bacteria inside the body after the infection. To test this, both the control and *parkin* mutant surviving larvae were isolated 6 h after the bacterial infection and homogenized before being plated on solid LB media. We found a large increase in the number of remaining bacteria in *parkin* mutant larvae compared to the controls (Fig. 1C and D). This is consistent with a previous finding where Manzanillo et al. have attributed this increased bacterial burden in *parkin* mutants to perturbed autophagy [12], but didn't examine whether parkin also mediates classical anti-bacterial responses, such as *Diptericin (Dipt)* up-regulation. Therefore, we measured the *Dipt* mRNA levels and compared them between controls and *parkin* mutants before and after the bacterial infection. Interestingly, the *Dipt* level between the control and *parkin* mutants were relatively similar at 2 and 4 h after infection, though its level was considerably lower in *parkin* mutants by 6 h after the infection (Fig. 1E). Taken together, these data suggest perturbed responses to immune challenges and wound infliction in *parkin* mutants.

3.2. Parkin and mitochondrial protein TSPO genetically interact in mediating immune response

Parkin is known to translocate to the mitochondria under stressful conditions as a quality control system [13]. Thus, we wondered whether parkin mediates innate immunity and injury responses through its interaction with mitochondrial proteins. As a possible mitochondrial mediator, we examined a mitochondrial protein TSPO that has recently been suggested to play a role in immunity [16] and also in parkin-mediated autophagy that is discrete from the infection condition [18]. We verified that *Drosophila* TSPO has a close amino acid sequence homology with mammalian TSPO (Fig. S1A), and all of its five putative transmembrane domains are conserved (Fig. S1B). In order to investigate possible genetic interaction between *TSPO* and *parkin*, we initially used *TSPO* deficiency allele, Df(2L)BSC107 (*TSPO*^{Df}) (Fig. S2A).

First, we tested whether TSPO could modify parkin-mediated immune response by measuring basal *Dipt* level. Significantly lower level of *Dipt* was observed in *parkin* homozygous mutants compared to the controls (Fig. 2A). Intriguingly, trans-heterozygous mutations in *parkin* and *TSPO* also led to similarly decreased level of basal *Dipt* compared to the controls (Fig. 2B) as in *parkin* mutants (Fig. 2A). Moreover, the trans-heterozygotes showed lower survival rate after the bacterial infection compared to the controls (Fig. 2C).

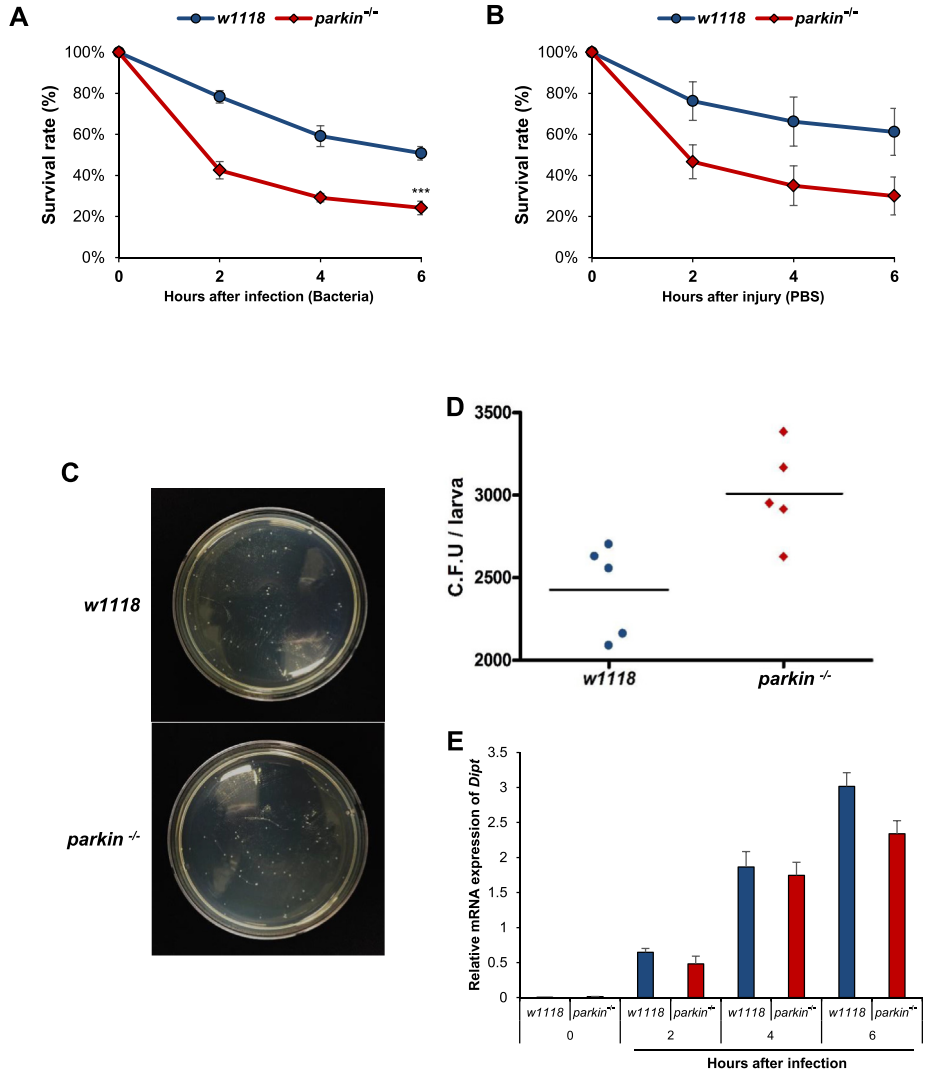

Fig. 1. Perturbed responses to infection and wound in *parkin* loss-of-function mutants. (A) Comparison of survival rate between *parkin* mutants (*parkin* $^{-/-}$) and controls (*w1118*) after bacterial infection. $^{***}P < 0.001$; error bars indicate SEM (n ≥ 3). (B) Comparison of survival rate between *parkin* $^{-/-}$ and *w1118* after injury only. (C) Comparison of *E. coli* colony formation on nutrient agar plate between *parkin* $^{-/-}$ and *w1118* 6 h after bacterial infection. (D) Comparison of colony forming unit (C.F.U.) of *E. coli* between *parkin* $^{-/-}$ and *w1118* 6 h after bacterial infection. (E) Quantified qPCR data of mRNA expression levels of an AMP (anti-microbial peptides) gene, *Dipt*, relative to *rp49* in the course of time after bacterial infection in *parkin* $^{-/-}$ and *w1118* larvae.

These evidences suggest that *parkin* and *TSPO* genetically interact to mediate innate immune response.

3.3. Loss of TSPO function shows similar defects to parkin mutants in responses to infection and wound

To investigate whether TSPO by itself plays a significant role in mediating bacterial infection, we first tested the effectiveness of $TSPO^{EY00814}$ allele and *TSPO* RNAi construct in reducing *TSPO* transcripts (Fig. S2A, S2B, and S2B). The $TSPO^{EY00814}$ homozygous larvae displayed a hypomorphic phenotype showing significant decrease in the *TSPO* transcripts (Fig. S2B). *TSPO* RNAi ubiquitously driven by *tubulin*-gal4 showed even more drastic reduction in *TSPO* transcripts compared to the *tubulin*-gal4 control (Fig. S2C). Therefore, we decided to use *TSPO* RNAi construct driven by *tubulin*-gal4 for the majority of the following experiments.

We then tested whether knockdown of *TSPO* could also have a significant impact on the survival rate after the infection as was observed in *parkin* mutants. *TSPO* knockdown increased lethality compared to the controls after the bacterial infection (Fig. 3A) in similar manner to *parkin* mutants (Fig. 1A). Comparable results were obtained with $TSPO^{EY00814}$ homozygous larvae, albeit the difference of increase in lethality was smaller (Fig. S2D). Knockdown of *TSPO* could also increase lethality after wound infliction (mock infection) (Fig. 3B), comparable to *parkin* mutants (Fig. 1B). Substantial increase in the bacterial burden was observed at 6 h after the infection in *TSPO* knockdown larvae compared to the controls, in line with observed increased lethality (Fig. 3C and D). Interestingly, this increased bacterial burden in *TSPO* knockdown larvae was significantly higher compared to *parkin* mutants at 6 h after infection (Figs. 3D and 1D). Analogous to *parkin* mutants (Fig. 1E), the induction of *Dipt* 6 h after the infection was markedly suppressed by *TSPO* knockdown compared to the controls (Fig. 3E). Curiously, the knockdown of *TSPO* showed a more appreciable difference in *Dipt* levels compared to the controls than was shown by *parkin* mutants (Figs. 3E and 1E). These data demonstrate phenotypic similarity between *TSPO* knockdown and *parkin* mutants in responding to immune challenges and wound infliction.

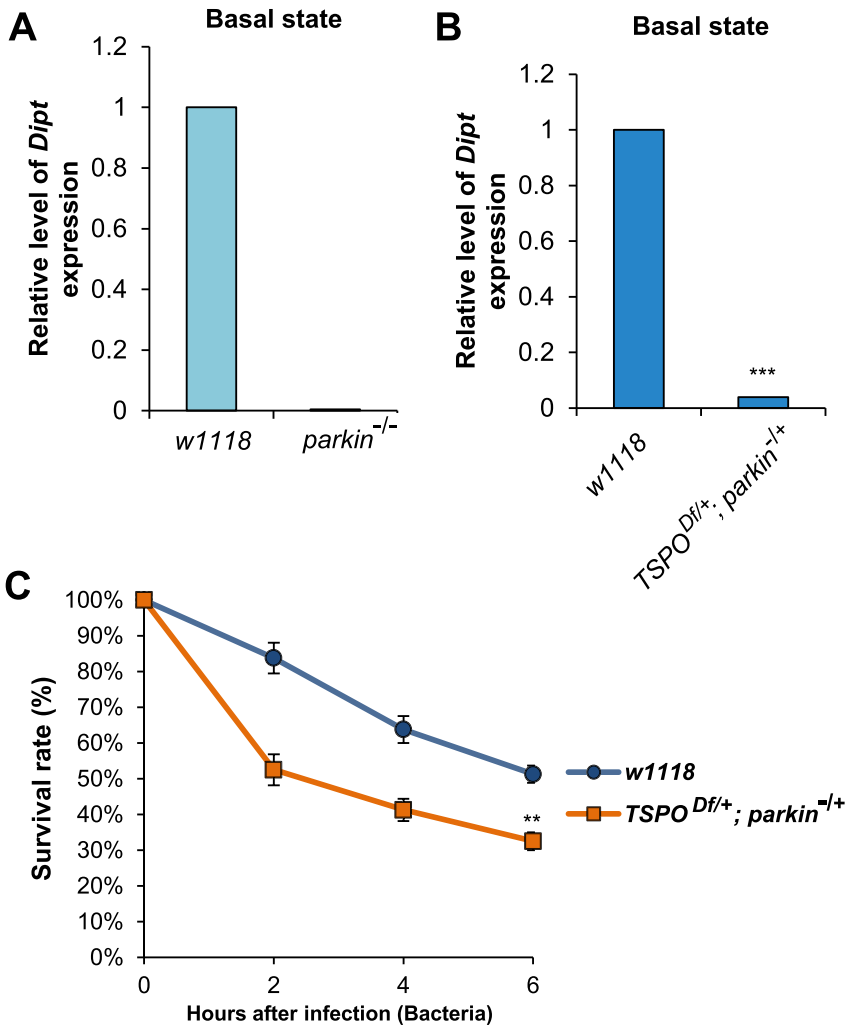

Fig. 2. *Parkin* and mitochondrial *TSPO* interact genetically to mediate innate immunity. (A) Quantified qPCR data of basal mRNA expression levels of *Dipt* before the bacterial infection in *parkin* $^{-/-}$ and *w1118* wandering third instar larvae. (B) Quantified qPCR data of basal mRNA expression levels of *Dipt* before the bacterial infection of *parkin* and *TSPO* trans-heterozygotic wandering third instar larvae. ***$P < 0.001$; error bars indicate SEM ($n \geq 3$). (C) Comparison of survival rate between *parkin* and *TSPO* trans-heterozygotes and *w1118*.

3.4. Mitochondrial TSPO-VDAC complex is involved in parkin-mediated immune defense

We wondered whether a genetic hierarchy exists between *parkin* and mitochondrial *TSPO* in mediating immune response, given that parkin is well-known to translocate to mitochondria. To explore this possibility, we examined whether *parkin* over-expression could rescue the increased post-infection lethality of *TSPO* knockdown larvae. Increased expression of *parkin* had no rescue effect on lethality of *TSPO* knockdown larvae after the infection (Fig. 4A), indicating that parkin does not act downstream of TSPO in mediating immune response. This suggests that parkin may either function upstream of, or in parallel to TSPO in mediating innate immunity.

TSPO is known to form a complex on the mitochondrial outer membrane with VDAC (porin in *Drosophila*) [21–23], but their function as a complex still remains disputable [24]. We therefore investigated whether TSPO-VDAC complex is involved in parkin-mediated immune response. The combinatorial trans-heterozygotic mutations in *porin* and *parkin* showed similar pattern of lethality with homozygous *parkin* mutants (Fig. 4B). Taken together, we propose TSPO-VDAC complex as a novel modulator of parkin-mediated innate immunity in *Drosophila*.

4. Discussion

In this study, we demonstrated a novel link between parkin and mitochondrial TSPO-VDAC complex in mediating response against septic injury. Recent studies highlighted parkin-mediated innate defense mechanisms through autophagic removal of bacteria [12] and its involvement in NF-kB signaling pathway [25–27]. Interestingly, however, a mitochondrial role of parkin associated with innate defense has not been explored. Here, we provide evidences to support the mitochondrial function of parkin in modulating innate defense mechanism through its interaction with mitochondrial TSPO-VDAC complex.

TSPO and VDAC have been shown to physically interact with each other [21–23], but their putative functions such as mediating steroidogenesis and apoptosis have remained highly controversial [24]. Recently, TSPO and VDAC have also been suggested to cooperatively inhibit parkin-mediated mitochondrial quality control through increased ROS production [18]. However, how parkin interact with mitochondrial TSPO-VDAC complex to mediate the innate immune response has not hitherto been investigated. Pallanck's group also showed synthetic pupal lethality between *parkin* and *TSPO* mutants [28], but without making any inferences relating to innate immunity. Our results indicated that homozygotic

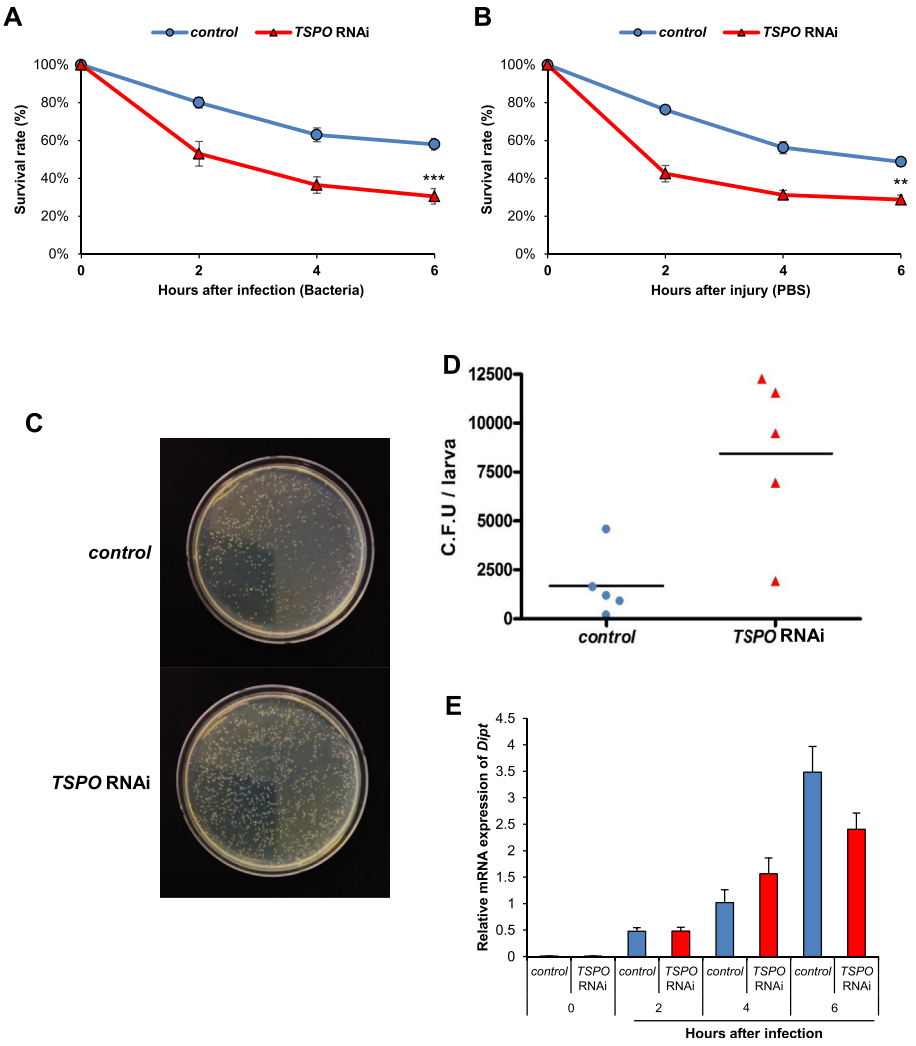

Fig. 3. *TSPO* knockdown perturbs infection and wound response, similar to *parkin* mutants. (A) Comparison of survival rate between *TSPO* knockdown larvae and controls (*tubulin*-gal4/+) after bacterial infection. $^{***}P < 0.001$; error bars indicate SEM ($n \geq 3$). (B) Comparison of survival rate between *TSPO* knockdown larvae and controls after injury only. $^{**}P < 0.005$; error bars indicate SEM ($n \geq 3$). (C) Comparison of *E. coli* colony formation on nutrient agar plate between *TSPO* knockdown larvae and controls 6 h after bacterial infection. (D) Comparison of colony forming unit (C.F.U) of *E. coli* between *TSPO* knockdown larvae and controls 6 h after bacterial infection. (E) Quantified qPCR data of mRNA expression levels of an AMP gene, *Dipt*, relative to *rp49* in the course of time after bacterial infection in *TSPO* knockdown larvae and controls.

mutations in *parkin* and *TSPO* can by themselves perturb innate defense system. Intriguingly, the heterozygotic mutant combination between *parkin* and *TSPO* or *parkin* and *VDAC* could also perturb the innate defense system against septic injury. Thus we suggest here that parkin and TSPO-VDAC complex cooperate to mediate innate immunity.

Functional hierarchy between parkin and TSPO-VDAC complex in modulating the immune response still remains elusive. Previous studies have shown that over-expression of *parkin* can functionally overcome the loss of its direct upstream regulator, *Pink1* [29,30]. Therefore, we reasoned that over-expression of *parkin* could rescue increased lethality caused by septic injury in *TSPO* knockdown larvae if parkin functions downstream of TSPO. However, *parkin* over-expression failed to rescue lethality caused by septic injury in *TSPO* knockdown larvae, which suggests that parkin seemingly does not function downstream of TSPO. Rather, we envision that parkin, a highly versatile ubiquitin ligase, ubiquitinates VDAC, and or TSPO to facilitate a signaling pathway towards invoking immune response. Indeed, it has been shown that parkin ubiquitinates VDAC in the context of mitophagy [18]. Whether parkin-mediated ubiquitination of VDAC or TSPO is functionally relevant in the context of innate immune response remains to be seen.

Finally, we envisage an association between parkin and TSPO-VDAC complex with neurodegenerative diseases and innate immunity, given the overwhelming evidences for both *parkin* and TSPO to mediate neurodegeneration [31–33]. Notably, mutations in the *PARK2/parkin* gene have been directly linked to AR-JP [4]. Additionally, multiple lines of evidence suggest that TSPO expression correlates with neuronal inflammation, and that it is closely associated with the progression of various types of neurodegenerative diseases including Alzheimer's and Parkinson's diseases [32]. Intriguingly, previous studies have made unexpected discovery that various neurodegenerative diseases are exacerbated by cases of infections [34,35]. Here, we have identified mitochondrial TSPO-VDAC complex to modulate parkin-mediated immune defense system in *Drosophila*. It is therefore conceivable for the TSPO-VDAC complex to coalesce neurodegeneration and infection at the mitochondria through interacting with other mitochondrial molecules associated with neurodegeneration besides parkin. Further experimental scrutiny on the role of TSPO-VDAC on both

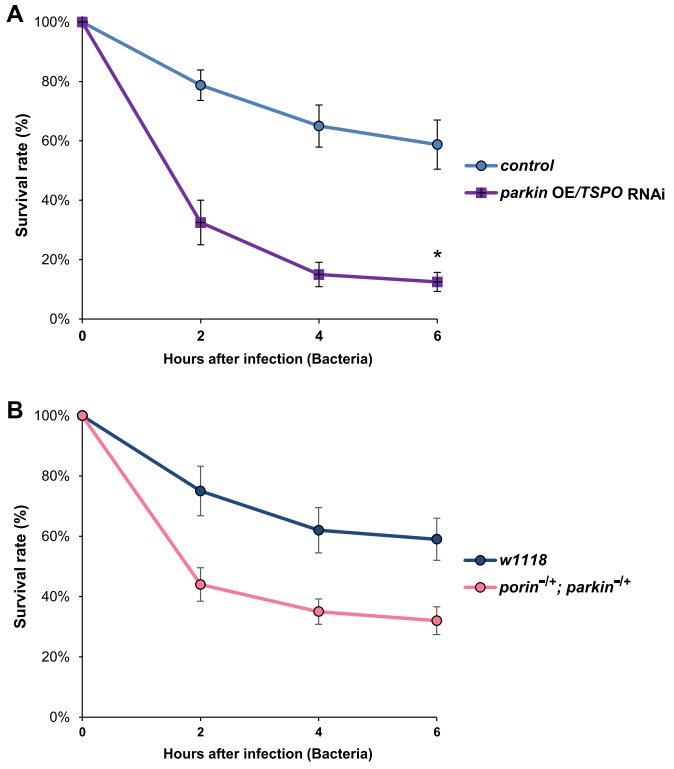

Fig. 4. Parkin-mediated immune response is genetically linked with mitochondrial TSPO-VDAC complex. (A) Comparison of survival rate between *parkin* overexpression-*TSPO* knockdown larvae and the controls (*tubulin*-gal4/+) after bacterial infection. $^{*}P < 0.01$; error bars indicate SEM ($n \geq 3$). (B) Comparison of survival rate between combinatorial *parkin* and *porin* trans-heterozygotes, and *w1118* after bacterial infection. $^{***}P < 0.001$; error bars indicate SEM ($n \geq 3$).

neurodegeneration and innate immunity is therefore warranted. Thus, we cautiously propose that parkin mediates innate immune responses with mitochondrial TSPO-VDAC complex in *Drosophila* with considerable implications in linking innate immunity with neurodegenerative diseases.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.006.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.006.

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