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Low-dose Radiation Induces *Drosophila* Innate Immunity through Toll Pathway Activation

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Low-dose radiation/Innate immunity/Toll/JNK/*Drosophila*.

Numerous studies report that exposing certain organisms to low-dose radiation induces beneficial effects on lifespan, tumorigenesis, and immunity. By analyzing survival after bacterial infection and antimicrobial peptide gene expression in irradiated flies, we demonstrate that low-dose irradiation of *Drosophila* enhances innate immunity. Low-dose irradiation of flies significantly increased resistance against gram-positive and gram-negative bacterial infections, as well as expression of several antimicrobial peptide genes. Additionally, low-dose irradiation also resulted in a specific increase in expression of key proteins of the Toll signaling pathway and phosphorylated forms of p38 and JNK. These results indicate that innate immunity is activated after low-dose irradiation through Toll signaling pathway in *Drosophila*.

INTRODUCTION

Over the past several decades, an accumulating body of data supports hormesis, the concept that exposure to low doses of toxins or stressors elicits favorable biological responses in organisms.[1–4)] Although high-dose radiation (HDR) is hazardous to organisms, low-dose radiation (LDR) induces beneficial effects such as inhibiting the development of cancer, activating immunity, reducing mutation and extending longevity.[5–8)] For example, a number of immunological parameters are stimulated in mice following low-dose irradiation (< 0.1 Gy).[9)] Although the hormetic effects of LDR are expressed as several distinct phenotypes, these phenotypes may be tightly linked. Previous microarray analysis by our group indicates that LDR not only extends fruit fly longevity, but also activates the expression of genes related to immune function.[10)] However, no clear evidence exists yet that LDR directly activates immune responses in *Drosophila*. Furthermore, the exact mechanism by which LDR mediates immune activation in any species is not yet fully understood.

Drosophila is an excellent model system for the study of innate immunity since key elements of the innate immune system are highly conserved.[11)] Identification of the Toll signaling pathway as a mediator of gram-positive bacterial and fungal infections in *Drosophila* led to the identification of the Toll-like receptor (TLR) signaling pathway in higher vertebrates. Additionally, the *Drosophila* immune deficiency (Imd) signaling pathway, which is principally activated in response to gram-negative bacteria, resembles the mammalian tumor necrosis factor (TNF) pathway.[12)] In fruit flies, systemic and local microbial infections induce robust antimicrobial peptide (AMP) responses. Seven classes of AMPs have been classified to date: *drosomycin* and *metchnikowin* are active against fungi; *defensin* is active against gram-positive bacteria; and *diptericin*, *drosocin*, *attacin*, and *cecropin* are active against gram-negative bacteria. Following infection, these AMPs are rapidly generated in the fat body, which is the functional equivalent of the mammalian liver. AMPs are then secreted into the hemolymph and ultimately mediate the removal of pathogens.[13)]

In this study, we tested whether LDR enhances the function of *Drosophila* innate immunity by monitoring the survival of irradiated flies after bacterial infection and the expression of AMP after irradiation. We also measured changes in other signaling pathways, including AKT, p38 mitogen-activated protein kinase (MAPK), and c-jun N-

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terminal kinase (JNK). Elucidating the innate immune response that ionizing radiation triggers in *Drosophila* is important because radiation induces the same canonical pathways as infectious agents.[14] We therefore sought to identify the signaling molecules that contribute to LDR-mediated activation of innate immune responses.

MATERIALS AND METHODS

Stock and cultivation conditions

All flies were cultivated and maintained in bottles at 25°C and 70% relative humidity on a standard cornmeal sugar-yeast-agar medium. The chamber was exposed to light for 12 hr per day. Canton S flies were used and considered wild type. Dr. W. J. Lee generously provided transgenic drosomycin-GFP and diptericin-GFP flies (Ewha Womans University, Seoul, Korea). These strains carry the gene for green fluorescence protein (GFP) fused to the drosomycin and diptericin gene promoters, respectively.[15,16]

γ-irradiation

Eggs were collected from 5-day-old female flies over 12–14 hr and reared on standard medium. Larvae (3rd instar) were irradiated in a γ-irradiation machine (^{137}Cs, IBL 437N; CIS Bio International, Gifsur-Yvette, France) at 0.2 Gy (a dose-rate of 0.8 Gy/min with 5 mm plumbum shields) and 10 Gy (a dose-rate of 4.3 Gy/min without shields). For the validation of irradiated dose, thermoluminescent dosimeters were placed within the exposure field. Following irradiation, non-irradiated and irradiated flies were maintained contemporaneously in the same incubator at 25°C.

Fluorescence microscopy

Live adults/larvae were anesthetized (adults with CO_2, larvae on ice) at the indicated times and viewed under epifluorescent illumination with a Nikon Eclipse E600 microscope equipped with CCD camera. Photographs were taken and processed with NIS-Elements BR imaging software (Nikon, Tokyo, Japan).

Bacterial infections and survival measurements

Flies were infected with bacteria as previously reported.[17] Briefly, *Staphylococcus aureus* was grown in an overnight culture and then diluted with 1x PBS to OD_{600} = 0.5. *Pseudomonoas aeruginosa* was prepared in a manner similar to *S. aureus*, but to an OD_{600} = 0.008 due to *P. aeruginosa* being extremely toxic when administered to *Drosophila* at a dose equivalent to *S. aureus*. For infection of flies, 3-day-old males were picked in the upper thorax with the same volume of bacterial solution using a fine tungsten needle. Mock-infected control flies were picked with 1x PBS. Survival of infected flies was monitored in a vial every 6 hr post-infection. Each vial contained 10 flies and each group comprised 10 vials. All data presented are representative of at least three independent experiments. Statistical significance was determined by log-rank test using Prism GraphPad 5.0 (GraphPad Software, San Diego, CA).

Table 1. Oligonucleotide sequences used as primers for quantitative real-time PCR.

Primer name	Sequence (5'→3')	Gene	CG number
gapdh1 forward	TTCGTGTGCGGCGTTAACCT	gapdh1	CG12055
gapdh1 reverse	GATGACCTTGGCCAGGGGAG		
drosomycin forward	AGCGCGGATGGAACGATATT	drosomycin	CG10810
drosomycin reverse	CACAATGCCCACGCTCTTGT		
defensin forward	GTGGATCCAATTCCAGAGGA	defensin	CG1385
defensin reverse	CACAGAGCGAAACGAAATCA		
drosocin forward	ATTTGTCCACCACTCCAAGC	drosocin	CG10816
drosocin reverse	GGCAGCTTGAGTCAGGTGAT		
diptericin forward	AAGAGCAGCGAACTGACCAT	diptericin	CG12763
diptericin reverse	GCGGCGTCTTAAAAGCTATG		
attacin C forward	CCAATGGCTTCAAGTTCGAT	attacin C	CG4740
attacin C reverse	AGGGTCCACTTGTCCACTTG		
metchnikowin forward	CCACCGAGCTAAGATGCAA	metchinikowin	CG8175
metchnikowin reverse	AATAAATTGGACCCGGTCTTG		
cecropin A1 forward	CATCAGTCGCTCAGACCTCA	cecropin A1	CG1365
cecropin A1 reverse	CGTTCGATTTTCTTGCCAAT		

Quantitative RT-PCR analysis

Trizol reagent (Invitrogen, Carlsbad, CA) was used to extract total RNAs from at least five replicates per group, with twenty 3-day-old males comprising each replicate. The purity and amount of RNA was determined spectrophotometrically (ND-1000, NanoDrop, Wilmington, DE). Quantitative RT-PCR was performed with ABI Prism 7300 Sequence Detection System and SYBR Green PCR Core reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The relative mRNA expression level was normalized to GAPDH1 mRNA. The comparative cycle threshold (Ct) method was used to analyze the data. Primers used for quantification of mRNA are listed in Table 1.

Protein extraction and immunoblotting analysis

Protein extracts were prepared either from at least five replicates per group, with twenty larvae or twenty 3-day-old adult flies. Fly tissue was homogenized in 100 µl buffer (100 mM KCl, 20 mM HEPES pH 7.5, 5% glycerol, 10 mM EDTA, 0.1% Triton X-100, 1 mM DTT) containing a protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) with a Pellet Pestle® motor. Extracts were cleared by centrifugation at 13,000 rpm for 30 min at 4°C, resolved on an SDS-polyacrylamide gel, and subjected to immunoblotting assay with specific antibodies for each protein. Antibodies were purchased from Santacruz biotechnology (Santa Cruz, CA; Toll, Dorsal, Pelle, GSK3β, phopho-JNK, JNK, p38, tubulin), Cell signaling technology (Danver, MA; phospho-Akt, Akt, phospho-GSK3β, phospho-p38), and Developmental Studies Hybridoma Bank (University of Iowa; Cactus, Relish).

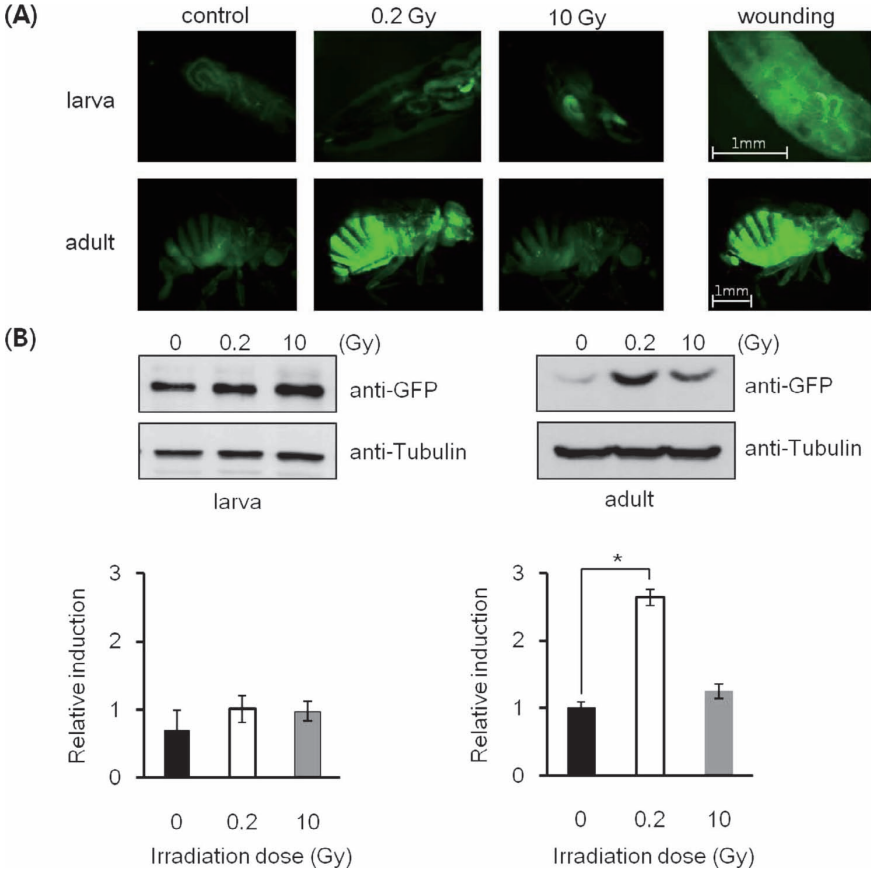

Fig. 1. Low-dose radiation induces expression of drosomycin-GFP in *Drosophila*. (A) Expression of drosomycin after irradiation. Transgenic fruit flies were irradiated at the 3rd instar larval stage. Larvae pictures were captured 8 hr after stabilization and adult pictures were taken in 3-day-old adults with a fluorescence microscope. Wounding was performed as a fluorescence positive control by nicking the body using a fine tungsten needle. (B) Immunoblot analysis for GFP. Protein was extracted from either 20 larvae or 20 adults at the same time as described in (A) and subjected to immunoblotting with antibody specific for GFP. This experiment was repeatedly carried out with at least five replicates. Relative induction is presented as mean ± S.E. and an asterisk indicates significant difference compared to control (t-test, * $p < 0.05$).

RESULTS

LDR induces expression of drosomycin, but not diptericin

To assess the expression of AMPs following irradiation, the fluorescence emitted from drosomycin-GFP and diptericin-GFP flies was measured in response to radiation treatment. *Drosomycin* and *diptericin* are AMPs in the *Drosophila* Toll and Imd signaling pathways, respectively, and were considered representative of the activation of each pathway. Although only a small amount of green fluorescence was detectable in control and irradiated drosomycin-GFP adult flies at 10 Gy, high levels of green fluorescence were repeatedly detected when flies were irradiated at 0.2 Gy (Fig. 1A). However, radiation had no effect on the green fluorescence measured in drosomycin-GFP larvae 8 hrs after irradiation (Fig. 1A). Radiation also did not induce green fluorescence in diptericin-GFP adult flies or larvae (data not shown). Immunoblotting analysis with antibodies specific for GFP was conducted to validate GFP expression induced in drosomycin-GFP flies as a result of LDR. The expression of GFP was significantly elevated in the adult flies irradiated with 0.2 Gy, compared to non-irradiated control adult flies or adult flies irradiated at 10 Gy (Fig. 1B). GFP expression did not change significantly in the larvae 8 hrs after irradiation.

LDR enhances survival of infected flies

To determine whether drosomycin expression induced by LDR contributes physiologically to the defense against microbial infection, wild type flies were irradiated at 0.2, 2, or 10 Gy at the 3rd instar larvae stage and were infected either with gram-positive (*Staphylococcus aureus)* or gram-negative bacteria as adults (*Pseudomonas aeruginosa*). Survival was monitored following infection. Interestingly, flies irradiated at 0.2 Gy exhibited significantly prolonged survival after infection with both types of microorganisms compared to non-irradiated control flies (Fig. 2; *S. aureus*, χ^2 = 6.739, p = 0.0094; *P. aeruginosa*, χ^2 = 29.09, p < 0.0001).

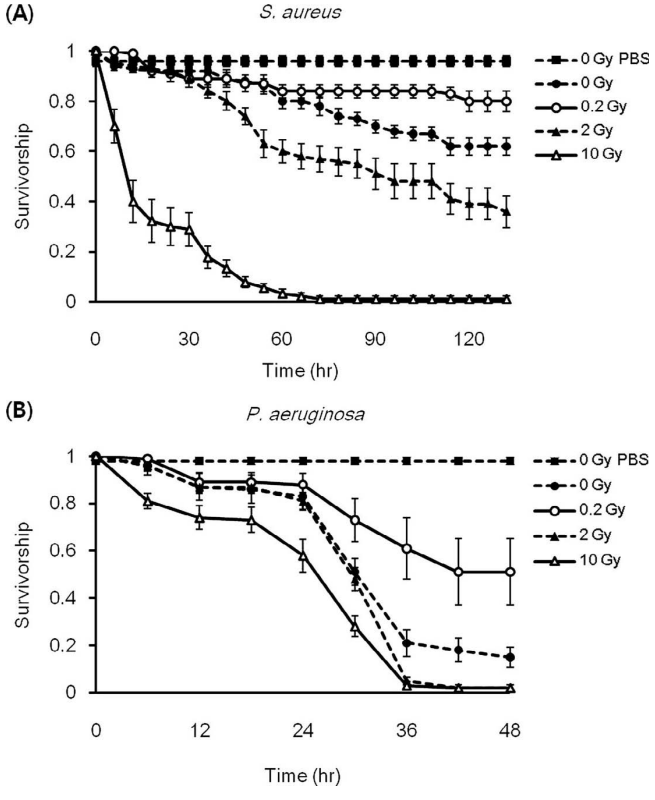

Fig. 2. Low-dose radiation enhances survival after bacterial infection. Flies were infected with (A) *Staphylococcus aureus* or (B) *Pseudomonas aeruginosa*, which were used as representative of gram-positive or gram-negative bacteria, respectively. Irradiated fruit flies were infected using a needle soaked in respective pathogenic cells as described in materials and methods. PBS was used as a negative control.

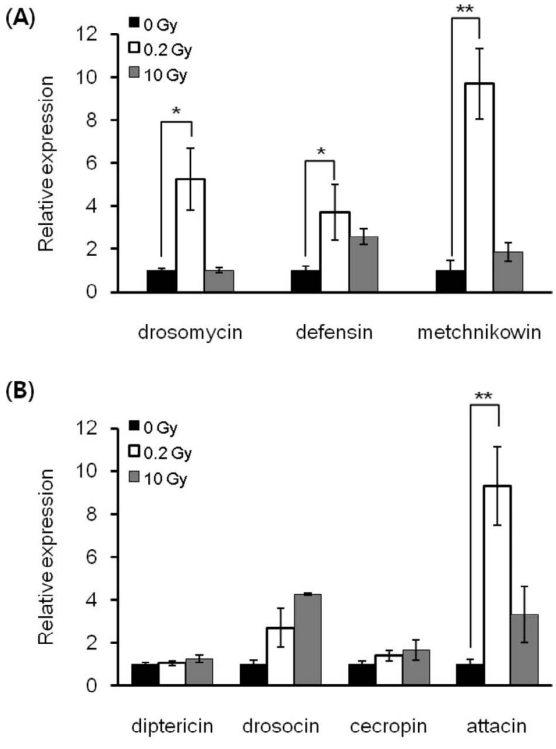

Fig. 3. Low-dose radiation specifically induces expression of several antimicrobial peptides. Quantitative RT-PCR using specific primers was conducted to evaluate the expression of antimicrobial peptide genes. For this experiment, total RNAs were extracted from irradiated 3-day-old flies as described in materials and methods. (A) All antimicrobial peptide genes triggered by the Toll pathway were increased in the flies irradiated with 0.2 Gy. (B) Low-dose radiation did not alter expression of most of the antimicrobial peptide genes, with the exception of *attacin C*. Data are presented as means ± S.E. and an asterisk indicates significant difference compared to control (t-test, * p < 0.05, ** p < 0.01).

In contrast, HDR at a dose of 2 or 10 Gy increased the susceptibility of flies to both types of microbial infection (*S. aureus*. $\chi^2 = 10.32$, $p = 0.0013$ for 2 Gy; $\chi^2 = 160.2$, $p < 0.0001$ for 10 Gy. *P. aeruginosa*, $\chi^2 = 2.843$, $p = 0.0918$ for 2 Gy; $\chi^2 = 17.52$, $p < 0.0001$ for 10 Gy).

LDR increases expression of several AMPs

Data thus far indicate that *drosomycin* expression induced by LDR contributes to the resistance against microbial infection (Figs. 1 and 2). To determine whether LDR activates other AMPs in addition to *drosomycin*, we evaluated the expression of other AMP genes after irradiation by quantitative real-time PCR (Fig. 3). Compared to non-irradiated control flies or flies irradiated at 10 Gy, the expression of all AMP genes belonging to the Toll pathway, such as *drosomycin*, *defensin*, and *metchnikowin*, was significantly increased in flies irradiated at 0.2 Gy (Fig. 3A). However, most of the AMP genes associated with the Imd pathway did not exhibit an LDR-specific expression increase (Fig. 3B). The levels of *diptericin* and *cecropin* were not significantly altered by irradiation. Irradiation increased the expression of drosocin in a radiation dose-dependent manner. *Attacin C* was the only gene of the Imd pathway that exhibited an LDR-specific increase in expression. These results indicate that the influence of LDR on expression of AMP genes in the Toll pathway is different from the influence of AMP genes in the Imd pathway.

LDR specifically activates key components of the Toll pathway

LDR prolonged the survival of flies after bacterial infection and increased the expression of AMP genes belonging to the Toll pathway. We then sought to determine if LDR activates components of the Toll pathway in fruit flies. Immunoblotting with antibodies specific for components of the Toll pathway indicated that, compared to non-irradiated control or flies irradiated at 10 Gy, protein levels of the NF-κB transcriptional activator Dorsal and the IRAK-like protein kinase Pelle were significantly increased in flies irradiated at 0.2 Gy (Fig. 4A). However, LDR did not alter expression of the transmembrane receptor Toll and Cactus, a homologue of mammalian IκBs (Fig. 4A). Additionally, LDR had no effect on the expression of Relish, a central NF-κB transcription factor in the Imd pathway (Fig. 4B). Irradiation did not change the expression of either the intact or cleaved form of Relish. Taken together, these results indicate that in the absence of microbial infection, LDR activates the expression of intracellular proteins involved in the Toll pathway, but not the Imd pathway.

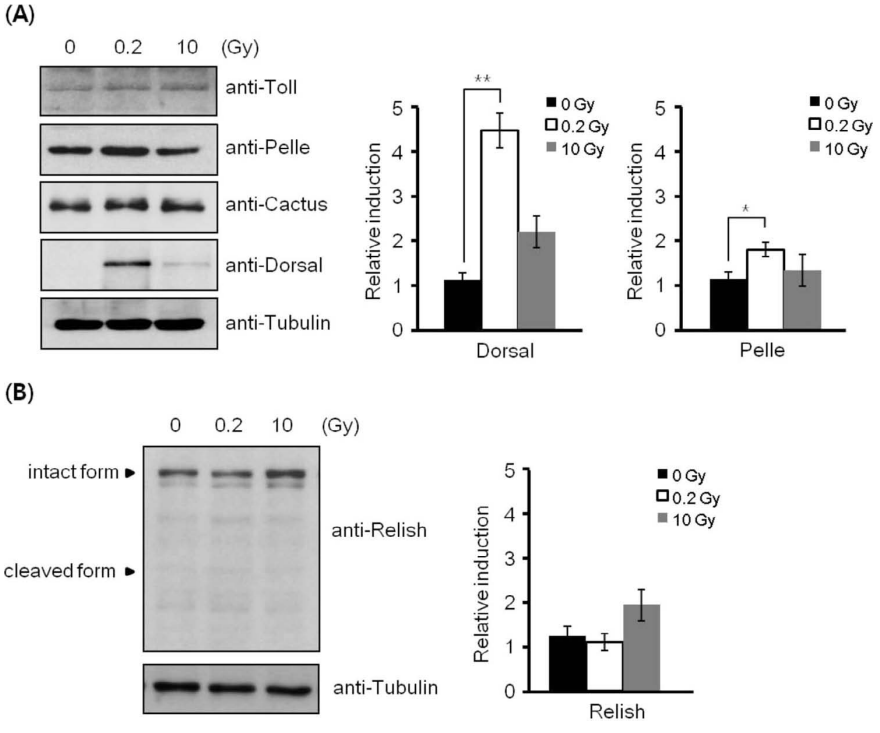

Fig. 4. Low-dose radiation induces expression of key Toll pathway components. Immunoblot analysis of (A) components of the Toll pathway, including Toll receptor, the IRAK family kinase Pelle, the IκB protein Cactus, and the NF-κB transcription factor Dorsal or (B) Components of the Imd pathway, including the NF-κB transcription factor Relish. Data presented are means ± S.E. and an asterisk indicates significant difference compared to control (t-test, * $p < 0.05$, ** $p < 0.01$).

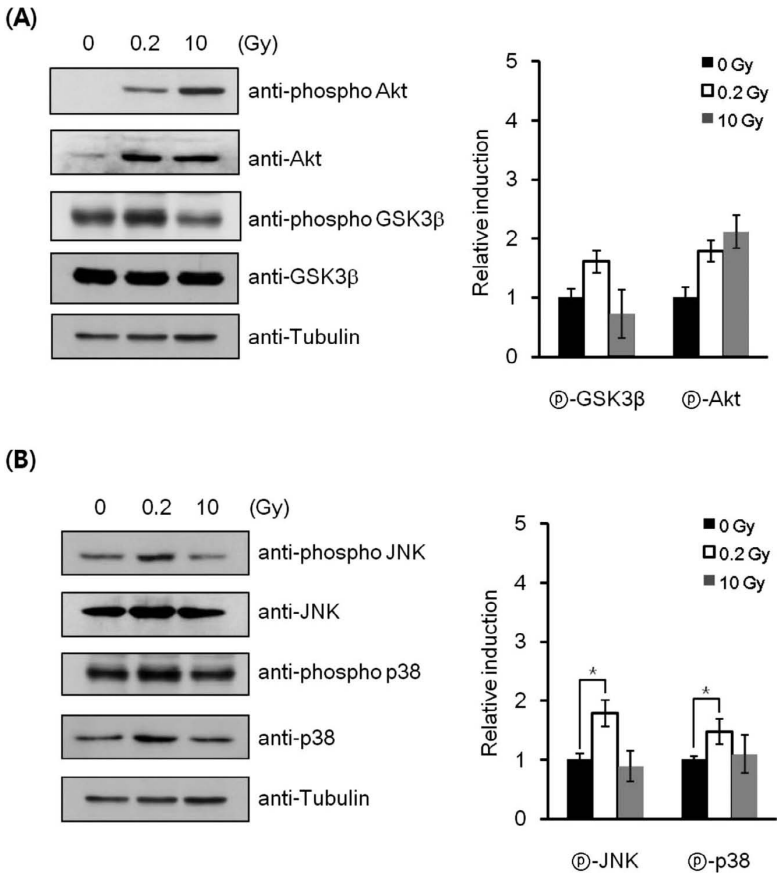

Fig. 5. Low-dose radiation activates MAPK/JNK pathway. Immunoblot analysis of stress regulators of (A) the AKT pathway or (B) the p38 MAPK, and JNK pathways. Stress regulators in the AKT pathway are activated in a radiation dose-dependent manner. Phosphorylated forms of p38 and JNK were elevated in the flies irradiated at a low dose. Data presented are means ± S.E. and an asterisk indicates significant difference compared to control (*t-test*, * $p < 0.05$).

The JNK pathway contributes LDR-mediated innate immunity

LDR increased the survival of flies after gram-negative bacterial infection, but did not increase the expression of most AMPs belonging to the Imd pathway or the level of Relish protein (Figs. 2 and 4). This discrepancy led us to examine other signal pathway(s) triggered by LDR that enhance the defense against gram-negative bacteria. The role of AKT in modulating the cellular response to ionizing radiation is well known.[18] We first investigated the role of this pathway in LDR-mediated microbial defenses. Ionizing radiation stimulated the expression of AKT kinase in a dose-dependent manner. LDR also triggered a small amount of glycogen synthase kinase 3β (GSK-3β) expression (Fig. 5A). We then examined the p38 MAPK and JNK pathways involved in host defense against pathogenic bacteria. Interestingly, the phosphorylated forms of both JNK and p38 MAPK were specifically increased by LDR (Fig. 5B). These results indicate that p38 MAPK and JNK signaling pathways may also contribute the activation of host defenses mediated by LDR in *Drosophila*.

DISCUSSION

Innate immune responses are conserved in metazoan organisms from insects to humans and serve as a first-line defense system against infection with pathogenic microorganisms. To characterize the effect of LDR on *Drosophila* innate immune responses, we have 1) monitored the expression of GFP in drosomycin-GFP and diptericin-GFP flies after low-dose (0.2 Gy) and high-dose (10 Gy) radiation; 2) compared the survival of bacterially-infected flies irradiated at a low dose (0.2 Gy) to those that were not irradiated and those irradiated at a high dose (10 Gy); and 3) monitored whether LDR changes the expression of AMP genes and proteins in signaling pathways related to immune activation. We found that GFP expression was increased in drosomycin-GFP adult flies irradiated at a low dose. As GFP expression was not increased in drosomycin-GFP larvae 8 hrs after irradiation, LDR-specific increases in *drosomycin* expression

seems to be a slow response that takes longer than 8 hrs. We also found that LDR specifically prolonged the survival of flies after gram-positive and gram-negative bacterial infections. Compared to the effects of HDR, which is known to weaken host defenses, our data indicate that LDR activates host defenses against microbial infection. The different effects between LDR and HDR have been extensively discussed in diverse fields, including mutation frequency, immunity, cancer development, and longevity.[8,9,19,20] The present study is the first report showing the stimulating effect of LDR on innate immunity of Drosophila. Similar to our results, LDR has been reported to activate various immune cells such as T cells , B cells and NK cells, orchestrating the immunological network.[21] In addition, secretion of several immune cytokines by dendritic cells was augmented by only LDR, but not HDR.[22] Our results also showed that the AMP genes of the Toll pathway, including *drosomycin*, *defensin*, and *metchnikowin*, were significantly increased in the flies irradiated at a low dose. Moreover, expression of key molecules in the Toll pathway, such as Pelle and Dorsal, were increased in flies irradiated at a low dose. However, LDR did not change the expression levels of the transmembrane receptor Toll or Cactus, the inhibitor of Dorsal. Additionally, LDR did not alter the expression or proteolysis rate of Relish. Taken together, these data suggest that LDR activates innate immune responses in *Drosophila* through the Toll pathway.

Compared to the highly-expressed AMPs triggered in the Toll pathway, LDR did not significantly change the expression of most AMP genes of the Imd pathway. The exception was expression of *attacin C*, which was the only Imd-associated gene affected by LDR. Additionally, LDR did not increase Relish expression or proteolysis, which is necessary to activate gene expression of downstream targets. It is puzzling that LDR increased the survival of flies after gram-negative bacterial infection without changing the expression of most AMP genes triggered by Imd pathway. There are two possible explanations for these results. First, LDR-mediated Toll pathway activation may confer resistance to gram-negative bacterial infection without changing the expression of Imd-related AMP genes. Recent finding indicate that the Toll pathway plays a direct role in the resistance to gram-negative bacterial infections and that the effect is independent of Relish.[23] Second, high expression of *attacin C*, which is induced by LDR, may be enough to confer the resistance to gram-negative bacterial infection. The previous finding that overexpression of *attacin C* and/or *diptericin* increased the survival of infected flies with *E. coli* supports this hypothesis.[24] Similarly, overexpression of a single AMP gene in fruit flies enhanced the resistance to hyperoxia-induced oxidative stress.[25] However, we should further investigate these possibilities since *attacin C* was known to be regulated by both Toll and Imd pathways.[23]

In addition to Toll activation, LDR specifically activated the p38 MAPK and JNK signaling pathways (Fig. 5). In particular, LDR significantly altered JNK phosphorylation. Interestingly, both the Toll and Imd pathways activate the JNK pathway in *Drosophila*.[26,27] Experiments using mutant Toll fruit flies demonstrated that JNK acts downstream of Toll to activate septic injury-induced target genes.[28,29] In addition to the Toll and Imd pathways, the JNK pathway may independently contribute to the expression of genes induced in response to microbial challenge.[27] Therefore, LDR-induced JNK activation may be involved in the induction of host defenses, including cytoskeletal regulation. Another LDR-activated signal pathway, p38 MAPK is thought to be crucial for host defenses against pathogenic bacteria. Similar to data presented here, previous results indicate that activated p38 modulates JNK activation through a cascade involving Toll, TRAF6 (TNF receptor associated factor 6), and TAB (TAK1 binding protein).[30–32] However, the relationship between p38-JNK with respect to immune activation is not yet clear because p38 has been also reported to suppress JNK activity in *Drosophila*.[33] We cannot also exclude the possibility that activation of innate immunity by LDR is resulted from the generation of low levels of reactive oxygen species (ROS) by LDR. However, the relationships among LDR, ROS generation and immune activation are poorly understood in any species yet.

Our biochemical and survival data were obtained at adult stage while flies are irradiated at 3rd instar larvae. It implies that LDR stimulating hormetic effects are not temporary but sustainable, at least in fruit flies. It is possible that exposure to LDR during developing stages (i.e., larvae) reprograms adult gene expression.[10] Elucidating the mechanism of gene expression reprogramming by LDR would be interesting experiment in future.

Although evidence supporting LDR-mediated hormesis was first uncovered in the early 20th century, most data have been based on epidemiological studies because sensitive methods to detect biological responses to LDR had been lacking.[19] However, it is difficult to elucidate the molecular mechanisms of radiation hormesis with epidemiological studies. Here, we identified molecular evidence of LDR-induced hormesis (stimulating effects) in terms of *Drosophila* innate immune responses. We propose that LDR specifically activates p38, JNK, and Toll pathways to activate these innate immune responses in fruit flies. Together with epidemiological studies, evidence from animal studies will help to solidify the hypothesis of radiation hormesis.

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