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Chlorogenic acid inhibits *Pseudomonas* toxin pyocyanin and activates mitochondrial UPR to protect host against pathogen infection

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Mitochondria are required for protecting host against pathogenic bacteria by activating mitochondrial unfolded protein response (UPR^{mt}). Chlorogenic acid (CGA), a phenolic acid compound of green coffee extracts and tea has been shown to exhibit activities such as antioxidant, antibacterial, hepatoprotective, cardioprotective, anti-inflammatory, neuroprotective, anti-obesity. However, whether CGA regulates innate immunity and the underlying molecular mechanisms remain unknown. In this study, we found that CGA increased resistance to Gram-negative pathogen *Pseudomonas aeruginosa* PA14 in dose dependent manner. Meanwhile, CGA enhanced innate immunity in *Caenorhabditis elegans* by reducing intestinal bacterial burden. CGA also inhibited the proliferation of pathogenic bacteria. Importantly, CGA inhibited the production of *Pseudomonas* toxin pyocyanin (PYO) to protect *C. elegans* from *P. aeruginosa* PA14 infection. Furthermore, CGA activated the UPR^{mt} and expression of antibacterial peptide genes to promote innate immunity in *C. elegans* via transcription factor ATFS-1(activating transcription factor associated with stress-1). Unexpectedly, CGA enhanced innate immunity independently of other known innate immune pathways. Intriguingly, CGA also protected mice from *P. aeruginosa* PA14 infection and activated UPR^{mt}. Our work revealed a conserved mechanism by which CGA promoted innate immunity and boosted its therapeutic application in the treatment of pathogen infection.

Keywords Chlorogenic acid, Innate immunity, Mitochondrial unfolded protein response (UPR^{mt}), Mice, *Caenorhabditis elegans*

With the growing concern regarding the drug resistance of using antibiotic, the attention towards natural and herbal substances that enhancing immune defenses against pathogens by strengthening host innate immunity has been increasing every day. Chlorogenic acid (CGA) is one of the most available acids among phenolic acid compounds, which widely distributes in plants and utilizes in traditional Chinese medicines[1]. CGA possesses a wide range of pharmacological effects such as antioxidant activity, antibacterial, hepatoprotective, cardioprotective, anti-inflammatory, antipyretic, neuroprotective, anti-obesity, antiviral, anti-microbial, anti-hypertension[2]. Meanwhile, CGA improves efficacy in the cancer immunotherapy via T cell regulation[1]. Recent study has shown that CGA extends lifespan in *Caenorhabditis elegans*[3,4]. Furthermore, CGA influences lipid metabolism via liver PPAR-α (Peroxisome Proliferator-activated Receptor α)[5]. However, whether CGA regulates innate immunity and the underlying molecular mechanisms are largely unknown.

Innate immunity is at the front line of our defense system against invading pathogenic microorganisms, which is evolutionarily conserved from nematodes to mammals[6,7]. During pathogens infection, the innate immunity is activated, and aroused antimicrobial response to invading pathogenic microorganisms[6,8–11]. *Caenorhabditis elegans* has been developed as a valuable genetic model for research on the animal immune response. In contrast,

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pathogenic bacteria produce different metabolites that are involved in growth and virulence[12,13]. *P. aeruginosa* is a common opportunistic pathogen in clinical practice. *P. aeruginosa* causes infection in patients by secreting a wide array of virulence factors, such as pyocyanin[14], phenazine-1-carboxamide[15], hydrogen cyanide[16] and iron chelating siderophores[16].

UPR^{mt} refers to the stress response of mitochondria to activate the transcriptional activation program of mitochondrial heat shock proteins and proteases, which plays important role in antibacterial immunity[16,17]. In general, UPR^{mt} sends signals to the nucleus through the mitochondrial peptide exporter HAF-1 and up-regulates transcription factor ATFS-1(activating transcription factor associated with stress-1)[18]. In addition, UPR^{mt} also induces transcription of ubiquitin-like protein 5 (UBL-5), which binds to DVE-1 (defective proventriculus protein 1) to form a complex that promotes the expression of mitochondrial HSP60[19]. During *P. aeruginosa* infection, ATFS-1-dependent UPR^{mt} is activated, which is followed by the increased expression of mitochondrial protective genes and antibacterial peptide genes[16]. ATF5 (activating transcription factor 5), homologous gene of ATFS-1 in *C. elegans*, also regulates mitochondrial stress response in mammalian cells[20].

Our study investigated the role of CGA in the host defenses against pathogen infection. CGA inhibited *Pseudomonas* toxin pyocyanin to protect host from *P. aeruginosa* PA14 infection. Furthermore, CGA activated ATFS-1-dependent UPR^{mt}, which is followed by the increased expression of mitochondrial protective and antibacterial peptide genes. CGA also increased resistance to *P. aeruginosa* PA14 infection and activated UPR^{mt} in mice. These findings revealed that the CGA properties related to innate immunity might significantly boost its application to infectious diseases.

Results

Chlorogenic acid promotes innate immunity in *C. Elegans*

To investigate whether Chlorogenic acid (CGA) promoted innate immunity, animals were exposed to *P. aeruginosa* PA14. We found that wild-type worms treated with CGA (0 μM, 1 μM, 10 μM, 100 μM) exhibited increased resistance to *P. aeruginosa* PA14 in dose dependent manner (Fig. 1A; Table S1). These results suggested that CGA enhanced innate immunity in *C. elegans*. Meanwhile, we observed that CGA (1 μM, 10 μM, 100 μM) also extended lifespan of wild-type animals that were fed heat-killed *P. aeruginosa* PA14 in dose dependent manner (Fig. 1B; Table S1). After CGA treatment, worms exposed to *Staphylococcus aureus*, and *Listeria monocytogenes* exhibited a higher survival rate (Figure S1A and S1B). These results suggested that CGA exhibited a broad spectrum of pathogen resistance. Furthermore, we performed the bacterial growth assay and found that 100 μM Chlorogenic acid significantly inhibited the proliferation of *P. aeruginosa* PA14 ($^*P<0.05$) (Fig. 1C). Because clearance of the intestinal pathogenic bacteria loads is part of host defense against pathogens infection[21]. Subsequently, we detected bacteria accumulation in the intestine after CGA treatment, and found that CGA significantly decreased the number of bacterial cells in the intestine compared to control animals ($^*P<0.05$) (Fig. 1D). These results indicated that CGA enhanced the resistance to pathogen infection.

Chlorogenic acid inhibits *Pseudomonas* toxin pyocyanin to protect *C. Elegans* from pathogen infection

Pseudomonas toxin pyocyanin (PYO) is an important virulence factor in *P. aeruginosa* PA14[22], which is synthesized from phenazine-1-carboxylic acid, a process that is mediated by phenazine-specific methytransferase, *PhzM*[23]. We found that CGA significantly inhibited the production of PYO ($^*P<0.05$) (Fig. 2A). Furthermore, worms infected with the *ΔphzM* mutant increased the survival, compared with those infected with the wild-type (WT) PA14 strain (Fig. 2B). However, PYO (25 μg/ml)[15] supplement rescued the long-live phenotype in animals infected with the *ΔphzM* mutant (Fig. 2B). Meanwhile, CGA increased the survival in worms infected with the wild-type PA14 strain and animals infected with the *ΔphzM* mutant + PYO (25 μg/ml) (Fig. 2B). CGA partially prolonged the survival rate of worms infected with the *ΔphzM* mutant (Fig. 2B). In addition, CGA reduced the intestinal bacteria loads in worms infected with the wild-type PA14 strain, or animals infected with the *ΔphzM* mutant, and animals infected with the *ΔphzM* mutant + PYO (25 μg/ml) (Fig. 2C). These results indicated that CGA enhanced the resistance to *P. aeruginosa* PA14 infection by inhibiting the production of PYO.

Chlorogenic acid activates mitochondrial unfolded protein response (UPRmt) to enhance innate immunity through ATFS-1

Mitochondria play key roles in antibacterial innate immunity by regulating UPR^{mt}[16,24]. Next, we asked if CGA treatment induced mitochondrial stress capable of activating the UPR^{mt}. We found that CGA significantly increased mitochondrial chaperone reporter *hsp-6::gfp* activation in the intestine ($^*P<0.05$) (Fig. 3A). In general, UPR^{mt} induces transcription of *atfs-1*, *ubl-5*, and *dve-1*. Quantitative real-time PCR analysis demonstrated that 100 μM CGA significantly increased the mRNA levels of *atfs-1*rather than *ubl-5*, and *dve-1* compared with the control ($^*P<0.05$) (Fig. 3B). To further confirm whether CGA activated the transcription factor ATFS-1, we detected the cellular translocation of ATFS-1 using transgenic worms that express a functional ATFS-1::GFP fusion protein. We found that 100 μM CGA significantly induced ATFS-1 nuclear localization in the intestine ($^*P<0.05$) (Fig. 3C). Furthermore, we tested the expression of ATFS-1-targeted immune response genes such as *abf-2*, *lys-2*, *clec-4*, and *clec-65* and mitochondrial protective genes such as *hsp-6* and *hsp-60*. We found that ATFS-1-targeted immune response genes and mitochondrial protective genes were up-regulated in 100 μM CGA-treated worms, compared with the control (Fig. 3D). However, 100 μM CGA failed to increase these genes expression in *atfs-1(gk3094)* mutant worms (Fig. 3D). In addition, we found that 100 μM CGA failed to enhance the resistance to *P. aeruginosa* PA14 infection in *atfs-1(gk3094)* mutant worms (Fig. 3E). These results suggested that CGA activated UPR^{mt} to enhance innate immunity by ATFS-1.

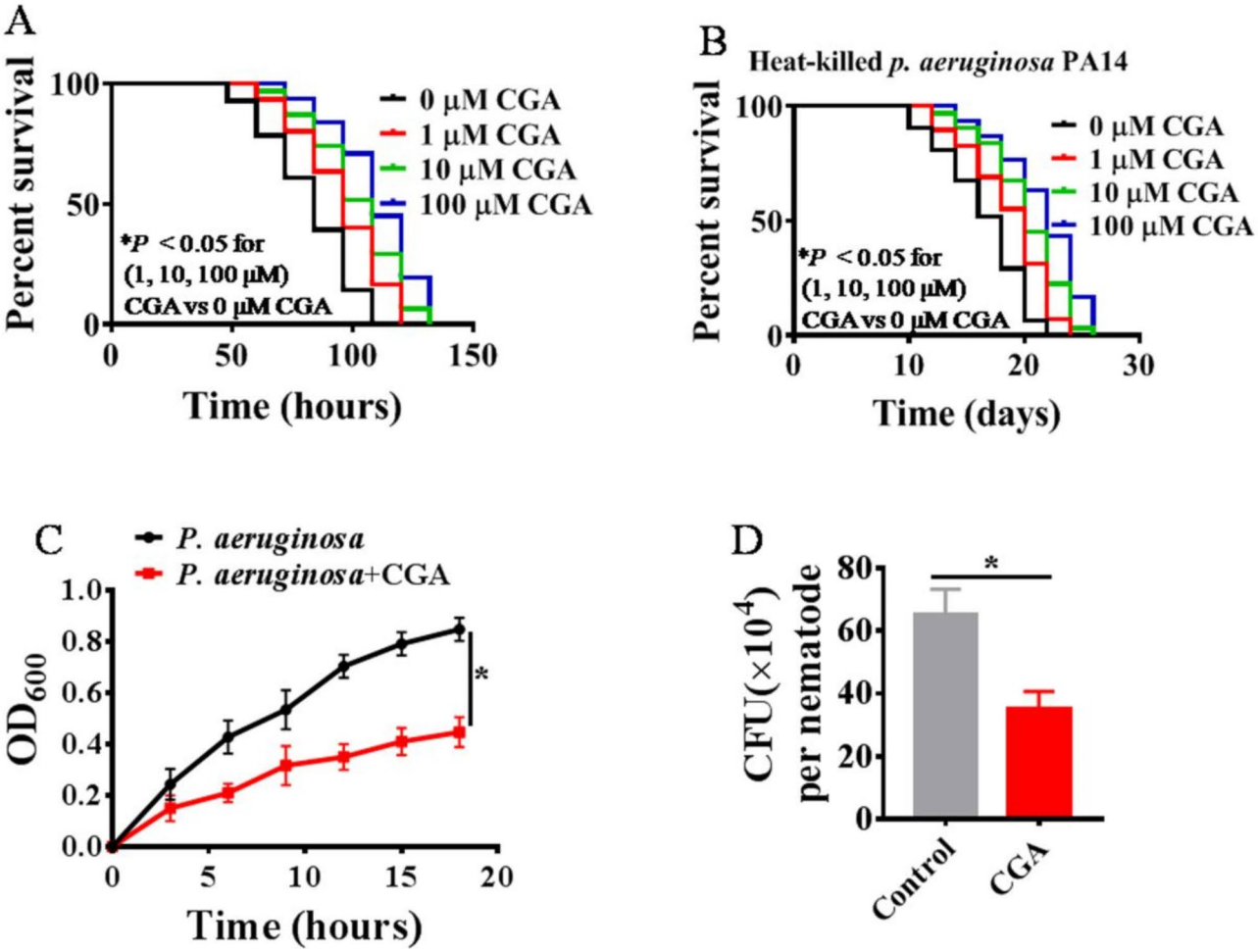

Fig. 1. Chlorogenic acid promotes innate immunity in *C. elegans*. (A) CGA promoted innate immunity in dose-dependent manner. (*$P < 0.05$, log-rank test). See Table S1 for survival data. (B) Survival of N2 animals exposed to heat-killed *P. aeruginosa* PA14 after CGA treatment. (*$P < 0.05$; log-rank test). See Table S1 for survival data. (C) 100 μM CGA significantly inhibited the proliferation of *P. aeruginosa* PA14. (*$P < 0.05$, log-rank test). Error bars represent mean ± SEM of three independent biological replicates. (D) 100 μM CGA decreased the intestinal bacteria burden in WT worms after *P. aeruginosa* PA14 infection 48 h. ($n \geq 20$). These results are mean ± SEM of three independent experiments. (*$P < 0.05$, unpaired t-test).

Chlorogenic acid extends survival independently of other known innate immune pathways

Next, we investigated if CGA interacted with established *C. elegans* innate immunity pathways, which included PMK-1/p38 MAPK[25], MPK-1/ERK MAPK[26], the MLK-1/MEK-1/KGB-1 c-Jun kinase pathway[27,28], transcription factor ZIP-2[29,30], and endoplasmic reticulum unfolded protein response XBP-1[31]. We found that 100 μM CGA increased the survival rates of N2, *pmk-1(km25)*, *mpk-1(n2521)*, *mlk-1(ok2471)*, *zip-2(ok3730)*, and *xbp-1(zc12)* mutant worms, after *P. aeruginosa* PA14 infection (Fig. 4A–F; Table S1). These results suggested that CGA promoted innate immunity in *C. elegans* independently of other known innate immune pathways.

Chlorogenic acid protects mice from *P. aeruginosa* PA14 infection

Next, we asked if CGA promoted innate immunity in mice. CGA treatment mice (50 mg/kg body weight) and control mice were infected with *P. aeruginosa* PA14 (1.0×10^6 CFUs/mouse). We found that CGA enhanced resistance to *P. aeruginosa* PA14 infection in wild-type mice (Fig. 5A). Meanwhile, we tested bacterial loads in lung tissue by quantifying colony-forming units (CFUs) of live bacterial cells. We found that CGA reduced the CFUs of *P. aeruginosa* PA14 than control mice (Fig. 5B). Interestingly, we found that CGA increased the mRNA levels of mouse UPR^{mt} genes HSPD1, HSPA9, LONP1, and YME1L1 in lung tissue (Fig. 5C). Furthermore, consistent with the role in *C. elegans*, CGA also significantly increased the protein levels of ATF5 (homologous gene of ATFS-1 in *C. elegans*) in lung tissue (*$P < 0.05$) (Fig. 5D). These results suggested that CGA promoted innate immunity and activated UPR^{mt} in mice.

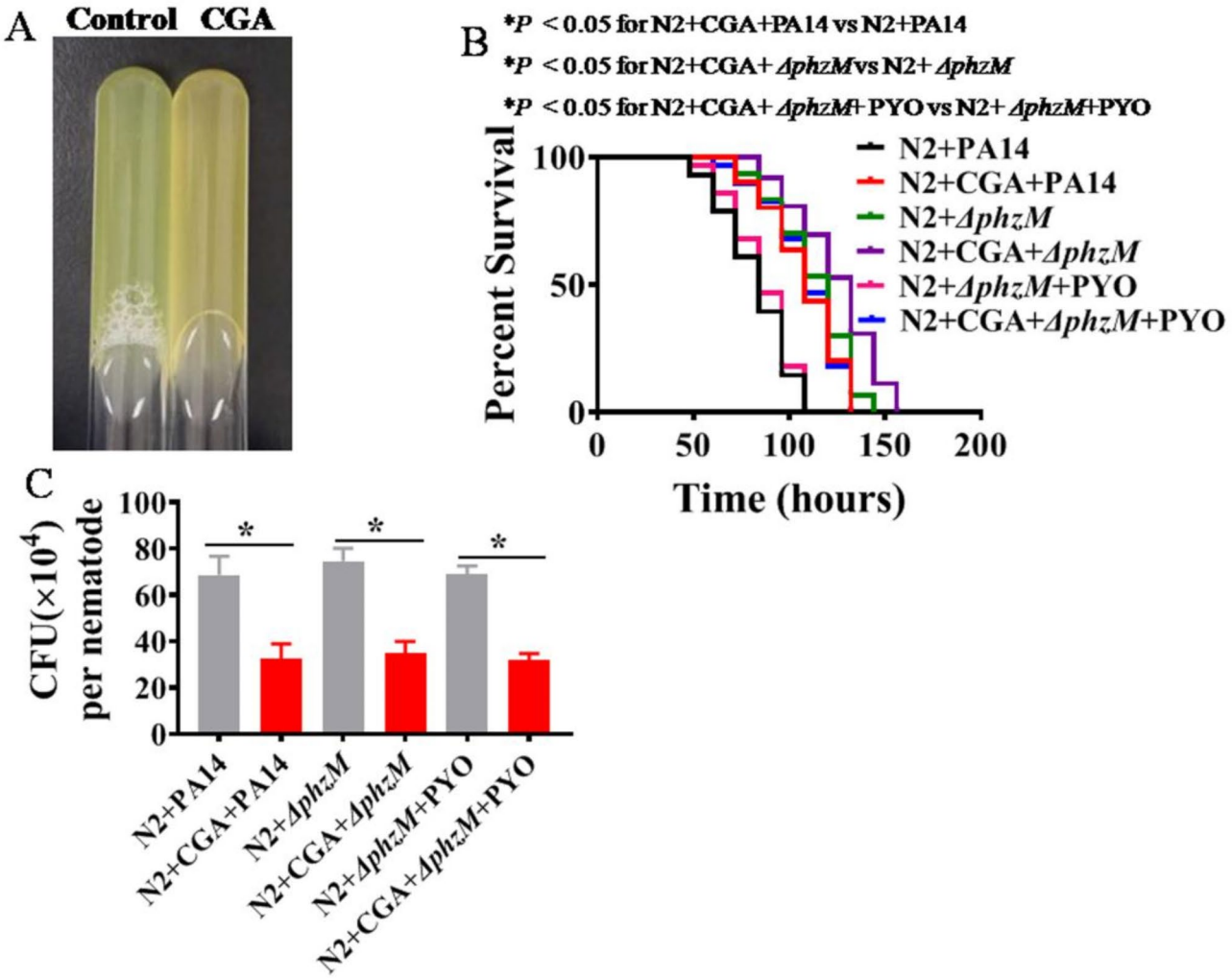

Fig. 2. Chlorogenic acid inhibits the prodction of *Pseudomonas* toxin pyocyanin to protect *C. elegans* from pathogen infection. (A) 100 μM CGA inhibited the production of PYO. (B) Survival of N2 worms exposed to wild type *P. aeruginosa* PA14, *ΔphzM* mutant, and *ΔphzM* mutant + PYO (25 μg/ml) after CGA treatment. (*$P < 0.05$; log-rank test). See Table S1 for survival data. (C) The CFUs of N2 worms exposed to wild type *P. aeruginosa* PA14, *ΔphzM* mutant, and *ΔphzM* mutant + PYO (25 μg/ml) after CGA treatment. These results are mean ± SEM of three independent experiments. (*$P < 0.05$, unpaired t-test).

Discussion

Chlorogenic acid (CGA) is one of the most available acids among phenolic acid compounds[1], which exhibits a wide range of pharmacological activities, such as antioxidant activity, antibacterial, hepatoprotective, cardioprotective, anti-inflammatory, antipyretic, neuroprotective, anti-obesity, antiviral, and, anti-hypertension[2]. However, the molecular mechanisms by which it promotes innate immunity remain unknown. Here, we demonstrated that preventive application of CGA protected *C. elegans* from Gram-negative pathogen *Pseudomonas aeruginosa* PA14 and Gram-positive pathogens *Staphylococcus aureus* and *Listeria monocytogenes* infection. Through innate immune pathways screening, this effect appeared to involve a conserved mechanism that influenced innate immunity in host, including ATFS-1 dependent UPR^{mt}. Importantly, Our findings provided an evidence that CGA inhibited PYO, which was an important virulence factor in *Pseudomonas aeruginosa* PA14[12,13]. It should be noted that CGA partially prolonged the survival rate of worms infected with the *ΔphzM* mutant. In addition, CGA reduced the intestinal bacteria loads in worms infected with the wild-type PA14 strain, or animals infected with the *ΔphzM* mutant, and animals infected with the *ΔphzM* mutant + PYO (25 μg/ml), suggesting that PYO is not the only factor that is inhibited by CGA. Furthermore, our results indicated that the enhancement of immune responses by CGA required the activation of immune response genes such as *abf-2*, *lys-2*, *clec-4*, and *clec-65* and mitochondrial protective genes such as *hsp-6* and *hsp-60*. Thus, our findings provided an alternative mechanism by which antibiotic action like CGA defended host against pathogen infection.

Accumulating evidence indicates that mitochondria play key roles in the resistance of host against pathogen infection by activation UPR^{mt}. For example, mitochondrial aconitase ACO-2 inhibits innate immunity against pathogenic bacteria in *C. elegans*[32]. Mitochondrial chaperone HSP-60/HSPD1 enhances antibacterial immunity

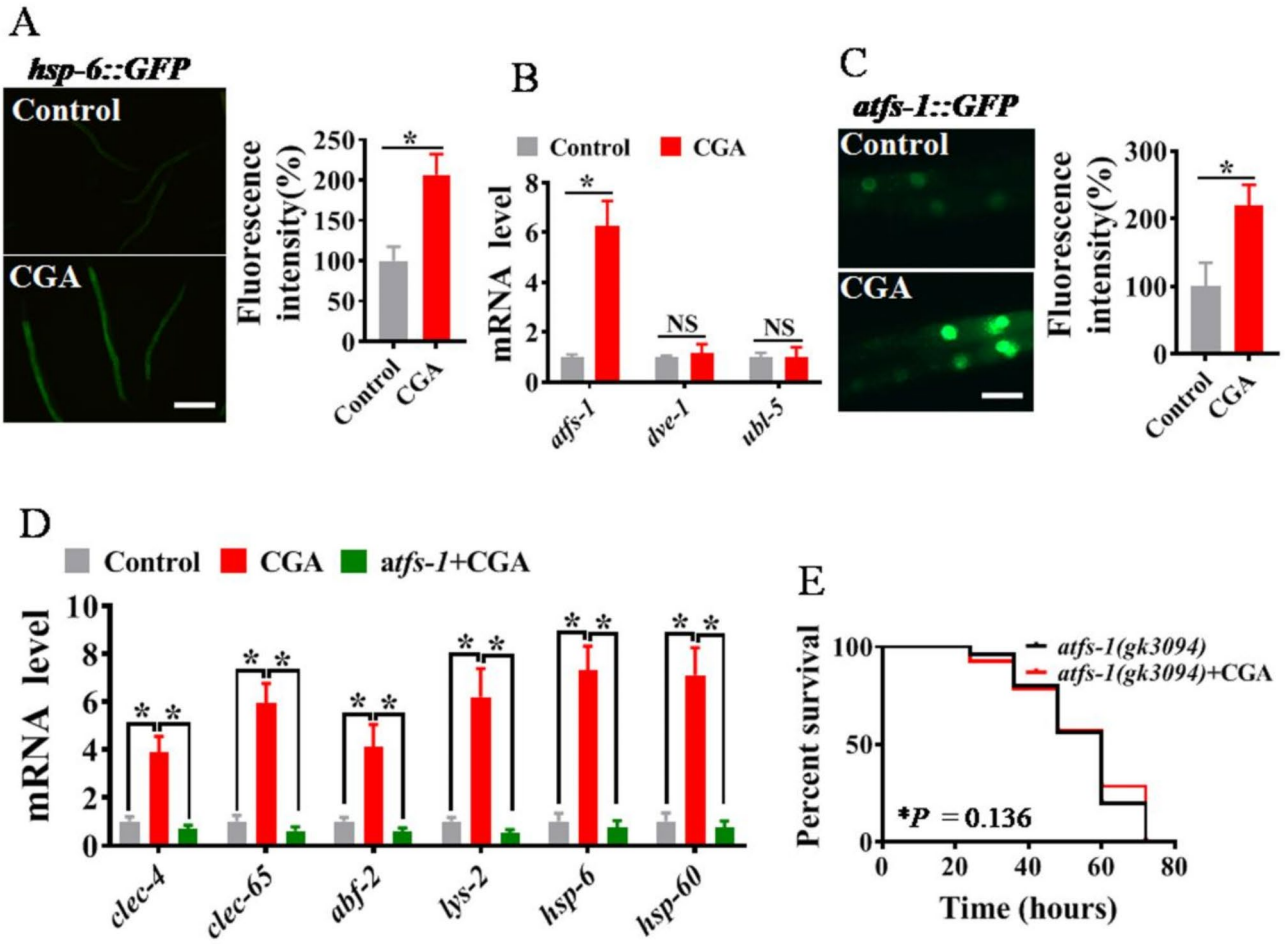

Fig. 3. Chlorogenic acid activates mitochondrial unfolded protein response (UPR^{mt}) to enhance innate immunity through ATFS-1. (A) 100 μM CGA increased the expression of *hsp-6::GFP*. The right panel shows quantification of *hsp-6::GFP*. ($n \geq 30$). Scale bars: 100 μm. These results are mean ± SEM of three independent experiments performed in triplicate. (*$P < 0.05$, one-way ANOVA). (B) The mRNA levels of *atfs-1*, *dve-1* and *ubl-5* in worms exposed to 100 μM CGA. These results are mean ± SEM of three independent experiments performed in triplicate. (*$P < 0.05$, one-way ANOVA). NS, no significance. (C) 100 μM CGA significantly induced ATFS-1 nuclear localization. The right panel shows the quantification of ATFS-1::GFP ($n \geq 30$). Scale bars: 50 μm. These results are mean ± SEM of three independent experiments performed in triplicate. (*$P < 0.05$, unpaired t-test). (D) The mRNA levels of ATFS-1-targeted immune response genes such as *abf-2*, *lys-2*, *clec-4*, and *clec-65* and mitochondrial protective genes such as *hsp-6* and *hsp-60* in worms expose to 100 μM CGA. These results are mean ± SEM of three independent experiments performed in triplicate. (*$P < 0.05$, one-way ANOVA). (E) 100 μM CGA failed to enhance resistance to *P. aeruginosa* PA14 infection in *atfs-1(gk3094)* mutant animals. (log-rank test). See Table S1 for survival data.

in *C. elegans* and human cells by targeting p38 MAPK pathway[24]. UPR^{mt} also increases the expression of antimicrobial genes in *C. elegans* in an ATFS-1-dependent manner and contributes to antibacterial innate immunity[16]. Furthermore, the increased expression of HSP60 in intestinal epithelial cells of colitis model animals and inflammatory bowel disease patients also indicates that UPR^{mt} response may be a key response for intestinal mucosal cells to detect pathogenic infection and improve the innate immunity of cells[33]. In this study, we found that CGA enhanced resistance to *P. aeruginosa* PA14 infection in mice by clearance bacteria loads of lung tissue. Importantly, ATF5/ATFS-1 not only induced UPR^{mt} in nematodes, but also induced high expression of mitochondrial heat shock protein HSP60/HSPD1, HSP70/HSPA9, and mitochondrial protease LONP1 and antimicrobial peptide HD-5 (human defensin 5) in mammalian cells[20]. Intriguingly, we found that CGA increased the mRNA levels of mouse UPR^{mt} genes HSPD1, HSPA9, LONP1, and YME1L1 in lung tissue. Furthermore, consistent with the role in *C. elegans*, CGA also increased the protein levels of ATF5 in lung tissue, suggesting that CGA might activate UPR^{mt} to protect mice from pathogen infection. Based on the observations that CGA can promote antibacterial immunity in worms and mice, our findings suggest that CGA may exhibit significant benefits by improving infectious diseases in mammals.

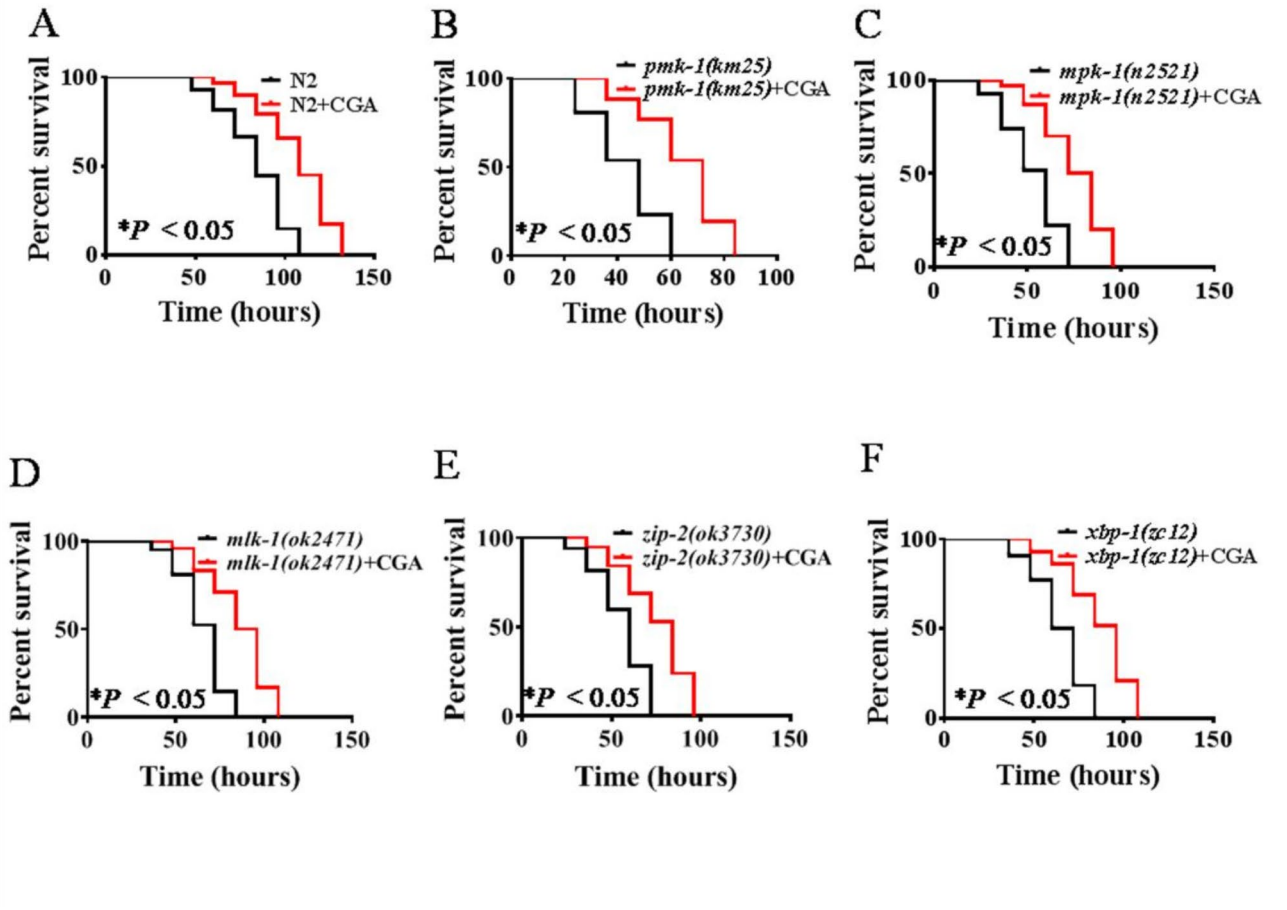

Fig. 4. Chlorogenic acid extends survival independently of other known innate immune pathways. (A–F) Survival of (N2) (A), *pmk-1(km25)* (B), *mpk-1(n2521)* (C), *mlk-1(ok2471)* (D), *zip-2(ok3730)* (E), and *xbp-1(zc12)* (F) mutant worms exposed to *P. aeruginosa* PA14 after 100 µM CGA treatment. (*$P < 0.05$, log-rank test). See Table S1 for survival data.

Materials and methods

Chemicals

Chlorogenic acid was obtained from Sigma Chemical Co. (St. Louis, MO) and dissolved in Dimethyl sulfoxide (DMSO) as a stock solution at a 100 mM concentration and stored in aliquots at – 20 °C.

Nematode strains

N2 Bristol wild-type, VC3056 *zip-2(ok3730)*, RB1908 *mlk-1(ok2471)*, KU25 *pmk-1(km25)*, SJ17 *xbp-1(zc12)*, VC3201 *atfs-1(gk3094)*, SD184 *mpk-1(n2521)*, OP675 *atfs-1::GFP*, and SJ4100 *hsp-6p::GFP* were obtained from the Caenorhabditis Genetics Center (CGC).

Slow-killing assay

P. aeruginosa PA14 was grown in LB (Lauria Bertani) broth at 37 °C and 180 rpm overnight, and 100 µL of the bacterial solution was spread to the NGM plates supplemented with or without Chlorogenic acid (0, 1, 10, 100 µM) and PYO (25 µg/ml). When the bacteria liquid was dried, the plates were incubated at 37 °C in a constant temperature incubator for 16–18 h and subsequently moved to a 25 °C constant temperature incubator for 3 h. The synchronized worms were cultivated on NGM plates containing *E. coli* OP50 until the young adult stage and 40–80 worms were transferred to the plates containing *P. aeruginosa* PA14. The samples were observed at 12 h intervals, and touch with picker no response worms were recorded as dead[34]. Each set of experiments were conducted on three plates, and all experiments were repeated three times independently.

Bacterial proliferation assay

Bacterial colonies were inoculated into LB broth, and equal volumes of the bacterial suspension was added to LB broth (with or without 100 µM Chlorogenic acid) and maintained at 37 °C and 180 rpm. The optical density (OD

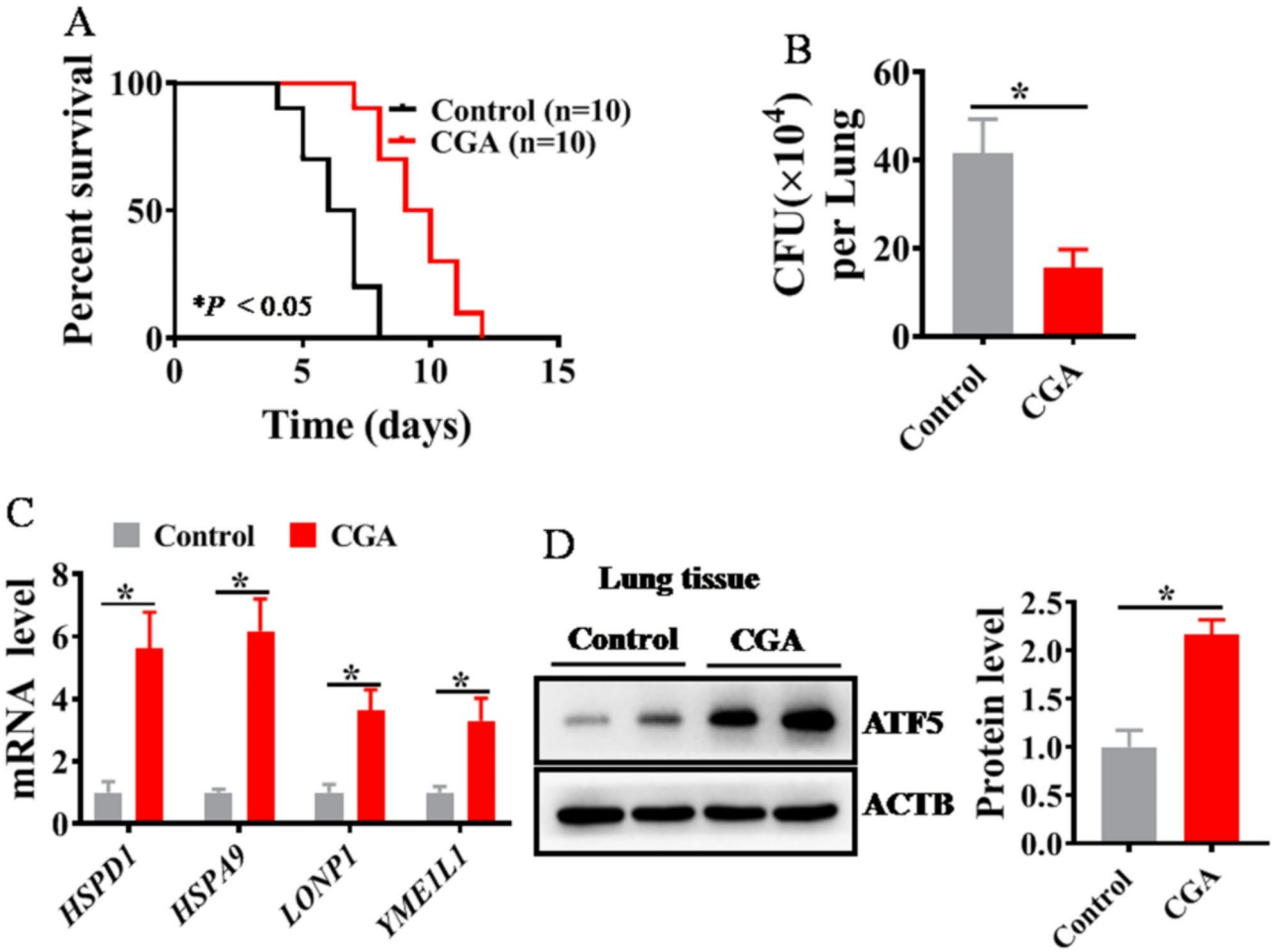

Fig. 5. Chlorogenic acid protects mice from *P. aeruginosa* PA14 infection. (A) CGA treatment (50 mg/kg body weight) enhanced resistance to *P. aeruginosa* PA14 infection in wild-type mice. (*$P < 0.05$, log-rank test). (B) The CFUs of *P. aeruginosa* from lung homogenates plated on LB agar plates after CGA treatment. (C) CGA increased the mRNA levels of mouse UPR^{mt} genes HSPD1, HSPA9, LONP1, and YME1L1 in lung tissue. These results are mean ± SEM of three independent experiments performed in triplicate. (*$P < 0.05$, one-way ANOVA). (D) CGA increased the protein levels of ATF5 in lung tissue. The right panel shows quantification of ATF5. These results are mean ± SEM of three independent experiments performed in triplicate. (*$P < 0.05$, one-way ANOVA).

600 nm) was measured every 3 h. Three replicates were tested in each group, and all experiments were repeated three times independently.

Quantification of intestinal bacterial loads

The synchronized worms were cultivated on *E.coli* OP50 at 20 °C until the young adult stage. Next, animals were transferred to NGM agar plates (supplemented with or without 100 μM Chlorogenic acid) and PYO (25 μg/ml) containing *P. aeruginosa*/GFP for two days at 25 °C[7,35]. Twenty worms were transferred into 100 uL Phosphate Buffer Saline (PBS) containing 0.1% Triton and ground. After 24 h of incubation at 37 °C, the colonies of *P. aeruginosa* /GFP were counted. Three plates were detected per assay and all experiments were repeated three times independently.

Fluorescence microscopy

The synchronized worms were cultivated on *E. coli* OP50 at 20 °C until the young adult stage supplemented with or without 100 μM Chlorogenic acid. The images were obtained by using Zeiss Axioskop 2 plus fluorescence microscope. Fluorescence intensity quantification was analyzed by using Image J software. Three plates were used for each group of experiments, and three independent replications were performed for each experiment.

Quantitative real-time PCR

Total RNA was extracted from worms or lung tissue with TRIzol Reagent (Invitrogen) as previously described[7]. cDNA was synthesized with reverse transcription kits (Invitrogen) and qPCR analysis was carried out using SYBR Premix-Ex TagTM (Takara, Dalian, China). For quantification, *C. elegans* transcripts were normalized to

pmp-3[36], and transcripts from lung tissue were normalized to ACTB. Primer sequences for qPCR were listed in Table S2.

Western blotting

Lung tissue were homogenized in liquid nitrogen, and added to protein lysate and placed on ice for 30 min. Samples were separated on a 10% SDS polyacrylamide gel and transferred to PVDF membrane (Millipore, Bedford, MA). Primary antibodies against ATF5 (Abcam, ab184923, 1:1000 dilution), and ACTB (Abcam, ab227387, 1:1000 dilution) were used. Membranes were developed with Supersignal chemiluminescence substrate (Pierce). The band intensity was measured by Image J software.

Animal study and colony counting of lung in mice

C57BL/6 mice were inoculated with *P. aeruginosa* PA14-laden agarose beads contained 1.0×10^6 CFUs/mouse, as previously described[7,37]. Some of animals received daily doses of 50 mg/kg body weight CGA through intraperitoneal injection for 6 d. Lung tissue homogenates were taken from the mice, and the number of colonies of *P. aeruginosa* PA14 was determined by serial dilution.

ARRIVE guidelines Compliance: All methods are reported in accordance with ARRIVE guidelines. All mouse studies were carried out under standard conditions and in accordance with Zunyi Medical University Animal Care Committee (ZMU21-2305-003) guidelines. All animals were obtained from Zunyi Medical University.

After the experiment, the animals were killed by cervical dislocation after anesthesia. The anesthetic was administered with pentobarbital sodium with a concentration of 2% and a dose of 50 mg/kg, which was injected into the abdomen.

Statistics

Statistical analyses were performed with GraphPad Prism 7.0. Data were expressed as mean ± SEM. Statistical analyses for all data were carried out using Student's t-test (unpaired, two-tailed) or ANOVA after testing for equal distribution of the data and equal variances within the data set. Survival data were analyzed by using the log-rank (Mantel-Cox) test. $^*P < 0.05$ was considered significant.

Data availability

The data that support the fingings of this study are not openly available due to reasons of sensitivity and are available from the corresponding author upon reasonable request.

Received: 19 August 2024; Accepted: 11 February 2025
Published online: 14 February 2025

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Author contributions

Y.X. , Q.X. and F.L. designed the study. Y.X. wrote the paper. L.L., C.H., T.H. S.R. and X.W. participated in experiments.

Funding

This study is supported by National Natural Science Foundation of China (32160037, 32060033). The Science and Technology Plan Project of Guizhou (QKHJC-ZK[2024]ZD071).

Competing interests

The authors declare no competing interests.

Consent for publication

All authors read and approved the final manuscript.

Ethical approval

All mouse studies were carried out under standard conditions and in accordance with Zunyi Medical University Animal Care Committee (ZMU21-2305-003) guidelines. This study protocol was approved by Zunyi Medical University Animal Care Committee.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-90255-1.

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