Report

A Glutamate-Dependent Redox System in Blood Cells Is Integral for Phagocytosis in *Drosophila melanogaster*

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Summary

Glutamate transport is highly regulated as glutamate directly acts as a neurotransmitter [1–3] and indirectly regulates the synthesis of antioxidants [4, 5]. Although glutamate deregulation has been repeatedly linked to serious human diseases such as HIV infection and Alzheimer's [6–8], glutamate's role in the immune system is still poorly understood. We find that a putative glutamate transporter in *Drosophila melanogaster*, *polyphemus* (*polyph*), plays an integral part in the fly's immune response. Flies with a disrupted *polyph* gene exhibit decreased phagocytosis of microbial-derived bioparticles. When infected with *S. aureus*, *polyph* flies show an increase in both susceptibility and bacterial growth. Additionally, the expression of two known glutamate transporters, *gender-blind* and *excitatory amino acid transporter 1*, in blood cells affects the flies' ability to phagocytose and survive after an infection. Consistent with previous data showing a regulatory role for glutamate transport in the synthesis of the major antioxidant glutathione, *polyph* flies produce more reactive oxygen species (ROS) as compared to wild-type flies when exposed to *S. aureus*. In conclusion, we demonstrate that a *polyph*-dependent redox system in blood cells is necessary to maintain the cells' immune-related functions. Furthermore, our model provides insight into how deregulation of glutamate transport may play a role in disease.

Results and Discussion

The Putative Amino Acid Transporter *polyph* Is Required in Blood Cells for Phagocytosis of Microbes

From a genetic screen, we identified a transposon line, Mi[ET1] CG12943[MB02238], with a striking defect in phagocytosis. The disrupted gene, CG12943 (Figure 1A), is predicted to contain an amino acid transmembrane transporter domain and be related to *Drosophila excitatory amino acid transporter 1*(*eaat1*) with ~20% amino acid similarity. CG12943 was named *polyphemus* (*polyph*) after the Cyclops in Greek mythology who failed to guard against Odysseus as *polyph* flies are similarly unable to defend against an intruding microbe.

The phagocytic capacity of *polyph* flies was measured using an in vivo adult phagocytosis assay [9]. Flies are injected with fluorescently labeled bioparticles. The amount of fluorescence in the dorsal vessel area where sessile phagocytes accumulate is visualized and quantified (Figure 1B). *polyph* flies were deficient in phagocytosis of *S. aureus* (Figure 1C), *E. coli* (Figure 1D), and zymosan (data not shown), while phagocytosis

of latex beads was normal (Figure 1E). *polyph* flies do not have fewer blood cells (Figure S3B available online), which indicates that *polyph* flies have adequate numbers of blood cells with functional phagocytic machinery. Hence, the defect in *polyph* is specific to the inability to phagocytose microbial-derived bioparticles.

Drosophila blood cells can circulate in larvae, while in adult flies they become mainly sessile and difficult to separate from the surrounding tissue [10]. Therefore, blood cell expression of *polyph* was measured by collecting larval hemolymph which includes circulating blood cells. FlyAtlas does not examine gene expression in the blood cells, but did report an enriched expression of *polyph* in the testes [11], so the sexes were evaluated separately.

In females, the blood cells have an enriched *polyph* expression compared to the carcass (Figure S1A). Males also express *polyph* in their blood cells, with similar levels in their carcass presumably due to testes expression. Adult *polyph* expression was measured in whole animals. Male expression of the gene was higher than in females, but neither sex showed significant upregulation with *S. aureus* infection. Immunostaining of Flag-tagged Polyph protein confirmed that the putative amino acid transporter localized to the plasma membrane consistent with a role in phagocytosis (Figure S1B).

As blood cells are a major source of *polyph* mRNA, an RNAi construct against *polyph* was specifically expressed in blood cells (Figure S2A), which recapitulated the decrease in *S. aureus* phagocytosis observed in the original mutant (Figure S2B). Furthermore, the phagocytosis defect in the original mutant could be rescued by expressing *polyph* in blood cells (Figure 2A), confirming that the defect is due to a lack of *polyph* expression in blood cells.

polyph Flies Have Decreased Resistance to an *S. aureus* Infection

polyph flies showed increased susceptibility to *S. aureus* infection (Figures 2B and 2C), which could be rescued by expression of *polyph* in blood cells (Figure 2D). This susceptibility was substantiated by expression of RNAi against the gene specifically in blood cells (Figure S2C). The gene is likely playing a role in resistance rather than tolerance, as there is an increased bacterial load in *polyph* flies as compared to wild-type files (Figure 2E). Blood-cell-specific expression of the gene rescues the bacterial growth indicating that *polyph* is required in the blood cells to control the growth of bacteria during an infection (Figure 2F).

As Polyph is predicted to be an amino acid transporter, the loss of this protein may be affecting fly growth either directly or through a nutrient-sensing mechanism. However, there was no difference in weight compared to the wild-type (Figure S3C). Additionally, *polyph* flies are not generally weak as they are not more susceptible to wounding (Figures 2B and 2C), being kept at 30°C (Figure S3A), or an *E. coli* infection (data not shown).

polyph Does Not Play a Role in the Induction of *drosomycin* or *diptericin*

When activated by microbes, the Toll and Imd pathways upregulate the production of antimicrobial peptides (AMPs), which

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CrossMark

A

polyph Expression

B

C

S. aureus

Phagocytosis of *S. aureus*

D

E. coli

Phagocytosis of *E. coli*

E

Latex Beads
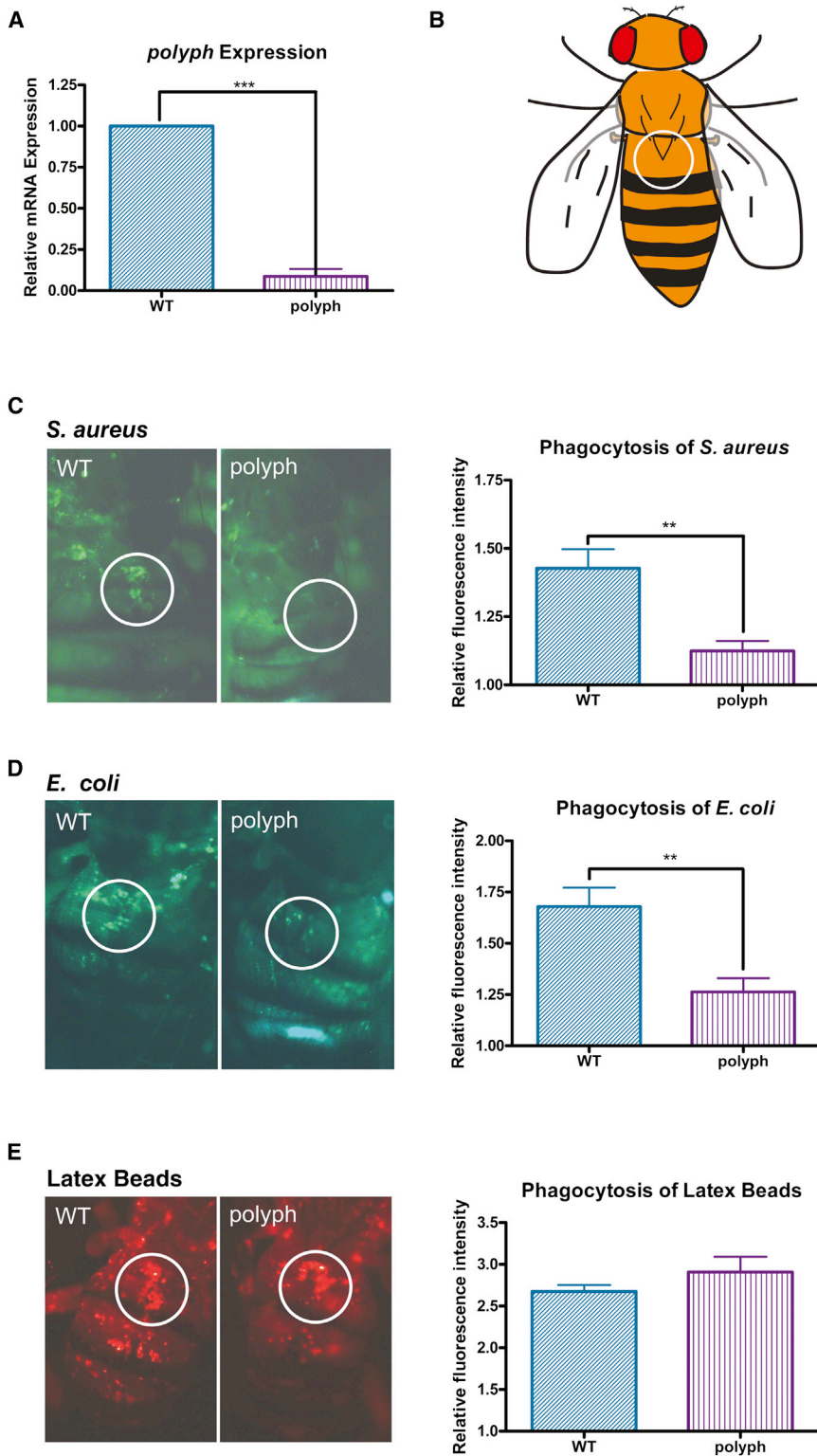

Phagocytosis of Latex Beads

Figure 1. *polyph*, a Putative Amino Acid Transporter, Is Required for Microbial Phagocytosis

(A) Comparison of *polyph* transcript levels via quantitative PCR in wild-type flies (WT) and flies containing a transposon insertion in the *polyph* gene (polyph). Relative expression was measured using *rp49* as an endogenous control. A pool of ten flies per genotype was used in each experiment.

(B) Representation of how the fly is visualized during the in vivo adult phagocytosis assay. The encircled area represents the area of the dorsal vein around which the sessile blood cells congregate.

(C–E) Representative pictures depicting phagocytosis in WT and *polyph* flies of fluorescein-labeled *S. aureus* bioparticles (C), fluorescein-labeled *E. coli* bioparticles (D), and red fluorescently labeled latex beads (E). Approximately six flies per genotype were used in each experiment. Quantification follows.

Error bars indicate ±SE. **p < 0.01; ***p < 0.001.

polyph does not play a major role in either of these pathways, consistent with data showing no effect on another AMP, *defensin*, when phagocytosis of *S. aureus* is decreased [14]. It appears that *polyph*'s primary role is modulating the cellular immune response.

polyph Flies Exhibit Increased ROS and Decreased Bead Phagocytosis when Exposed to *S. aureus*

According to HomoloGene, *polyph* is closely related to *eaat1*, a glutamate transporter in the central nervous system [15, 16]. Eaats transport extracellular glutamate into the cell using a sodium/potassium exchange system [17]. The other major branch of glutamate transporters, the X^-_c transporters, exchanges a cystine for a glutamate in a sodium-independent process. The direction of exchange is concentration dependent [18]. Normally, there is more glutamate inside the cell than outside, with the inverse being true for cystine, thus causing the antiporter to exchange an intracellular glutamate for an extracellular cystine. Once inside the cell, cystine is reduced to cysteine, the limiting reagent in glutathione (GSH) synthesis [4, 18]. GSH acts as a major antioxidant in the cell by reducing reactive oxygen species (ROS) [19]. Therefore, we measured ROS levels in *polyph* flies using the substrate CM-H2CDFDA, which becomes fluorescent when oxidized. When flies were first injected with PBS, followed by the substrate, *polyph* showed no significant difference in ROS levels as compared to the wild-type. However, when flies were first exposed to *S. aureus* and then injected with the substrate, *polyph* flies made significantly more ROS than did wild-type files (Figure 3A). For determination of whether exposure to

act to directly kill microbes or limit their growth. *S. aureus* infection strongly induces *drosomycin*, while *E. coli* causes a comparatively weak induction. The opposite is true with *diptericin*. When infected with either *S. aureus* or *E. coli*, *polyph* flies showed normal induction of both *drosomycin* and *diptericin*, which are primarily upregulated by the Toll and Imd pathways, respectively [12, 13] (Figures S2D and S2E). Hence,
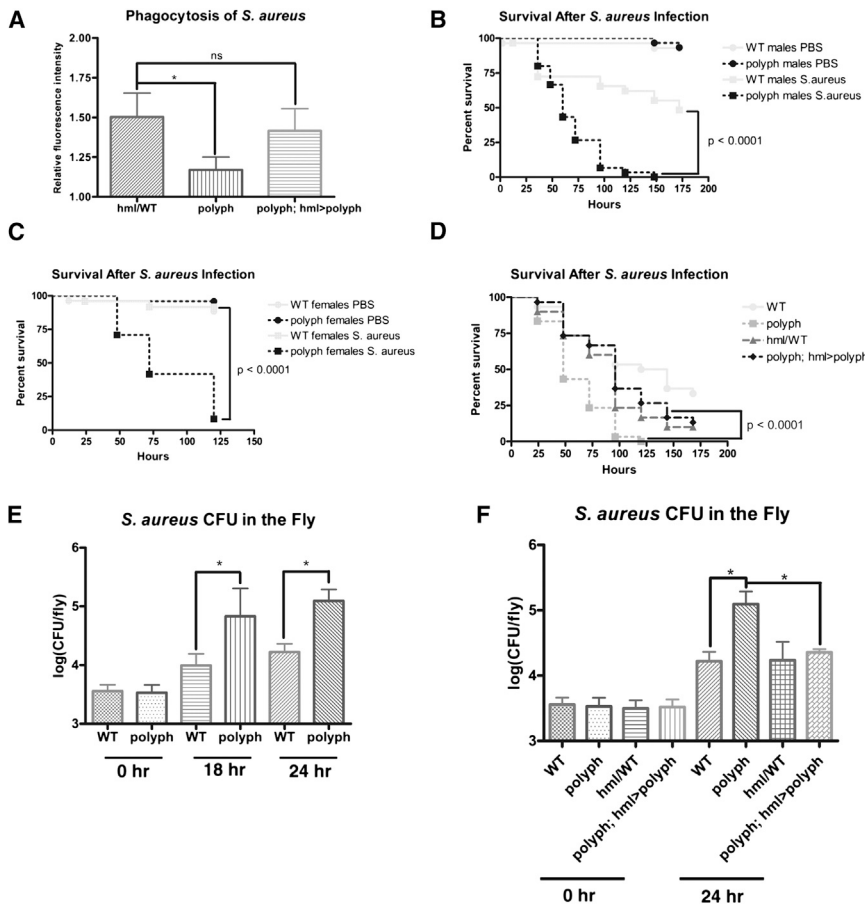
Figure 2. *polyph* Flies Have Decreased Resistance against an *S. aureus* Infection

(A) Quantification of the phagocytosis of fluorescein-labeled *S. aureus* bioparticles in *hml*/WT, *polyph,* and *polyph; hml>polyph* flies. Approximately six flies per genotype were used in each experiment.

(B and C) Representative survival curves of male (B) and female (C) WT and *polyph* flies after injection of *S. aureus* (optical density [OD] 0.5). n = 24–30 flies. Experiments were performed in triplicate.

(D) Representative survival curve of WT, *polyph, hml*/WT, and *polyph; hml>polyph* flies after injection of *S. aureus* (OD 0.5). The WT flies have *polyph*'s genetic background, while *hml*/WT flies have the same genetic background as the rescue flies in which *polyph* expression is driven by *hml*ΔGAL4. n = 28–30 flies. Experiments were performed in triplicate.

(E) Comparison of the *S. aureus* (OD 0.5) recovered in WT and *polyph* flies 0, 18, and 24 hr postinfection. Bacterial load was measured in eight individual flies per genotype at each time point in each experiment. The experiment was performed in quadruplicate.

(F) Comparison of the *S. aureus* (OD 0.5) recovered in WT, *polyph*, *hml*/WT, and *polyph; hml>polyph* flies 0 and 24 hr postinfection. Three replicate experiments were performed.

Error bars indicate ±SE. *p < 0.05; ns, not significantly different.

S. aureus causes an increase of ROS specifically in *polyph* blood cells, larval hemolymph was bled into PBS and incubated with CM-H2CDFDA and *S. aureus* ex vivo. These experiments were done with larval-derived hemolymph because of the difficulty collecting adult hemocytes poses. At 30 min, there was no difference between ROS levels in wild-type and *polyph* flies, indicating that *polyph* flies do not have a higher basal level of ROS. However, after 90 min, the ROS levels in *polyph* flies were significantly higher than that in wild-type flies (Figure 3B).

The increase in ROS corresponds with a decrease in *polyph* flies' phagocytic capacity upon exposure to *S. aureus*. When flies are injected with PBS and then latex beads, *polyph* flies show a phagocytic capacity equal to wild-type flies. However, when flies were preinjected with *S. aureus*, *polyph* flies take up significantly less latex beads than wild-type flies (Figure 3C). To examine whether *polyph* flies have a more limited phagocytic capacity that is overwhelmed by the double injection of bacteria and beads, we injected flies with yellow/green fluorescent beads and then red fluorescent beads. *polyph* flies were able to uptake the red beads in a manner indistinguishable from wild-type flies (Figure S3E). Hence, *polyph* flies do not have a more limited phagocytic capacity but instead become unable to phagocytose upon exposure to *S. aureus*.

To confirm that glutamate metabolism and ROS affect phagocytosis, we examined the role of glutamine synthetase-1 (GS1). GS1 converts glutamate and ammonia into glutamine and regulates ROS production by decreasing glutamate levels [20, 21]. *Drosophila* GS1 also regulates ROS, as flies

expressing *gs1* RNAi in blood cells have more ROS after infection with *S. aureus* (Figure S3F). *gs1* flies have impaired phagocytosis of *S. aureus* like *polyph* (Figure 3D), confirming that glutamate misregulation and increased ROS are associated with decreased phagocytosis.

polyph Flies Are Less Susceptible to the Intracellular Pathogen *Listeria monocytogenes*

L. monocytogenes is a facultative intracellular pathogen capable of invading phagocytic cells and subsequently escaping from the phagosome [22]. The bacteria then successfully replicate within the phagocyte and spread to other cells throughout the organism. Interestingly, *polyph* flies are less susceptible to an *L. monocytogenes* infection as compared to wild-type flies (Figure 3E). It is possible that the decreased phagocytosis and increased ROS result in reduced bacterial loads, allowing *polyph* flies to fight off infection more effectively than wild-type flies.

Phagocytosis Is Affected when Amino Acid Transport Is Either Blocked or Modulated in Blood Cells

As normal glutamate transport is essential for healthy nerve function, multiple drugs have been developed to modulate glutamate transport. Threo-beta-hydroxyaspartate (THA), an aspartate analog, acts as a general inhibitor of eaat transporters [4]. Hemolymph was collected from wild-type larvae, and after addition of either PBS or THA, the blood cells were assayed for uptake of *S. aureus*. Blood cells incubated with THA showed significantly less phagocytosis than the blood cells in PBS (Figure 4A), indicating that blocking glutamate transport in wild-type blood cells inhibits phagocytosis. This was supported by measurement of the phagocytic capacity
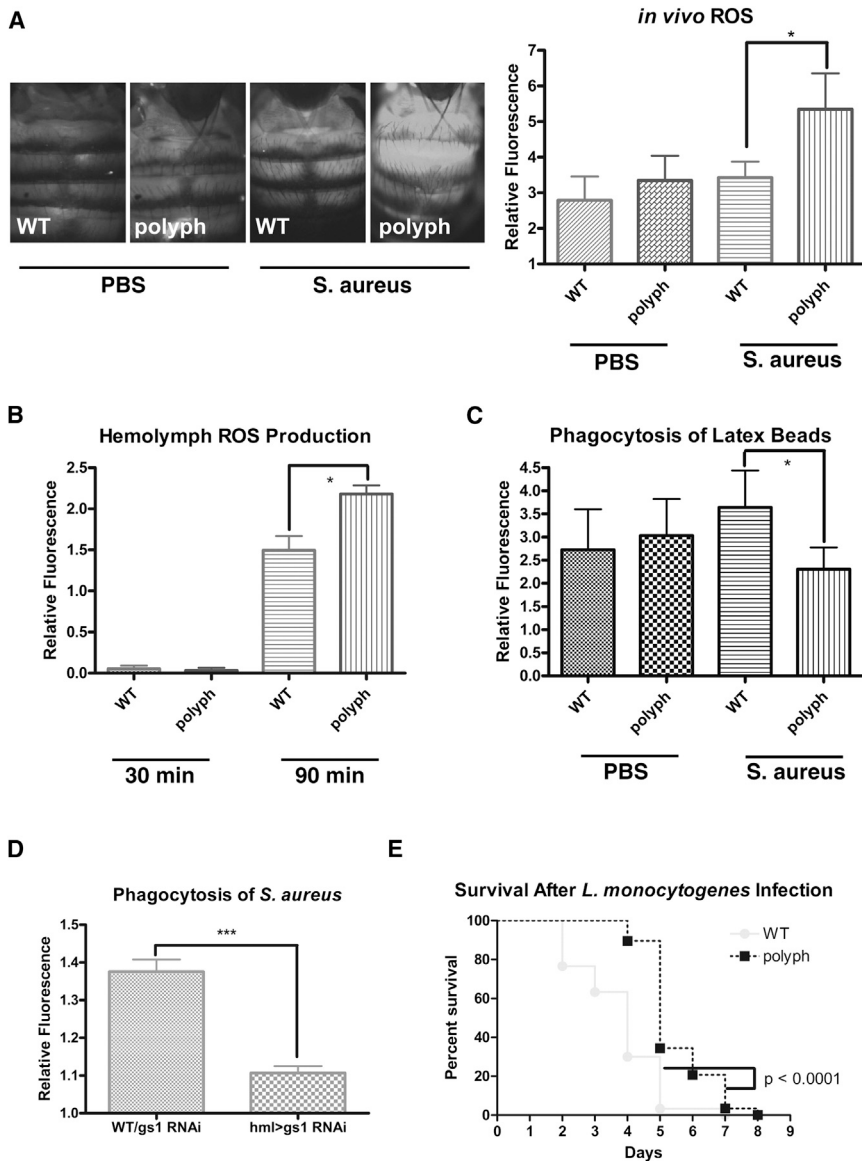
A

B

C

D

E

Figure 3. *polyph* Has Increased ROS in Its Hemolymph and Decreased Phagocytosis when Exposed to Bacteria

(A) Representative pictures of oxidized CM-H2CDFDA-derived fluorescence in WT and *polyph* flies after a 30 min preinjection of either PBS or overnight culture of *S. aureus*. Approximately six flies were used per genotype in each experiment. Quantification follows.

(B) Measurement of oxidized CM-H2CDFDA-derived fluorescence in WT and *polyph* blood cells incubated ex vivo with an overnight *S. aureus* culture.

(C) Quantification of the phagocytosis of red fluorescently labeled latex beads in WT and *polyph* flies after a 30 min preinjection of either PBS or overnight culture of *S. aureus*. Approximately six flies per genotype were used in each experiment.

(D) Quantification of the phagocytosis of fluorescein-labeled *S. aureus* bioparticles in WT/*gs1* RNAi and *hml>gs1* RNAi. Approximately six flies per genotype were used in each experiment.

(E) Representative survival curve of WT and *polyph* flies after injection of *L. monocytogenes* (OD 0.1). n = 28–30 flies.

All experiments were done in triplicate. Error bars indicate ±SE. *p < 0.05.

of cells in minimal media with glutamate, cystine, or both added. Cells showed significantly less phagocytosis when incubated with only glutamate and consistently more (albeit not significant, p = 0.061) when given cystine (Figure 4B). The base minimal media contained an extremely small amount of cystine (0.099 mM) and no glutamate. When excess glutamate was added extracellularly, this mimicked the presumed situation of *polyph* cells. By artificially forcing a high [glutamate]$_{extracellular}$ and a low [glutamate]$_{intracellular}$, this drives the glutamate/cystine antiporter to pump glutamate into the cell and cystine out, thereby depleting the cells' store of cystine and preventing them from making GSH. Without their ability to counteract bacteria-induced ROS, it is possible that the cells become unable to phagocytose efficiently. In contrast, when cells are given high levels of cystine, this drives the antiporter in the canonical direction, allowing the cell to continually make more GSH. These data indicate that glutamate transport into blood cells is a tightly regulated process that is necessary for blood cells to remain effective against an infection.
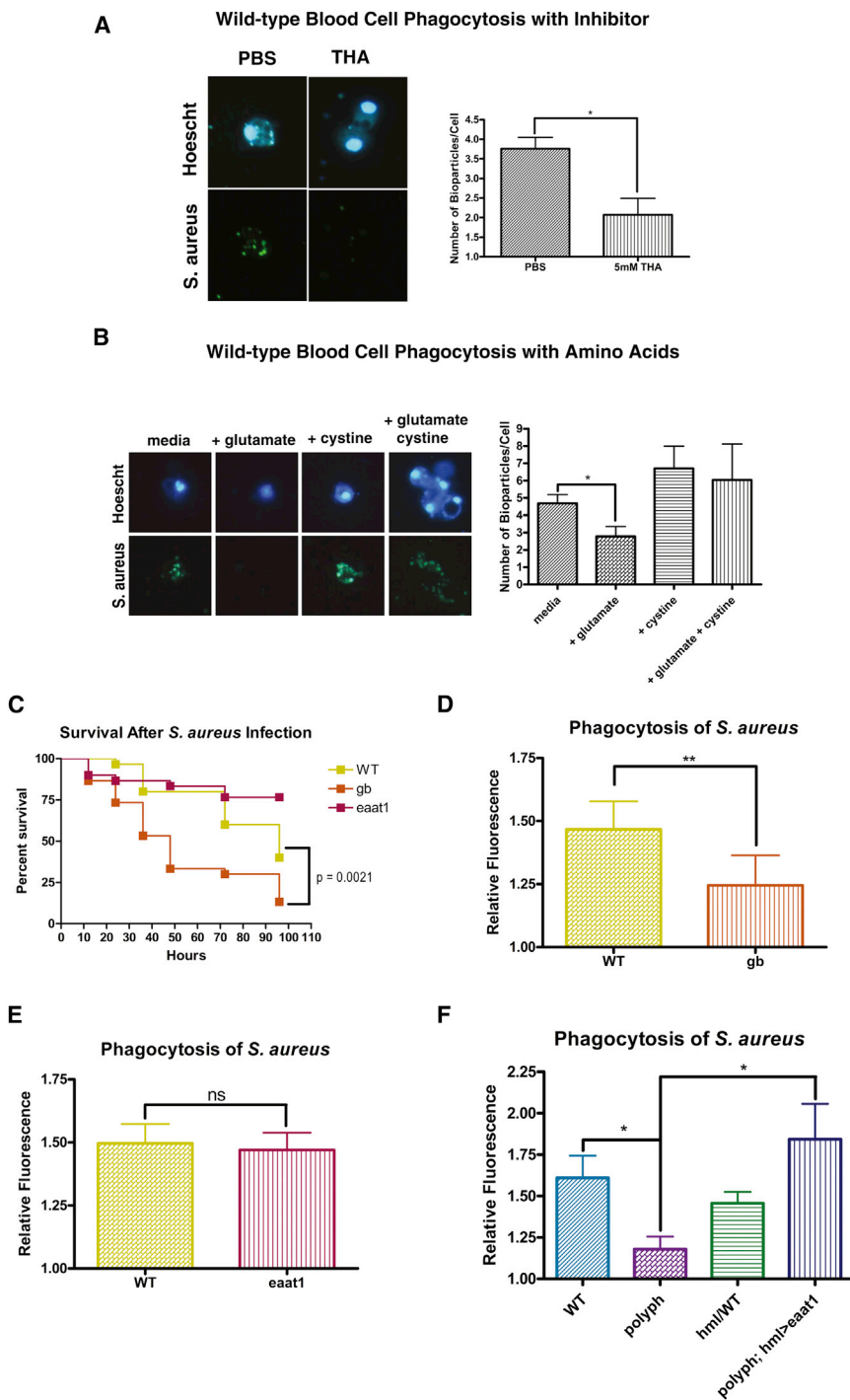
Other Amino Acid Transporters, when Expressed in Blood Cells, Play a Similar Role in the Immune Response as *polyph*

Glutamate, an important neurotransmitter, plays a complicated role in glutamate toxicity, a phenomenon where neurons become sick from increased intracellular ROS due to excess extracellular glutamate [23, 24]. The glutamate/cystine antiporter is concentration dependent, so if *eaat1* is functioning properly, there is more intracellular glutamate than extracellular, driving glutamate out and cystine in [18]. However, if *eaat1* or the glutamate/cystine antiporter, *genderblind* (*gb*), is mutated, then cystine is no longer transported in, GSH is limited, and ROS rises. The role of *gb* in glutamate transport and GSH synthesis is well established [25]. *gb* larvae had significantly less glutamate in their hemolymph compared to wild-type larvae [26]. However, the role of *gb* in an immunological response was unknown. We examined whether *eaat1* or *gb* was expressed on blood cells and whether mutants showed phenotypes similar to *polyph*.

gb (Figure S1C), but not *eaat1* (Figure S1D), was expressed in blood cells. Consistent with this, *gb* mutants showed both increased susceptibility to *S. aureus* infection and decreased phagocytosis of *S. aureus*, whereas *eaat1* mutants did not (Figures 4C–4E). Conversely, to determine whether *polyph* plays a role in the CNS, we performed a climbing assay. When knocked down, flies will instinctively climb upward. *eaat1* mutants have a motor defect due to inefficient glutamate transport in their CNS, and they climb more slowly than wild-type flies [15]. This motor defect was observed in *eaat1* but not in *polyph* flies (Figure S3D),

A
Wild-type Blood Cell Phagocytosis with Inhibitor

B
Wild-type Blood Cell Phagocytosis with Amino Acids

C

D

E

F

Figure 4. Survival and Phagocytosis Are Affected when Blood Cell Amino Acid Transport Is Modulated

(A and B) Wild-type blood cells in hemolymph were incubated ex vivo with fluorescein *S. aureus* bioparticles in PBS or 5 mM THA (A) or media plus nothing, glutamate, cystine, or glutamate and cystine (B). After 30 min, the number of bioparticles/cell was counted in approximately 20 cells per group in each experiment.

(C) Representative survival curves of WT, *gb*, and *eaat1* flies after injection of *S. aureus* (OD 0.05). n = 30 flies.

(D–F) Quantification of the phagocytosis of fluorescein-labeled *S. aureus* bioparticles in WT and *gb* flies (D), WT and *eaat1* flies (E), and WT, *polyph*, *hml*/WT, and *polyph; hml>eaat1* flies (F).

Approximately six flies per genotype were used in each experiment. All experiments were performed at least in triplicate. Error bars indicate ±SE. *p < 0.05; **p < 0.01; ns, not significantly different.

S1) indicates that *polyph* flies have increased extracellular glutamate in their hemolymph due to an inability to effectively transport it into the cell. This leads to a decrease in GSH and an increase in internal ROS. Without tight regulation of glutamate transport across the blood cell membrane, the fly loses its resistance to a pathogenic infection due to a decrease in phagocytic capacity.

With an unbalanced GSH redox system, there is potential for human disease. People with a glutathione reductase deficiency have impaired neutrophil function. When the antioxidant GSH reduces ROS, it is converted into its oxidized state, glutathione disulfide (GSSG). Glutathione reductase facilitates conversion of GSSG back to its reduced state, GSH. Without the enzyme, the redox system skews toward the oxidized state and is less able to reduce free reactive species. Neutrophils from individuals with the genetic disorder could maintain normal levels of ROS for a short period of time, but not after an extended incubation with bacteria

indicating that *polyph* does not play a major role in the nervous system.

If Polyph is functioning in a similar manner to Eaat1 but in a different tissue, then blood-cell-specific expression of *eaat1* in a *polyph* mutant background should rescue the phenotypes seen in *polyph* flies. There is indeed strong rescue of the phagocytosis phenotype in *polyph*; *hml>eaat1* flies (Figure 4F). These data indicate that Polyph is an important glutamate transporter expressed on blood cells. It works with the cystine/glutamate antiporter Gb to control internal ROS in order to maintain macrophage function. Our model (Movie

[27]. These findings support our model and show that understanding the delicate balance of this system has relevance to human disease. Additionally, increased glutamate levels in the plasma have been associated with other diseases, including amyotrophic lateral sclerosis [28], epilepsy [29], stroke-associated headache [30], HIV-associated dementia [31], Parkinson's disease [32], breast cancer, colorectal carcinoma, and AIDS [33]. Our results demonstrate that a tightly regulated redox system in the blood cells is necessary to maintain immune cell function and therefore for the health of the whole organism.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.09.061.

Acknowledgments

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