Nephrocytes remove microbiota-derived peptidoglycan from systemic circulation to maintain immune homeostasis

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Highlights

Nephrocytes prevent Toll pathway activation in response to microbiota

Nephrocytes uptake microbiota-derived peptidoglycan and degrade it inside lysosomes

Accretion of peptidoglycan and Toll pathway activation follow the loss of nephrocytes

Lack of nephrocytes results in increased resistance to infection and shorter lifespan

Keywords

Peptidoglycan scavenging, Toll pathway, microbiota, nephrocytes, podocytes, kidney, reticuloendothelial system, blood filtration

SUMMARY

Preventing aberrant immune responses against the microbiota is essential for organismal health. Here, we examined the role of hemolymph (insect blood) filtration in regulating systemic responses to microbiota-derived PAMPs. *Drosophila* deficient for the transcription factor *Klf15* are viable but lack nephrocytes—a cell type with molecular, anatomical, and functional similarity to the glomerular podocyte of the vertebrate kidney. *Klf15* mutants displayed constitutively elevated Toll pathway activity, and as a consequence, were both shorter-lived and more resistant to infection. We found that nephrocytes uptake Lys-type peptidoglycan from systemic circulation and target it to lysosomes. Without nephrocyte function, microbiota-derived peptidoglycan accumulated in circulation, triggering Toll pathway activation in the absence of infection. These results unveil a role for insect blood filtration in the maintenance of immune homeostasis in the presence of microbiota and identify a potential root cause for the chronic immune activation observed in animals suffering from impaired blood filtration.

INTRODUCTION

As the first line of defense against invading microorganisms, the innate immune system senses and responds to both pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Kim Newton, 2012). Peptidoglycan, a major constituent of the microbial cell wall, is an immune-stimulatory PAMP found in all bacteria, pathogenic or not. Following infection, detection of PAMPs in systemic circulation triggers a cascade of immune reactions that can ultimately lead to sepsis (Cecconi et al., 2018). Even in the absence of infection, organisms do not live in a sterile environment. For instance, the animal gut harbors numerous microbial species, which constitute the microbiota. A consequence of the presence of microbiota is the translocation of microbiota-shed PAMPs from the gut lumen into systemic circulation. This is a phenomenon that has been documented in a variety of organisms, including both *Drosophila* and mammals (Capo et al., 2017; Clarke et al., 2010; Corbitt et al., 2013; Gendrin et al., 2009; Paredes et al., 2011; Zaidman-Remy et al., 2006). Considering that microbiota-derived PAMPs, such as peptidoglycan, are found in circulation, regulating their systemic concentration is critical to the maintenance of immune homeostasis. The kidneys are key regulators of blood composition. Therefore, we hypothesized that they could play a major role in regulating systemic immune responses to circulating, microbiota-derived PAMPs. Here, we examined this hypothesis using the *Drosophila melanogaster* nephrocyte as a model.

Drosophila is a powerful system to study innate immune responses to both pathogens and gut microbes (Buchon et al., 2014; Liu et al., 2017). To resist infection, *Drosophila* relies on both cellular and humoral innate immune responses. The cellular response consists of encapsulation and phagocytosis, while the humoral response involves the melanization cascade and the synthesis

of antimicrobial peptides (AMPs) by the fat body (an organ analogous to the liver and adipose tissue of mammals). Production of AMPs is controlled by two principal signaling cascades, the Toll and Imd pathways. Both pathways are activated in response to peptidoglycan (PGN): Lys-type PGN from Gram-positive bacteria triggers the Toll pathway, while DAP-type PGN from Gram-negative bacteria and certain Gram-positive bacilli induces the Imd pathway (Buchon et al., 2014). In the fly, peptidoglycan is detected by peptidoglycan recognition proteins (PGRPs). PGRP-LC and PGRP-LE sense DAP-type PGN, and PGRP-SA recognizes Lys-type PGN (Kaneko et al., 2006; Michel et al., 2001). Additionally, *Drosophila* possesses several immune mechanisms to both shape the microbiota and prevent excessive immune responses upon detection of microbial stimuli (Basbous et al., 2011; Cao et al., 2013; de Gregorio et al., 2002; Gordon et al., 2008; Levashina et al., 1999; Maillet et al., 2008; Scherfer et al., 2008; Thevenon et al., 2009). In the case of the Imd pathway, these mechanisms include negative regulators that avert excessive immune activation in response to the microbiota. For instance, the transcription factor Caudal suppresses Imd-dependent expression of AMPs in the gut, thereby shaping microbiota composition (Ryu et al., 2008). Another regulator, Pirk, sequesters PGRP-LC to prevent its exposure to microbiota-derived PGN and subsequent activation of the Imd pathway (K. Aggarwal et al., 2008; Kleino et al., 2008; Lhocine et al., 2008). Finally, secreted PGRPs with amidase activity scavenge and degrade immunostimulatory DAP-type PGN in order to block Imd activation (Paredes et al., 2011). A notable example of this is PGRP-LB, which is released into the hemolymph to degrade translocated, microbiota-derived PGN, thus preventing systemic immune activation (Paredes et al., 2011; Zaidman-Remy et al., 2006). Although the Toll pathway also responds to the presence of peptidoglycan, little is known about the mechanisms that suppress Toll activation in response to microbiota.

The excretory system of *Drosophila* is composed of nephrocytes, which regulate hemolymph (extracellular fluid analogous to blood) composition by filtration followed by filtrate endocytosis, and Malpighian tubules, which modify and secrete urine (Denholm et al., 2009; Hartley et al., 2016). *Drosophila* nephrocytes can be divided into two distinct groups: the garland cells, which appear as a necklace-like structure surrounding the esophagus, and the pericardial cells that form two rows of cells flanking the heart (S. K. Aggarwal and King, 1967; Crossley, 1972; Na and Cagan, 2013). In the adult stage, pericardial nephrocytes serve as the primary filtration units (Zhang et al., 2013). Hemolymph filtration occurs in a stepwise manner. First, hemolymph is filtered across the nephrocytes' negatively charged basement membrane and a specialized filter known as the nephrocyte diaphragm. The filtrate then enters the lacunae, also known as the labyrinthine channels, which extend several microns into the nephrocyte's cortical region (Kosaka and Ikeda, 1983). It is in these chambers where the filtrate is finally endocytosed by nephrocytes (Denholm et al., 2009). Nephrocytes possess significant molecular, anatomical, and functional similarities to the glomerular podocyte, a cell type of the mammalian kidney important for the kidney's filtration function (Weavers et al., 2009; Zhuang et al., 2009). Both podocytes and nephrocytes possess a slit diaphragm and act as size- and charge-selective filters in the sequestration of materials from the blood and hemolymph (Reiser and Altintas, 2016; Weavers et al., 2009). The *Drosophila* ortholog of mammalian *Klf15*, a transcription factor required for podocyte differentiation (Mallipattu et al., 2012), is restricted to and essential for nephrocyte differentiation and function (Ivy et al., 2015). Flies mutant for *dKlf15* are viable but lack both garland and pericardial nephrocytes in the adult, making it an excellent tool to study the impact of hemolymph filtration on immune function (Ivy et al., 2015).

Here, we examined the role of blood (hemolymph) filtration by the fly's podocytes in the maintenance of immune homeostasis. We found that flies devoid of nephrocytes (*dKlf15* mutants), or with diminished nephrocyte function, were more resistant to a variety of microbial infections. Flies mutant for *dKlf15* exhibited improved survival upon infection but also a shorter lifespan stemming from abnormal Toll pathway activation. Aberrant Toll signaling in *dKlf15* mutant flies was dependent on the presence of microbiota carrying Lys-type peptidoglycan. Microbiota-derived PGN accumulated in the hemolymph of these flies, triggering chronic stimulation of the Toll pathway. In wildtype flies, microbiota-derived Lys-type peptidoglycan found in systemic circulation was taken up by nephrocytes via endocytosis and degraded within lysosomes. Thus, renal filtration of microbiota-derived peptidoglycan maintains immune homeostasis in *Drosophila*, a function likely conserved in mammals.

RESULTS

Klf15 mutants are less susceptible to microbial infection

In order to evaluate the role of hemolymph (analogous to mammalian blood) filtration in immune function and homeostasis, we turned to flies mutant for the transcription factor *Klf15* (*Klf15*^{NN} null allele), which lack nephrocytes. First, we confirmed the previously published result that *Klf15* mutants fail to develop nephrocytes (Figure 1A) (Ivy et al., 2015). Our data also showed that *Klf15* mutants exhibited a significantly shorter basal lifespan compared to wildtype (WT) controls of the same genetic background (Figure 1B). Despite having a curtailed life expectancy, *Klf15* mutants survived sterile wounding comparably to WT animals (Figure 1C-1G). To determine whether immune competence was affected by the loss of hemolymph filtration, we conducted survival

assays with *Klf15* mutants following systemic infection with the bacterial pathogens *Serratia marcescens* Type strain, *Salmonella typhimurium*, *Listeria innocua*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Providencia rettgeri*. *Klf15* mutants displayed significantly increased survival against five of these infections (Figures 1C-1G). The only exception was infection with *P. rettgeri*, to which the mutant proved more sensitive (Figure S1A). We did not observe a survival phenotype following challenge with two fungal agents, *Metarhizium anisopliae* and *Beauveria bassiana* (Figures S1B-S1C). Overall, these results suggest that *Klf15* mutants are broadly protected against systemic infection by bacterial pathogens.

To verify that the enhanced survival observed in the *Klf15* mutant was a direct consequence of the loss of nephrocytes, we generated nephrocyte-deficient flies through complementary means. Using the nephrocyte-specific driver *Dot-Gal4*, we knocked down *Klf15* expression throughout development (*Dot-Gal4 > UAS-Klf15-IR*), which results in adult flies lacking nephrocytes (Ivy et al., 2015). Upon infection with *S. aureus* and *E. faecalis*, these flies also displayed increased survival relative to the WT controls, confirming our earlier findings (Figure 1H and Figure S1D). Next, we set out to determine whether the survival phenotype of *Klf15* mutants resulted from loss of hemolymph filtration in the mutant or from a developmental defect associated with the loss of nephrocytes. To distinguish between the two possibilities, we took advantage of the fact that adult-specific loss of *Klf15* halts the endocytic function of mature nephrocytes (Ivy et al., 2015). We knocked down *Klf15* specifically during the adult stage using the conditional, nephrocyte- and heart-specific driver *Hand-Gal4^{ts}* (*Hand-Gal4^{ts} > UAS-Klf15-IR*) and infected these flies separately with *S. aureus* and *E. faecalis*. Diminishing the endocytic competence of adult nephrocytes by *Klf15* knockdown was sufficient to increase survival to infection with both bacteria

(Figure 1I and Figure S1E). Altogether, our results support the conclusion that loss of nephrocyte function generally increases survival against microbial infection.

Klf15 mutants are more resistant to infection, independent of phagocytosis and melanization

Multicellular organisms employ two complementary strategies to combat infection: resistance, to eliminate microbes, and disease tolerance, to allow them to withstand the infection and/or its deleterious consequences (Ayres and Schneider, 2011). To determine whether the improved survival of *Klf15* mutants was due to an increase in disease tolerance, we compared the *BLUD* of WT and *Klf15* mutants following infection with *S. aureus* and *E. faecalis*. *BLUD*, which stands for bacterial load upon death, represents the maximal quantity of bacteria that an infected fly can sustain before it dies (Duneau et al., 2017), and is therefore one measure of disease tolerance. We found that control flies and *Klf15* mutants die carrying similar numbers of each bacterium tested (Figure S2A), indicating that this marker of disease tolerance is not altered in the mutant. Next, we tested whether the survival advantage of the mutant stemmed from improved resistance to infection. We monitored bacterial load during the course of *S. aureus*, *E. faecalis*, *S. marcescens*, *L. innocua*, and *S. typhimurium* infections (Figure 2A-2B and Figure S2B-D). *Klf15* mutants carried significantly lower bacterial burdens than wildtype flies as soon as 3 h post-infection with *S. aureus*, 4.5 h after challenge with *E. faecalis*, and 6 h post-inoculation with *S. marcescens*, *L. innocua*, and *S. typhimurium*, demonstrating that flies without nephrocytes are more resistant to pathogens in the early stages of infection.

Drosophila relies primarily on three effector mechanisms to control bacterial growth: phagocytosis, melanization, and the production of AMPs. First, we evaluated a role for phagocytosis in the resistance phenotype of the *Klf15* mutant. We injected nephrocyte-deficient and WT flies with pH-sensitive pHrodo bacteria, which become fluorescent only after being engulfed into a fully mature, acidified phagosome (Guillou et al., 2016). After quantification, we observed close to 50% less fluorescence in *Klf15* mutant flies relative to controls (Figure S2E). Moreover, injection of flies with latex beads prior to systemic infection with both *S. aureus* and *E. faecalis*, a treatment that blocks phagocytosis (Elrod-Erickson et al., 2000), did not alter the survival phenotype of *Klf15* mutants (Figure S2F-S2G). These results demonstrate that phagocytic activity does not contribute meaningfully to the increased resistance of *Klf15* mutant flies. Assessment of phenoloxidase (PO) activity, the terminal enzymatic step driving melanization, revealed that while *Klf15* mutants had significantly higher PO activity in basal conditions, they also displayed significantly lower PO activity relative to controls 3 h post-infection with *S. aureus* and *E. faecalis* (Figure S2H). To clarify whether melanization played any role in the survival phenotype of *Klf15* mutants, we generated a mutant deficient for both *Klf15* and key genes required for the melanization response ($PPO1^{\Delta}$, 2^{Δ},3^{1}) (Binggeli et al., 2014; Dudzic et al., 2015). Upon infection with *S. aureus*, the quadruple mutant (*Klf15* ; $PPO1^{\Delta}$, 2^{Δ},3^{1}) exhibited improved survival relative to the triple mutant ($PPO1^{\Delta}$, 2^{Δ},3^{1}) (Figure S2I), suggesting that melanization is not required for the protection observed in nephrocyte-deficient flies. In sum, our data indicate that loss of nephrocytes confers increased resistance to hosts independent of phagocytosis and melanization.

The Toll pathway is constitutively active in *Klf15* mutants

Nephrocytes are major regulators of hemolymph (insect blood) content via filtration followed by filtrate endocytosis (Hartley et al., 2016; Soukup et al., 2009). Therefore, we considered whether changes in circulating proteins in the mutant could account for the increased resistance observed in *Klf15* mutant flies. Previously, we performed a proteomic analysis of hemolymph composition in both WT and *Klf15* unchallenged flies (Hartley et al., 2016). An in-depth analysis of this dataset revealed that amongst 130 proteins enriched (≥ 1.5-fold) or detected only in the hemolymph of nephrocyte-deficient mutants, 65 proteins were annotated as having an immune-related function (Figure 2C). All 65 proteins were predicted to have a signal sequence (SignalP 4.1), which is expected for secreted hemolymph proteins. Of these 65 proteins, we found that 19 are encoded by core genes of the *Drosophila* immune response (i.e., genes with increased transcription in response to most bacterial infections, see (Troha et al., 2018)), 30 are the products of genes that are induced only by a subset of microbial infections, and 16 are coded by genes that, while not regulated in response to infection themselves, have been ascribed an immune function. Interestingly, we also noted that a majority (33 of 65) of these proteins are known targets of the Toll pathway (e.g., the antimicrobial peptide genes *IM2*, *IM23*, and *CG15067*). Our data therefore established that the hemolymph of *Klf15* mutants is enriched in proteins of immune function primarily encoded by target genes of the Toll pathway and suggested that changes in Toll pathway activity could explain the increase in resistance to pathogens observed in *Klf15* mutant flies.

We developed two competing hypotheses to explain the accumulation of Toll pathway targets in the hemolymph of *Klf15* mutants. The first hypothesis posited that because nephrocytes are critical regulators of protein turnover in the hemolymph, the rise in immune effectors could be

the result of a decrease in protein turnover in these flies. Alternatively, the accumulation of immune gene products could be due to aberrant activation of the Toll and/or Imd pathways in nephrocyte-deficient flies. In agreement with the latter hypothesis, our proteomic analysis also identified proteins that were depleted (≥ 1.5-fold) in the hemolymph of *Klf15* mutants relative to controls (Figure S2J). Six of these proteins are encoded by genes that typically show decreased transcription in response to bacterial infection in a Toll-dependent manner (e.g., *Lsp1β* and *CG2233*) (Troha et al., 2018), arguing that changes in hemolymph protein content are due to Toll pathway activation rather than protein turnover. To test this idea directly, we surveyed the activation of the Toll and Imd pathways by measuring the mRNA expression of 5 Toll target genes and 4 Imd target genes in WT and *Klf15* mutant flies under basal conditions. RT-qPCR data from whole fly showed that the mRNA expression of all 5 Toll target genes—*IM2*, *CG15067*, *Drs*, *CG18067*, and *CG15293*—was significantly increased in *Klf15* mutants compared to controls (Figure 2D and Figure S2K). In contrast, we did not find any appreciable differences in gene expression between WT and *Klf15* mutants for the Imd target genes *Dpt*, *AttC*, and *TotA*; the exception was *AttD*, for which the mutant had significantly lower mRNA expression relative to WT (Figure 2E and Figure S2K). These data indicate that the Toll pathway, but not the Imd pathway, is constitutively activated in *Klf15* mutants in unchallenged conditions. In agreement with these data, we also detected abnormal Toll activation in flies in which *Klf15* was knocked-down by RNAi specifically during the adult stage (*Hand-Gal4^{ts} > UAS-Klf15-IR*), demonstrating that the loss of nephrocyte scavenging function is solely responsible for Toll activation in these flies (Figure 2F). In contrast to our results in unchallenged conditions, infection of *Klf15* mutants with either *S. aureus* or *E. faecalis* revealed no significant differences between WT and mutant in terms of Toll or Imd target gene expression at any of the time points surveyed (3, 8, and 12 h post-

challenge) (Figure S2L-S2M). In conclusion, our data establish that *Klf15* mutants present elevated basal mRNA expression of Toll target genes in conjunction with increased immune resistance to pathogens.

Increased pathogen resistance in *Klf15* mutants is contingent on higher baseline Toll activity

Next, we asked whether Toll pathway activity could be responsible for the increased resistance observed in *Klf15* mutant flies. We began by verifying that the increase in baseline Toll target gene expression was dependent on the Toll pathway itself. RT-qPCR of the Toll target genes *IM2* (Figure 3A), *CG15067,* and *Drs* (Figure S3A-S3B) showed that a null mutation in the gene coding for the Toll cytokine Spz completely abolished the increase in Toll target gene expression found in *Klf15* mutants (*Klf15* ;; spz^{rm7} double mutants). This was also true for a null mutation in the gene coding for SPE (*Klf15* ;; SPE^{SK6}), a key enzyme involved in the maturation of Spz and subsequent activation of the Toll pathway (Figure S3C-S3E), demonstrating that the increase in Toll target gene expression in *Klf15* hosts is due to elevated Toll pathway activity. Notably, suppression of the Toll pathway by either spz^{rm7} or SPE^{SK6} completely abrogated the survival advantage of *Klf15* mutants against pathogenic infection (Figure 3B-3C and Figure S3F-S3G). These results indicate that a surge in Toll pathway signaling is directly accountable for the augmented resistance of *Klf15* flies to infection.

The Toll pathway can be induced by endogenous DAMPs, which trigger the maturation of the circulating serine protease Persephone (Psh), or by the recognition of PAMPs, leading to the activation of the serine protease ModSP (Buchon et al., 2009; Gottar et al., 2006; Ming et al.,

2014). Consequently, we set out to investigate whether aberrant Toll signaling in *Klf15* mutants was dependent on the detection of DAMPs or PAMPs by the host. While a null allele of *psh* was unable to rescue the elevated basal expression of the Toll target genes *IM2* (Figure 3D), *CG15067*, and *Drs* (Figure S3H-S3I) in *Klf15* mutants (*Klf15* , psh^{1}), a null mutation in *modSP* fully reverted this increase (*Klf15* ;; $modSP^{1}$) (Figure 3G and Figure S3J- S3K). Accordingly, while the improved survival phenotype of the *Klf15* mutant was still present in *Klf15*, psh^{1} flies (Figure 3E-3F), *Klf15* ;; $modSP^{1}$ flies no longer exhibited it (Figure 3H-3I). Thus, our data support the conclusion that elevated Toll signaling in response to PAMPs is responsible for the *Klf15* phenotype.

ModSP activity, and therefore Toll pathway signaling, can be induced by the binding of pattern recognition receptors (PRRs) to two types of PAMPs: β-(1,3)-glucan derived from the fungal cell wall is recognized by GNBP3 (Gottar et al., 2006), while bacterial peptidoglycan (PGN) is detected by PGRP-SA (Michel et al., 2001). Thus, we moved to resolve whether the increase in Toll pathway activity observed in *Klf15* mutants was due to sensing of PGN by PGRP-SA or β-(1,3)-glucan by GNBP3. RT-qPCR of the Toll target genes *IM2*, *CG15067*, and *Drs* demonstrated that the increase in basal Toll pathway signaling present in *Klf15* flies was downstream of PGRP-SA (*Klf15* , $PGRP\text{-}SA^{seml}$) (Figure 3J and Figure S3L-S3M) but not GNBP3 (*Klf15* ;; $GNBP3^{Hades}$) (Figure S3N-S3P). The enhanced survival phenotype of *Klf15* mutants was also lost in the double mutant *Klf15* , $PGRP\text{-}SA^{seml}$ (Figure 3K-3L), but not in the double mutant *Klf15* ;; $GNBP3^{Hades}$ (Figure S3Q-S3R), indicating that the surge in Toll signaling observed in nephrocyte-deficient flies is likely downstream of peptidoglycan recognition. Finally, bacterial load data from the double mutants *Klf15* ;; $modSP^{1}$ and *Klf15* , $PGRP\text{-}SA^{seml}$ infected with *S. aureus* or *E. faecalis* showed that in absence of a functional Toll pathway, *Klf15* mutants no longer display reduced

pathogen load compared to WT (Figure S3S-S3T), confirming that the increase in resistance observed in *Klf15* mutants is dependent on the Toll pathway.

Microbiota-derived PAMPs trigger aberrant Toll pathway activation in *Klf15* mutants

Gut microbes are a source of PAMPs, such as peptidoglycan, and therefore can act as elicitors of the immune system (Clarke et al., 2010; Kaneko et al., 2004). In *Drosophila*, multiple mechanisms are in place to prevent undue activation of the Imd pathway in response to microbiota. These include the expression of a plethora of negative regulators (e.g., Caudal and Pirk) and enzymes that degrade DAP-type PGN (e.g., PGRP-LB and PGRP-SC). However, no similar mechanism has been described for the Toll pathway despite the fact that it also senses PGN (Lys-type) (Bischoff et al., 2004; Park et al., 2007; Wang et al., 2006). Because the increase in Toll pathway activity in *Klf15* flies depends on PGRP-SA, we hypothesized that the phenotype could stem from an errant immune response against the microbiota. To test this idea, we used RT-qPCR to measure the mRNA expression of the Toll target genes *IM2*, *CG15067* (Figure 4A), and *Drs* (Figure S4A) in wildtype and *Klf15* mutants raised in both conventionally reared (CR) and germ-free (GF) conditions. We found that the increase in Toll signaling in *Klf15* mutants was fully dependent on the presence of microbiota, as GF wildtype and GF *Klf15* flies displayed similar mRNA expression for all measured Toll target genes. Since *Klf15* mutants did not have a higher microbiota load or show any alteration in gut barrier integrity—as determined by the SMURF assay and measurements of both circulating bacteria in the hemolymph and whole fly microbiota (Figure S4B-S4D)—our results indicate that *Klf15* mutants display aberrant Toll pathway activation in response to microbiota.

Because the increase in Toll pathway activity found in nephrocyte-deficient flies is both downstream of PGRP-SA and microbiota-dependent, we postulated that this phenotype could arise from an abnormal response to microbiota-derived PAMPs. In agreement with this hypothesis, mono-colonization of GF *Klf15* flies with the Gram-positive, Lys-type PGN-carrying microbes *E. faecalis* and *Lactobacillus brevis* (*L. brevis*, like many other *Lactobacilli* spp., carries Lys-Asp-type PGN as previously described (Salvetti et al., 2012; Schleifer and Kandler, 1972)) triggered aberrant Toll pathway activity, while recolonization with the Gram-negative, DAP-type PGN-containing *Acetobacter pomorum* did not (Figure 4A-S4A). Of note, *E. faecalis*, *A. pomorum*, and *L. brevis* are normal constituents of the *Drosophila* gut microbiota (Broderick et al., 2014). This result suggested that the microbiota could act to elevate Toll pathway signaling in nephrocyte-deficient flies by providing a source of Lys-type PGN, thus stimulating PGRP-SA in the absence of infection. Additional experiments confirmed that gut microbiota-derived PAMPs were sufficient to trigger the Toll pathway in GF *Klf15* mutants. Feeding GF *Klf15* hosts with heat-killed *L. brevis* or *E. faecalis*, but not *A. pomorum*, was enough to elicit abnormal Toll pathway activity as measured by *IM2*, *CG15067*, and *Drs* expression (Figure 4B and Figure S4E). Altogether, these results established that in *Klf15* mutants, gut microbiota-derived Lys-type PGN induces an errant, Toll pathway-mediated immune response.

Next, we explored whether this abnormal response to the microbiota could be responsible for the increase in resistance to infection observed in *Klf15* mutants. Unlike flies raised in CR conditions, GF *Klf15* flies infected with the bacterial pathogens *S. aureus* and *E. faecalis* did not exhibit increased survival to infection relative to GF wildtype controls (Figures 4C-4D). These

results suggest that microbiota-derived Lys-type PGN primes the Toll pathway in *Klf15* mutants, leading to enhanced resistance. Chronic immune activation is costly and harmful to hosts (Charroux et al., 2018; Guo et al., 2014; Paredes et al., 2011). As we noted that flies devoid of nephrocytes had a shorter lifespan (Figure 1B), we asked whether this could also be due to chronic immune activation in response to the microbiota. *Klf15* mutant flies reared in GF conditions significantly outlived their CR counterparts, and no difference in lifespan was found between *Klf15* and WT flies raised in GF conditions (Figure 4E). Our results demonstrate that nephrocytes are part of a program that prevents microbiota-dependent Toll pathway activation, thus avoiding its deleterious effect on lifespan.

Nephrocytes endocytose peptidoglycan from the hemolymph to avert excessive immune activation in response to microbiota

We find that the Toll pathway is significantly activated in response to microbiota-derived PAMPs in the absence of nephrocytes. This could be the result of either the presence of elevated amounts of microbiota-shed PGN in the hemolymph (as microbiota-shed PAMPs are commonly translocated from the gut lumen into systemic circulation) or the hyper-reactivity of these flies to PAMPs. SPE, a signaling component of the Toll pathway, accumulates in the hemolymph of *Klf15* mutants (Figure 2C) despite its mRNA not being transcriptionally increased (Figure S4I). Overexpression of SPE is also sufficient to trigger Toll pathway activation (Jang et al., 2006). Consequently, we hypothesized that accretion of SPE could result in an aberrant response to the microbiota in *Klf15* mutant flies. While overexpression of SPE alone resulted in increased expression of three target genes of the Toll pathway, *IM2*, *CG15067*, and *Drs* (Figure S4F-S4H), the mRNA expression was identical between CR and GF conditions, suggesting that this effect

was not dependent on the presence of microbiota. It is therefore unlikely that the microbiota-dependent induction of Toll in *Klf15* flies is due to SPE accumulation. In light of this result, we moved on to the next hypothesis. As nephrocytes regulate hemolymph composition by filtration followed by filtrate endocytosis, we reasoned that in the absence of nephrocytes, microbiota-derived Lys-type PGN could accumulate in the hemolymph. We therefore measured the amount of PGN circulating in the hemolymph of WT and *Klf15* mutants in both CR and GF conditions. Using a colorimetric assay (SLP), we detected three times more circulating PGN in *Klf15* mutants than in WT controls under CR conditions, with no difference found between the two genotypes under GF conditions (Figure 5A). These data establish that nephrocytes participate in the removal of microbiota-shed PGN from systemic circulation.

Subsequently, we focused on determining what mechanisms underlie nephrocyte-mediated PGN removal from hemolymph. Nephrocytes are filtration devices. Their surface is covered by extensive membrane invaginations, which are sealed at the top by slit diaphragms. These chambers, known as lacunae or labyrinthine channels, are where most of their endocytic activity takes place. Once endocytosed, internalized cargo is either trafficked to lysosomes for degradation, metabolized and released back into circulation, or stored in vacuoles for the lifespan of the fly (Denholm et al., 2009; Psathaki et al., 2018a). To assess whether nephrocytes internalize circulating PGN, we immunostained nephrocytes with an anti-PGN antibody (raised against PGN from a Gram-positive Streptococcus sp.). Confocal sectioning of nephrocytes revealed a strong punctate signal pattern, indicating that nephrocytes do indeed internalize PGN (Figure 5B, specifically 5B-1). PGN staining disappeared in flies reared in GF conditions, suggesting that nephrocytes take up microbiota-derived PGN in order to remove it from the hemolymph (Figure

5B-2, see Figure S5A for quantification). Immunostaining against PGN in nephrocytes expressing either a reporter for the early endosomal marker Rab5 (*Hand-Gal4^{ts} > UAS-Rab5-YFP*) or a reporter for the late endosomal marker Rab7 (*Hand-Gal4^{ts} > UAS-Rab7-YFP*) showed co-localization of PGN with both markers (Figure 5C, see Figure S5B for Pearson correlation coefficients). We also detected co-localization of PGN with the lysosomal markers cathepsin L and Lamp1 (Lysosomal associated membrane protein 1, Figure 5C, S5B, and S5C), implying that PGN is internalized by endocytosis and routed to the lysosomal compartment.

We then moved to evaluate the role of endocytosis in the uptake of PGN by nephrocytes. The dynamin Shibire is involved in the early steps of endocytosis. Blocking Shibire triggers elongation of the lacunae/labyrinthine channels within the nephrocytes (Figure 5D, Duf labels the lacunae) (Kosaka and Ikeda, 1983; Psathaki et al., 2018b). Because the filtration and endocytic functions of nephrocytes are separate, blocking endocytosis but not filtration via expression of the thermosensitive *shibire^{ts1}* allele results in the accumulation of filtrate in the lacunae, a phenomenon previously observed with the circulating serpin Necrotic (Soukup et al., 2009). When we blocked the endocytic pathway using this same allele (*Hand-Gal4^{ts} > UAS-shi^{ts1}*), we observed the accumulation of PGN in the lacunae of nephrocytes (Figure 5B-3, 5D, S5D-1, and S5E), signifying that nephrocytes endocytose PGN by a Shibire-dependent mechanism. In addition, nephrocyte-specific expression of a dominant negative form of Rab5 (*Hand-Gal4^{ts} > UAS-Rab5^{DN}*, Rab5 is a key regulator of early endosomal trafficking) and Rab7 (*Hand-Gal4^{ts} > UAS-Rab7^{DN}*, Rab7^{DN} reroutes all traffic to clear vacuoles, thereby blocking access to the lysosome (Fu et al., 2017)) led to cytoplasmic accumulation of endocytosed PGN relative to controls (Figure 5B-4, 5B-5, and S5D-2, quantification in S5A). Since our data indicated that PGN is routed to the lysosomal

compartment inside nephrocytes, we next assessed whether acidification of the lysosome is important for PGN degradation. Nephrocyte-specific knockdown of two key components of the vacuolar proton pump V-ATPase (*Dot-Gal4 > UAS-Vha16-1-IR* and *Dot-Gal4 > UAS-Vha44-IR*), which functions to acidify the endo-lysososmal compartment (Mauvezin et al., 2015), led to substantial PGN accumulation (Figure 5B-6 and S5D-3, quantification in S5A). Taken together, these results demonstrate that PGN is endocytosed and degraded by nephrocytes in a Shibire-, Rab5-, Rab7-, and V-ATPase-dependent manner.

Finally, we examined the consequences of arresting the endocytic function of nephrocytes on Toll pathway activity. Blocking endocytosis in nephrocytes via expression of the same *shibire*^{ts1} and *Rab5*^{DN} alleles was sufficient to induce abnormally high transcription of Toll target genes (Figure 5E-5F). These results are in agreement with the increase in Toll pathway signaling we detected in flies in which *Klf15* was knocked down in adult nephrocytes (Figure 2F), a condition known to block their endocytic capability (Ivy et al., 2015). Altogether, our results establish that nephrocytes remove microbiota-derived PGN from systemic circulation, thus preventing deviant immune activation in response to gut microbes.

DISCUSSION

Regulation of circulating, microbiota-derived PAMPs is critical for maintenance of immune homeostasis. Toll and Imd, the two primary immune pathways in the fly, recognize the presence of invading bacteria through sensing of specific forms of peptidoglycan (PGN): the Toll pathway recognizes Lys-type PGN from Gram-positive bacteria, while the Imd pathway detects DAP-type PGN from Gram-negative bacteria (Lemaitre and Hoffmann, 2007). Because the gut is constantly

436 exposed to microbes and their PAMPs, it relies on specialized mechanisms to prevent local
437 immune activation against the microbiota. Given that microbiota-shed PGN translocates from the
438 gut lumen into general circulation (Capo et al., 2017; Clarke et al., 2010; Corbitt et al., 2013;
439 Gendrin et al., 2009; Paredes et al., 2011; Zaidman-Remy et al., 2006), additional mechanisms are
440 required to prevent systemic immune activation in response to the microbiota. Without such
441 processes, chronic immune induction can lead to abnormal development (Bischoff et al., 2006)
442 and/or a shortened lifespan, indicating that uncontrolled immune activity can be costly to the host's
443 health (Charroux et al., 2018; Guillou et al., 2016; Paredes et al., 2011). Mechanisms that prevent
444 the systemic activation of Imd in response to the microbiota include the secretion of amidase
445 PGRPs into the hemolymph, which act to degrade DAP-type peptidoglycan (Bischoff et al., 2006;
446 Charroux et al., 2018; Guo et al., 2014; Paredes et al., 2011; Zaidman-Remy et al., 2006). To date,
447 we have no knowledge of a parallel mechanism that would accomplish this for the Toll pathway,
448 although it seems like such mechanism should be necessary. Here, we propose that filtration of
449 blood (hemolymph) by nephrocytes takes charge of this Toll-specific, immune homeostatic
450 function. We found that nephrocytes endocytose Lys-type PGN from systemic circulation and
451 route it to lysosomes for degradation, thus maintaining immune homeostasis in the presence of
452 Lys-type PGN-carrying, Gram-positive microbiota.
453
454 Why would an organism evolve distinct mechanisms to eliminate 2 types of peptidoglycan?
455 One possibility is that efficient degradation of Lys-type PGN requires specialized enzymes, such
456 as lysozymes, that work best in the acidified environment of a mature lysosome than in circulation.
457 The optimal pH for *Drosophila* lysozyme activity is ~5 (Regel et al., 1998). By contrast,
458 hemolymph pH is considerably higher, with pH values ranging from 7.3 to 7.4 (Ghosh and

O'Connor, 2014). As nephrocytes are professional endocytic cells, they are well suited to rapidly and proficiently uptake Lys-type PGN from the hemolymph and route it for degradation to lysosomes. In support of this idea, it is worth noting that nephrocytes express at least 6 lysozyme-like genes (Chintapalli et al., 2007). Due to redundancy and a lack of genetic tools for all 6 lysozyme genes, we were not able to functionally test the role of these lysozymes in the degradation of PGN. Therefore, a role for lysozyme remains speculative. Interestingly, our data also established that the Imd pathway is not activated in the absence of nephrocytes. This may be a result of the efficient degradation of DAP-type PGN by amidase PGRPs, such that there is no remaining intact PGN of this class that needs to be filtered and endocytosed. Alternatively, the intrinsic negative charge of the nephrocyte basement membrane, which is known to act as a charge-selective filter (Denholm et al., 2009), may act to exclude passage of DAP-type but not Lys-type PGN.

Recent work by Soukup and colleagues shows that nephrocytes uptake Nec, a secreted serpin and negative regulator of the Toll pathway, and target it for lysosomal degradation (Soukup et al., 2009). Our work not only confirmed this finding, as Nec protein levels were higher in the hemolymph of *Klf15* mutants compared to wildtype, but also found that other key signaling components of the Toll pathway, such as SPE, accumulated in the hemolymph of *Klf15* mutants despite their transcripts not being increased themselves. These results suggest that hemolymph filtration by nephrocytes may serve to regulate Toll pathway homeostasis on multiple levels: regulating both Lys-type PGN hemolymph concentration and the amount of circulating Toll pathway components available in the hemolymph. Our results did not allow us to determine whether the accumulation of signaling components of the Toll pathway was also important for the

loss of immune homeostasis in flies lacking nephrocytes. However, the fact that GF *Klf15* mutants did not show an increase in Toll activity suggests that PGN filtration, rather than protein accumulation, is the critical mechanism at work. In addition, we note that the phenotype associated with a lack of nephrocytes is not easily predicted. At first glance, accumulation of Nec in flies devoid of nephrocytes would suggest a possible decrease in immune reactivity. However, our results proved the opposite, demonstrating that the loss of PGN filtration primes the immune system and increases resistance to infection.

Chronic kidney disease (CKD), characterized by a gradual loss of glomerular filtration rate, leads to alterations in plasma protein content similar to those observed in *Klf15* mutants. Specifically, proteomic analysis shows that patients with CKD progressively accumulate in their plasma high quantities of 24 proteins involved in the complement system, with a large number of these proteins belonging to the alternative complement pathway (Glorieux et al., 2015). Further activation of the innate immune system is also evident in CKD patients, as their plasma is enriched for 62 proteins associated with the acute phase response (Glorieux et al., 2015). Given the remarkable functional, structural, and molecular similarities between nephrocytes and the glomerular podocytes of the mammalian kidney, we propose that renal filtration by the kidneys could also act to regulate the amount of microbiota-derived PAMPs, such as peptidoglycan, in the blood, thus maintaining immune homeostasis. In support of this idea, we highlight that the alternative complement pathway, several components of which are enriched in the plasma of CKD patients, is activated by peptidoglycan, including Lys-type peptidoglycan (Kawasaki et al., 1987). In both *Klf15* mutants and CKD patients, proteomic studies also showed accumulation of lysozymes in circulation, with lysozyme C increasing in the plasma and the lysozyme encoded by

CG6426 rising in hemolymph. It is possible that lysozyme accumulation may result, in both cases, from induction of the immune system in response to PGN, especially as CG6426 is a target of the Toll pathway (de Gregorio et al., 2001; Troha et al., 2018). Finally, it has been proposed that nephrocytes are functionally analogous to endocytic scavenger cells of the mammalian reticuloendothelial system (Sørensen et al., 2012; Wigglesworth, 1970). Therefore, it is possible that additional cells with scavenging function, such as hepatocytes, may also be involved in the regulation of circulating, microbiota-shed PAMPs.

Altogether, our results have revealed an unexpected role for podocyte filtration in the maintenance of insect immune homeostasis. They suggest that renal clearance may be a major and conserved mechanism to remove peptidoglycan from circulation, thus preventing aberrant immune activation in response to the gut microbiota. Because of the parallels between the filtration systems of flies and mammals, as well as the similar consequences of altering renal function in both species, we propose that at least part of the immune activation observed in patients suffering from glomerular diseases stems from the accumulation of peptidoglycan in plasma.

AUTHOR CONTRIBUTIONS

Conceptualization: N.B., B.P.L., P.S.H. P.N. and K.T.
Data curation: K.T., P.N., and A.P.
Formal analysis: K.T. and P.N.
Methodology: K.T. and P.N.
Supervision: N.B. and B.P.L.
Validation: K.T. and P.N.
Visualization: K.T., P.S.H. and P.N.
Writing – original draft: K.T. and N.B.

Writing – review & editing: N.B., K.T., P.N., A.P., B.P.L., and P.S.H.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

FIGURE LEGENDS

Figure 1. Loss of nephrocyte function increases survival against infection

(A) Adult pericardial nephrocytes stained with WGA Alexa Fluor 594 conjugate (red). Phalloidin-FITC (green) marks the heart tube. Staining is shown for both wildtype (WT) and *Klf15* mutants.

(B) Lifespan curve comparing WT to *Klf15* flies.

(C-G) Survival curves over 7 days following infection of WT and *Klf15* mutants with the bacterial pathogens: *S. marcescens* Type strain (C), *S. typhimurium* (D), *L. innocua* (E), *E. faecalis* (F), and *S. aureus* (G).

(H-I) Survival of flies expressing nephrocyte-specific RNAi against *Klf15* throughout development (*Dot-Gal4 > UAS-Klf15-IR*) (H) or only during the adult stage (*Hand-Gal4^{ts} > UAS-Klf15-IR)* (I) after infection with *S. aureus*.

The curves represent the average percent survival ±SE of three or more biological replicates. **p<0.01 ***p<0.001 ****p<0.0001 in a Log-rank test.

Figure 2. The Toll pathway is activated in *Klf15* mutants

(A-B) Bacterial load time course of control and *Klf15* flies following infection with *S. aureus* (A) and *E. faecalis* (B). Three repeats are graphed together, with each symbol representing an individual fly's number of colony forming units (CFU). Horizontal lines represent median values for each time point. Data were normalized and then analyzed using a two-way ANOVA followed by Sidak's post-test for specific comparisons (*p<0.05 **p<0.01 ***p<0.001 ****p<0.0001).

(C) Heat map showing a list of circulating proteins enriched (≥ 1.5-fold) in the hemolymph (insect blood) of *Klf15* mutants over that of WT or only present in *Klf15* flies but not wildtype. A color scale on the left side of the heat map denotes whether the gene that encodes each protein is transcriptionally induced by infection (green), a target of the Toll pathway (blue), or predicted to possess a signal peptide (orange). Core genes are highlighted with a ★ symbol (Troha et al., 2018).

(D-E) Whole fly RT-qPCR of Toll target genes *IM2*, *CG15067*, and *Drs* (D) and Imd target genes *Dpt*, *AttC*, and *TotA* (E) using unchallenged wildtype and *Klf15* samples.

(F) Quantification of Toll target genes *IM2*, *CG15067*, and *Drs* via RT-qPCR in flies expressing RNAi against *Klf15* only during the adult stage (*Hand-Gal4^{ts} > UAS-Klf15-IR*). Mean values of three or more repeats are represented ±SE. *p<0.05 **p<0.01 ***p<0.001 in a Student's t-test.

Figure 3. Increased resistance to infection in *Klf15* mutants is PGRP-SA-dependent

(A-C) Comparison of *Klf15* ;; *spz^{rm7}* double mutants to wildtype, *Klf15*, and *spz^{rm7}* single mutants in experiments measuring *IM2* (Toll target) gene expression via RT-qPCR (A), survival against *S. aureus* (B), and survival against *E. faecalis* (C).

(D-F) Comparison of *Klf15* , psh^{1} double mutants to wildtype, *Klf15*, and psh^{1} single mutants in experiments measuring *IM2* gene expression via RT-qPCR (D), survival against *S. aureus* (E), and survival against *E. faecalis* (F).

(G-I) Comparison of *Klf15* ;; $modSP^{1}$ double mutants to wildtype, *Klf15*, and $modSP^{1}$ single mutants in experiments measuring *IM2* gene expression via RT-qPCR (G), survival against *S. aureus* (H), and survival against *E. faecalis* (I).

(J-L) Comparison of *Klf15* , $PGRP\text{-}SA^{seml}$ double mutants to wildtype, *Klf15*, and $PGRP\text{-}SA^{seml}$ single mutants in experiments measuring *IM2* gene expression via RT-qPCR (J), survival against *S. aureus* (K), and survival against *E. faecalis* (L).

For RT-qPCR experiments, mean values of three or more repeats are presented ±SE (*$p<0.05$ **$p<0.01$ ***$p<0.001$ in a Student's t-test). Survival curves show the average percent survival ±SE of three biological replicates (***$p<0.001$ ****$p<0.0001$ in a Log-rank test).

Figure 4. Nephrocytes prevent excessive immune activation against Gram-positive microbiota

(A) Quantification of mRNA transcripts in conventional (CR), germ-free (GF), and germ-free flies recolonized with either live *A. pomorum* (Gram-negative), live *L. brevis* (Gram-positive), or live *E. faecalis* (Gram-positive). RT-qPCR measurements of Toll target genes *IM2* and *CG15067* are shown.

(B) Quantification of mRNA transcripts in conventional (CR), germ-free (GF), and germ-free flies fed either heat-killed *A. pomorum* (Gram-negative), heat-killed *L. brevis* (Gram-positive), or heat-killed *E. faecalis* (Gram-positive). RT-qPCR measurements of Toll target genes *IM2* and *CG15067* are presented.

For RT-qPCR experiments, mean values of three or more repeats are given ±SE. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001 in a Student's t-test.

(C-D) Survival curve over 7 days following infection of WT and *Klf15* flies with *S. aureus* (C) and *E. faecalis* (D) in both conventional (CR) and germ-free (GF) conditions.

(E) Lifespan curve comparing WT to *Klf15* mutants in both conventional (CR) and germ-free (GF) conditions.

Survival curves give the average percent survival ±SE of three biological replicates (**p<0.01 ****p<0.0001 in a Log-rank test).

Figure 5. Nephrocytes endocytose peptidoglycan from systemic circulation

(A) Quantification of peptidoglycan quantity in the hemolymph (insect blood) of WT and *Klf15* flies in germ-free (GF) and conventionally reared (CR) conditions. *p<0.05 in a Student's t-test.

(B1-B2) Immunostaining of nephrocytes from CR and GF flies with PGN antibody demonstrates that nephrocytes internalize microbiota-derived PGN.

(B3-B6) Nephrocyte-specific expression of *shibire^{ts1}* (*Dot-Gal4 > UAS-shi^{ts1}*), *Rab5^{DN}* (*Hand-Gal4^{ts} > UAS-Rab5^{DN}*), *Rab7^{DN}* (*Hand-Gal4^{ts} > UAS-Rab7^{DN}*), and *Vha16-1-IR* (*Dot-Gal4 > UAS-Vha16-1-IR*) led to accumulation of PGN in nephrocytes when compared to control (see Figure 5B-1, 5D, and S5D-1-D-2 for additional controls). Scalebar = 10μm.

(C) Immunostaining against PGN reveals colocalization (yellow circles) with the early endosomal marker Rab5 (*Hand-Gal4^{ts} > UAS- Rab5-YFP*), the late endosomal marker Rab7 (*Hand-Gal4^{ts} > UAS-Rab7-YFP*), and the lysosomal compartment marker Cathepsin L. Scalebar = 10μm.

(D) Cortico-cytoplasmic view of nephrocytes immunostained with Duf (labels the lacunae) shows expansion of the lacunae (marked by the yellow dotted lines) in *shibire^{ts1}* (*Dot-Gal4 > UAS-shi^{ts1}*)

flies. Immunostaining with PGN antibody reveals that PGN is accumulating in the lacunae. Blue arrowheads are labeling the nuclei.

(E-F) Whole fly RT-qPCR of Toll target genes *IM2*, *CG15067*, and *Drs* in unchallenged conditions. Gene expression was measured in flies expressing $shibire^{ts1}$ and $Rab5^{DN}$ in a nephrocyte-specific manner (*Dot-Gal4 > UAS-shi*^{ts1} and *Hand-Gal4*^{ts} *> UAS-Rab5*^{DN}). *p<0.05 **p<0.01 ***p<0.001 in a Student's t-test.

STAR METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Nicolas Buchon (nicolas.buchon@cornell.edu).

This study did not generate new unique reagents.

The graphical abstract was created with BioRender.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Rearing of Drosophila melanogaster

Flies were maintained on standard sucrose-cornmeal-yeast medium: 50 g baker's yeast, 60 g cornmeal, 40 g sucrose, 7 g agar, 26.5 mL Moldex (10%), and 12 mL Acid Mix solution (4.2% phosphoric acid, 41.8% propionic acid) per 1L of deionized H_2O. Wildtype and mutant flies were raised at 24 °C. Flies originating from crosses that employ the UAS-Gal4-Gal80^{ts} gene expression system were raised at 18 °C (Gal80^{ts} On, Gal4 Off) and transferred to 29 °C (Gal80^{ts} Off, Gal4 On) 5 days after eclosion. Males were used for all experiments, with the exception of

immunostaining (larger female size is preferred for dissection and visualization of cells). For experiments with mutants, 5- to 8-day-old adult flies were used. For experiments with *UAS* transgenes, 10- to 14-day-old flies were used (to allow for the expression of the pertinent construct).

Drosophila melanogaster strains

Klf15^{NN}, *spz*^{rm7}, *SPE*^{SK6}, *psh*^{1}, *modSP*^{1}, *GNBP3*^{Hades}, *PGRP-SA*^{seml}, and *PPO1*$^{\Delta}$, *2*$^{\Delta}$,*3*^{1} mutants and the *dLamp-3xmCherry* lysosomal marker have been previously described (Buchon et al., 2009; Dudzic et al., 2015; Gobert et al., 2003; Gottar et al., 2006; Hegedűs et al., 2016; Ivy et al., 2015; Jang et al., 2006; Michel et al., 2001; Ming et al., 2014). The nephrocyte-specific drivers, *Dot-Gal4* and *Hand-Gal4*^{ts}, are detailed in (Ivy et al., 2015). The following lines were purchased from the Bloomington *Drosophila* Stock Center: *UAS-shi*^{ts1} (44222), *UAS-Rab5*^{DN} (9771), *UAS-Rab7*^{DN} (9778), *UAS-Rab5-YFP* (24616), *UAS-Rab7-YFP* (23270). The following lines were purchased from the Vienna *Drosophila* Resource Center: *UAS-Vha16-IR* (49290) and *UAS-Vha44-IR* (46563).

Culturing of microbes

The following bacteria were cultured overnight in LB broth and adjusted to the specified density: *Serratia marcescens* Type (OD_{600} =1), *Salmonella typhimurium* (OD_{600} =1), *Listeria innocua* (OD_{600} =1), *Enterococcus faecalis* (OD_{600} =1), *Staphylococcus aureus* (OD_{600} =1), and *Providencia rettgeri* (OD_{600} =1). *S. typhimurium* and *L. innocua* were grown at 37 °C. All other bacteria were grown at 29 °C. The fungi *Beauveria bassiana* and *Metarhizium anisopliae* were grown at 29 °C on YPG-agar plates.

METHOD DETAILS

Infection, survival, and lifespan experiments

Flies were systemically infected with bacteria via septic pinprick to the thorax. Pinprick infection with an OD_{600} =1 for the bacteria aforementioned results in inoculation with ~3,000 CFU/fly. For natural infections with fungi, CO_2-anaestethized flies were placed directly on the sporulating lawn of a fungal culture plate and the plate was shaken for ~15 seconds to coat the flies in spores. Flies were then transferred to a new, clean food vial to recover. All flies, regardless of infection method, were maintained at 29 °C for the duration of the experiments. For survival experiments, death was recorded daily following inoculation, with flies transferred to fresh vials every 2 to 3 days. For lifespan measurements, adults were transferred to 29 °C 5 days post-eclosion and remained at that temperature for the duration of the experiment. All experiments were performed at least 3 times.

Quantification of bacterial CFUs

At specified time points following inoculation, flies were individually homogenized by bead beating in 500 µl of sterile PBS using a tissue homogenizer (OPS Diagnostics). Dilutions of the homogenate were plated onto LB agar plates using a WASP II autoplate spiral plater (Microbiology International), incubated overnight at 29 °C, and CFUs were counted. All experiments were performed at least 3 times.

RT-qPCR

For all experiments involving RT-qPCR, total RNA was extracted from pools of 20 flies using the standard TRIzol (Invitrogen) extraction. RNA samples were treated with DNase (Promega), and

cDNA was generated using murine leukemia virus reverse transcriptase (MLV-RT-Promega). qPCR was performed using the SSO Advanced SYBR green kit (Bio-Rad) in a Bio-Rad CFX-Connect instrument. Data represent the relative ratio of the target gene and that of the reference gene *RpL32*. Mean values of at least three biological replicates are represented ±SE. The primer sequences used can be found in Table 1.

Phagocytosis assays

To assay phagocytosis, flies were injected in the thorax with 69 nl of pHrodo Red Bioparticles™ (Invitrogen) using a Nanoject (Drummond Scientific). The fluorescence within the abdomen of the flies was then imaged at 3 h post-injection with a Leica MZFLIII fluorescent microscope and quantified using ImageJ (NIH) as previously described (Guillou et al., 2016). To block phagocytosis, adult flies were pre-injected with a solution containing latex beads as previously described in (Elrod-Erickson et al., 2000). Twenty-four hours post injection, the flies were subjected to systemic infection as described above.

Hemolymph extraction

Hemolymph was collected using a centrifugation or capillary method. In the first method, ~100 anesthetized flies are loaded into a modified spin column (Qiagen), in which the filter was removed and thoroughly washed with water before use, and 2 metal beads are placed on top of the flies. Flies are then centrifuged twice at 5,000 g for 5 minutes at 4 °C. For more details, see (Troha and Buchon, 2019). For the capillary method, a pulled glass needle is used to prick flies in the thorax. Hemolymph is extracted into the needle by capillary action.

DOPA assay

Extracted hemolymph was immediately diluted in a 1:10 ratio using a protease inhibitor cocktail (Sigma: 11697498001) and kept on ice. Briefly, 50 µL of diluted hemolymph was combined with 150 µl of a 5 mM $CaCl_2$ solution and 800 µL of L-DOPA (Sigma: D9628) reagent. Following thorough mixing, 200 µl of sample/well was loaded into a 96-well plate. Using a spectrophotometer set to 29 °C, a kinetic assay was performed at OD_{492}.

Generation of GF and mono-colonized flies

Collected eggs were surface sterilized by immersion in 70% ethanol for 2 min. Eggs were then dechorionated via treatment with a 10% bleach solution for 10 min. This was followed by rinsing the eggs in sterile water 3 times to remove any leftover bleach. The eggs were then transferred to pre-autoclaved media vials, where they were allowed to develop. The entire procedure was performed using sterile technique in a laminar flow hood. For mono-colonized flies, pre-autoclaved media vials were seeded with 200 µL of the desired individual bacterial culture (OD_{600} =200). After the bacterial solution was absorbed into the media, adult germ-free flies were flipped into the mono-colonized media vial. Experiments with mono-colonized flies were carried out 5 days after the flies were first exposed to the bacteria.

PGN detection by SLP assay

The Silkworm Larvae Plasma (SLP) assay was used. After diluting extracted hemolymph (1:10 ratio), 50 µL of hemolymph sample/condition was used for the SLP assay (Fujifilm Wako Pure Chemical Corporation: 297-51501) following the manufacturer's instructions.

Gut barrier integrity (Smurf) assay

Adult flies were fed standard medium supplemented with Blue Dye No. 1 (2.5%). A fly was counted as a Smurf when the blue dye could be observed outside of the digestive tract.

UAS/GAL4/GAL80^{ts} gene expression system

For RNAi and overexpression experiments, we used the UAS/GAL4 gene expression system in combination with GAL80ts to restrict the expression of the constructs specifically to the adult stage. Flies were collected 5 to 8 days after eclosion from the pupal case and shifted to 29 °C for an additional 8 days prior to any experiments. See our *Rearing of Drosophila melanogaster* section for additional details.

Immunohistochemistry and fluorescence imaging

Dissected nephrocytes were fixed in a 4% paraformaldehyde solution in PBST (PBS with 0.5% Triton X-100) for 1 h. After repeated washes in PBST, samples were blocked in 3% BSA PBST for 3 h and incubated overnight with primary antibodies in 1% BSA PBST at 4 °C. Samples were labeled with secondary antibodies in 1% BSA PBST for 2 h. Samples were washed after each antibody labeling step with PBST containing 4% NaCl to reduce non-specific background labeling. The primary antibodies used in this study were: mouse anti-peptidoglycan (GeneTex: GTX39437) diluted 1:200, chicken anti-GFP (Invitrogen: A10262) diluted 1:1500, rabbit anti-Cathepsin L (Abcam: ab58991) diluted 1:1000, rabbit anti-Dumbfounded diluted 1:100 (Psathaki et al., 2018b), and rabbit anti-RFP (Invitrogen: R10367) diluted 1:2000. The secondary antibodies were: Alexa Fluor 488 anti-chicken (A11039), Alexa Fluor 488 anti-rabbit (A21206), Alexa Fluor 488 anti-mouse (A21202), Alexa Fluor 555 anti-rabbit (A31572), and Alexa Fluor 555 anti-mouse

(A31570), all diluted 1:1500 and from Invitrogen. Imaging was performed on a Zeiss LSM 700 confocal inverted microscope. Pearson correlation coefficients were calculated using ImageJ (FIJI, version: 2.0.0-rc-69/1.52n).

QUANTIFICATION AND STATISTICAL ANALYSIS

Aside from one exception, all analyses were performed in Prism (GraphPad Prism V7.0a, GraphPad Software, La Jolla, CA, USA). For survival assays, the curves represent the average percent survival ±SE of three or more biological replicates (n=20 flies for each biological replicate). A Log-rank test was used to determine significance (*p<0.05 **p<0.01 ***p<0.001 ****p<0.0001). In bacterial load quantification assays, the horizontal lines represent median values for each time point. Three biological replicates were included. Following normalization, results were analyzed using a two-way ANOVA followed by Sidak's post-test for specific comparisons (*p<0.05 **p<0.01 ***p<0.001 ****p<0.0001). For all other experiments, mean values of three or more biological repeats are presented ±SE. Significance was calculated by a Student's t-test following normalization (*p<0.05 **p<0.01 ***p<0.001 ****p<0.0001). Whenever survival curves crossed, a Cox's proportional-hazards model was used instead of a Log-rank test to assay significance. In this case, SPSS (IBM Corp. Released 2017. IBM SPSS Statistics for Mac OS X, Armonk, NY: IBM Corp.) was used for the analysis.

SUPPLEMENTAL INFORMATION

Supplemental information includes 5 figures and 1 table.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1 (Related to Figure 1). *Klf15* flies display improved survival against a variety of microbial infections

(A) Survival curve of *Klf15* and WT flies infected with *P. rettgeri*.

(B-C) Survival curves over 14 days following natural infection of WT and *Klf15* flies with the fungal pathogens *M. anisopliae* (B) and *B. bassiana* (C).

(D-E) Survival of flies expressing nephrocyte-specific RNAi against *Klf15* throughout development (*Dot-Gal4 > UAS-Klf15-IR*) (D) or only during the adult stage (*Hand-Gal4^{ts} > UAS-Klf15-IR*) (E) after infection with *E. faecalis*.

The curves represent the average percent survival ±SE of three biological replicates. *p<0.05 ***p<0.001 ****p<0.0001 in a Log-rank test.

Supplementary Figure 2 (Related to Figure 2). The Toll pathway is turned on in *Klf15* mutants

(A) Bacterial load upon death (*BLUD*) of wildtype and *Klf15* mutants infected with *S. aureus* and *E. faecalis.*

(B-D) Bacterial load data of control and *Klf15* flies following infection with *S. marcescens* Type strain, *L. innocua*, and *S. typhimurium*. Three repeats are graphed together, with each symbol representing an individual fly's number of colony forming units (CFU). Horizontal lines represent median values for each time point. Results were analyzed using a two-way ANOVA followed by Sidak's post-test for specific comparisons (*p<0.05 **p<0.01).

(E) Representative fluorescence images of the abdomens of control and *Klf15* mutants 3 h post-injection with pHrodo bacteria. Fluorescence was quantified and the average plotted ±SE. **p<0.01 in a Student's t-test.

(F-G) Survival curves over 7 days of WT and *Klf15* flies that were pre-injected with latex beads 24 h prior to infection with the pathogens *S. aureus* (D) and *E. faecalis* I. **p<0.01 ***p<0.001 ****p<0.0001 in a Log-rank test.

(H) Phenoloxidase activity was measured using the L-DOPA assay. WT and *Klf15* samples were measured in unchallenged conditions as well as following infection with *E. faecalis* and *S. aureus*. *p<0.05 **p<0.01 in a Student's t-test.

(I) Comparison of *Klf15* ; $PPO1^{\Delta}$, 2^{Δ},3^{1} quadruple mutants to WT, *Klf15*, and $PPO1^{\Delta}$, 2^{Δ},3^{1} mutants in experiments measuring survival against *S. aureus*. ***p<0.001 ****p<0.0001 in a Log-rank test.

(J) Heat map showing a list of circulating proteins depleted (≥ 1.5-fold) in the hemolymph (insect blood) of *Klf15* mutants relative to WT. A color scale on the left side of the heat map denotes whether the gene that encodes each protein is transcriptionally decreased by infection (pink), a target of the Toll pathway (blue), or predicted to possess a signal peptide (beige). Core genes are highlighted with a ★ symbol (Troha et al., 2018).

(K) Whole fly RT-qPCR of Toll target genes *CG18067* and *CG15293* and Imd target gene *AttD* using unchallenged WT and *Klf15* samples. *p<0.05 in a Student's t-test.

(L-M) Whole fly RT-qPCR of Toll target genes *IM2*, *CG15067*, and *Drs* and Imd target gene *Dpt* following infection with *S. aureus* (J) and *E. faecalis* (K). *p<0.05 **p<0.01 ****p<0.0001 in a Student's t-test.

Supplementary Figure 3 (Related to Figure 3). Increased resistance to infection in *Klf15* flies is Toll-dependent

(A-B) Comparison of *Klf15* ;; spz^{rm7} double mutants to WT, *Klf15*, and spz^{rm7} single mutants in experiments measuring mRNA expression of Toll target genes *CG15067* (A) and *Drs* (B) via RT-qPCR.

(C-G) Comparison of *Klf15* ;; SPE^{SK6} double mutants to WT, *Klf15*, and SPE^{SK6} single mutants in experiments measuring mRNA expression of Toll target genes *IM2* (C), *CG15067* (D), and *Drs* (E) via RT-qPCR as well as survival against *S. aureus* (F) and *E. faecalis* (G).

(H-I) Comparison of *Klf15* , psh^{1} double mutants to WT, *Klf15*, and psh^{1} single mutants in experiments measuring mRNA expression of Toll target genes *CG15067* (H) and *Drs* (I) via RT-qPCR.

(J-K) Comparison of *Klf15* ;; $modSP^{1}$ double mutants to WT, *Klf15*, and $modSP^{1}$ single mutants in experiments measuring mRNA expression of Toll target genes *CG15067* (J) and *Drs* (K) via RT-qPCR.

(L-M) Comparison of *Klf15* , $PGRP\text{-}SA^{seml}$ double mutants to WT, *Klf15*, and $PGRP\text{-}SA^{seml}$ single mutants in experiments measuring mRNA expression of Toll target genes *CG15067* (L) and *Drs* (M) via RT-qPCR.

(N-R) Comparison of *Klf15* ;; $GNBP3^{Hades}$ double mutants to WT, *Klf15*, and $GNBP3^{Hades}$ single mutants in experiments measuring mRNA expression of Toll target genes *IM2* (N), *CG15067* (O), and *Drs* (P) via RT-qPCR as well as survival against *S. aureus* (Q) and *E. faecalis* (R).

For RT-qPCR experiments, mean values of three or more repeats are given ±SE (*$p<0.05$ **$p<0.01$ ***$p<0.001$****$p<0.0001$ in a Student's t-test). Survival curves show the average

848 percent survival ±SE of three biological replicates (**p<0.01 ***p<0.001 ****p<0.0001 in a Log-
849 rank test).
850 (S-T) Bacterial load data of WT, *Klf15*, *modSP*^{1}, *Klf15* ;; *modSP*^{1}, *PGRP-SA*^{seml}, and *Klf15* ,
851 *PGRP-SA*^{seml} flies following infection with *S. aureus* and *E. faecalis*. Three repeats are graphed
852 together, with each symbol representing an individual fly's number of colony forming units (CFU).
853 Horizontal lines represent median values for each time point. Results were analyzed using a two-
854 way ANOVA followed by Sidak's post-test for specific comparisons (****p<0.0001).
855
856 **Supplementary Figure 4 (Related to Figure 4). Nephrocytes prevent overactive immune**
857 **responses to gut microbes**
858 (A) Quantification of mRNA expression in conventional (CR), germ-free (GF), and germ-free flies
859 recolonized with either live *A. pomorum* (DAP-type PGN), live *L. brevis* (Lys-type PGN), or live
860 *E. faecalis* (Lys-type PGN). RT-qPCR measurements of Toll target gene *Drs* are shown.
861 (B) Percent SMURF flies found after feeding a diet containing 2.5% Blue #1 Dye for both WT
862 and *Klf15* flies.
863 (C) Comparison of circulating (hemolymph) bacteria between *Klf15* and control flies. Samples
864 were plated on three separate media: De Man, Rogosa, and Sharpe (MRS), Luria-Bertani (LB),
865 and yeast-peptone-glucose (YPG) agar.
866 (D) Comparison of whole fly microbiota between *Klf15* and control flies. Samples were plated on
867 three separate media: De Man, Rogosa, and Sharpe (MRS), Luria-Bertani (LB), and yeast-peptone-
868 glucose (YPG) agar.
869 (E) Quantification of mRNA expression in conventional (CR), germ-free (GF), and germ-free flies
870 fed either heat-killed *A. pomorum* (DAP-type PGN), heat-killed *L. brevis* (Lys-type PGN), or heat-

killed *E. faecalis* (Lys-type PGN). RT-qPCR measurements of Toll target gene *Drs* are presented. For RT-qPCR experiments, mean values of three or more repeats are given ±SE. *p<0.05 **p<0.01 in a Student's t-test.

(F-H) Quantification of mRNA expression in flies overexpressing *SPE* (*c564-Gal4^{ts} > UAS-SPE*) in both conventional (CR) and germ-free (GF) conditions. RT-qPCR measurements of Toll target gene *IM2* (F), *CG15067* (G), and *Drs* (H) are shown.

(I) Whole fly RT-qPCR of *SPE* using unchallenged wildtype and *Klf15* samples.

Supplementary Figure 5 (Related to Figure 5). Nephrocytes uptake peptidoglycan from the hemolymph

(A) Quantification and comparison of PGN puncta per cell for CR and GF flies and flies expressing *shibire^{ts1}* (*Dot-Gal4 > UAS-shi^{ts1}*), *Rab5^{DN}* (*Hand-Gal4^{ts} > UAS-Rab5^{DN}*), *Rab7^{DN}* (*Hand-Gal4^{ts} > UAS-Rab7^{DN}*), *Vha16-1-IR* (*Dot-Gal4 > UAS-Vha16-1-IR*), and *Vha44-IR* (*Dot-Gal4 > UAS-Vha44-IR*) in a nephrocyte-specific manner. ****p<0.0001 in a Student's t-test.

(B) Pearson correlation coefficients (PCC) from co-localization experiments.

(C) Immunostaining against PGN reveals colocalization (yellow circles) with the lysosomal marker Lamp1. Scalebar: 10μm

(D1-D2) Control images (18°C) for *shibire^{ts1}* (*Dot-Gal4 > UAS-shi^{ts1}*) and *Rab7^{DN}* (*Hand-Gal4^{ts} > UAS-Rab7^{DN}*) experiments.

(D3) Nephrocyte-specific expression of *Vha44-IR* (*Dot-Gal4 > UAS-Vha44-IR*) led to accumulation of PGN in nephrocytes when compared to control. Scalebars: 10μm

(E) Surface view: Immunostaining against Duf (labels the lacunae) shows expansion of the lacunae in *shibire^{ts1}* (*Dot-Gal4 > UAS-shi^{ts1}*) flies. Immunostaining with PGN antibody reveals that PGN is accumulating in the lacunae.

DATA AND CODE AVAILABILITY

This study did not generate datasets/code.

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