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Iron sequestration by transferrin 1 mediates nutritional immunity in *Drosophila melanogaster*

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Iron sequestration is a recognized innate immune mechanism against invading pathogens mediated by iron-binding proteins called transferrins. Despite many studies on antimicrobial activity of transferrins in vitro, their specific in vivo functions are poorly understood. Here we use *Drosophila melanogaster* as an in vivo model to investigate the role of transferrins in host defense. We find that systemic infections with a variety of pathogens trigger a hypoferremic response in flies, namely, iron withdrawal from the hemolymph and accumulation in the fat body. Notably, this hypoferremia to infection requires *Drosophila* nuclear factor κB (NF-κB) immune pathways, Toll and Imd, revealing that these pathways also mediate nutritional immunity in flies. Next, we show that the iron transporter Tsf1 is induced by infections downstream of the Toll and Imd pathways and is necessary for iron relocation from the hemolymph to the fat body. Consistent with elevated iron levels in the hemolymph, *Tsf1* mutants exhibited increased susceptibility to *Pseudomonas* bacteria and Mucorales fungi, which could be rescued by chemical chelation of iron. Furthermore, using siderophore-deficient *Pseudomonas aeruginosa*, we discover that the siderophore pyoverdine is necessary for pathogenesis in wild-type flies, but it becomes dispensable in *Tsf1* mutants due to excessive iron present in the hemolymph of these flies. As such, our study reveals that, similar to mammals, *Drosophila* uses iron limitation as an immune defense mechanism mediated by conserved iron-transporting proteins transferrins. Our in vivo work, together with accumulating in vitro studies, supports the immune role of insect transferrins against infections via an iron withholding strategy.

transferrin | *Drosophila* | nutritional immunity | iron sequestration | innate immunity

Iron plays an indispensable role in numerous physiological processes, such as respiration, the trichloroacetic acid cycle, oxygen transport, gene regulation, and DNA biosynthesis. Owing to its versatile biological utility, iron is an essential element in the biological processes of all living organisms, and is central to metabolic function. As a consequence, iron sequestration by the host is a potent defense against bacterial pathogens, a process termed nutritional immunity (1–7). Early reports dating back to the 1940s documented that intramuscular inoculation of dogs with *Staphylococcus aureus* leads to a precipitous drop in plasma iron levels, which was named hypoferremia of infection (8). This hypoferremic response is an important facet of the innate immune system aimed at limiting iron availability to invading microbes by withholding iron within the cells and tissues. In line with this, individuals who suffer from iron overload due to mutations affecting iron metabolism have an enhanced risk of infection (9). To sequester iron from pathogens, the host relies on a number of iron-binding proteins, among which members of the transferrin family frequently play a prominent role (1, 10). Transferrins are monomeric glycoproteins that are ubiquitous in metazoans. Mammals have four types of transferrin: serum transferrin, lactoferrin, melanotransferrin, and the inhibitor of carbonic anhydrase (11, 12). Among these, serum transferrin and lactoferrin have been implicated in nutritional immunity via iron sequestration from invading pathogens (5, 12). Serum transferrin is abundant in the blood of mammals and primarily functions as an iron transporter by shuttling the iron from the gut to peripheral sites of storage and use (13). Lactoferrin is found on mucosal surfaces, and in biological fluids including milk and saliva, indicating that it is part of the innate immune response; however, there is no functional in vivo data supporting this role (14–16). Due to their high affinity to iron, transferrins have been shown to inhibit the growth of certain microbes (17). While numerous studies reported the potent antimicrobial activity of purified transferrins in vitro, in vivo studies addressing transferrin function are rather limited (10, 15, 18–24). Although hypotransferrinemic (hpx) mice devoid of serum transferrin exist, how they respond to microbial infection has yet to be examined (25). Hence, the in vivo role of transferrins awaits further investigation.

Due to its genetic tractability, *Drosophila melanogaster* has been a model of choice to study innate host defense mechanisms (26). The systemic antimicrobial response is probably the best-characterized immune mechanism in *Drosophila*. It involves the fat body, and, to a lesser extent, hemocytes, producing antimicrobial peptides that are secreted into the hemolymph. This response is regulated at the transcriptional level by two nuclear factor κB (NF-κB) pathways, Toll and Imd, whose inactivation causes a high susceptibility to infection (26–29). However,

Significance

Hosts sequester iron as a strategy to limit pathogen acquisition of this essential nutrient in a process termed nutritional immunity. Due to their in vitro antimicrobial activity, iron-binding proteins transferrins are suspected to play a role in iron sequestration. However, little is known about the in vivo role of transferrins. Here, we found that *Drosophila melanogaster* exhibits infection-induced hypoferremia mediated by Transferrin 1. Due to excessive iron in hemolymph, *Transferrin 1* (*Tsf1*)-deficient flies are hypersusceptible to certain infections. Our study reveals that nutritional immunity is an important, previously unrecognized arm of immune defense in *Drosophila*. Using fly and bacterial genetics, we show that Tsf1 mediates nutritional immunity by sequestering iron from the pathogens in vivo on the whole-organism level.

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The authors declare no competing interest.

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whether nutritional immunity via iron sequestration constitutes a part of the insect defense response has not been studied. There are three transferrin homologs in *Drosophila*: Tsf1, Tsf2, and Tsf3. Tsf2 is a component of epithelial septate junctions (30) and is unlikely to play an antimicrobial role, whereas Tsf3 has not been functionally characterized yet but might play a role in circadian rhythms (31). Tsf1 was recently shown to function as an iron transporter in the hemolymph (the insect blood) similar to mammalian serum transferrin (32). Specifically, fat body-derived Tsf1 is secreted into the hemolymph and transports iron from the gut and hemolymph to the fat body. The *Tsf1* gene is induced upon infections, pointing to its role in host defense (33–35). Proteomic analysis of hemolymph also revealed Tsf1 up-regulation after infection with the fungus *Beauveria bassiana* (36). Transferrin genes have been shown to be up-regulated in response to infection in other insect species, including representatives from Diptera, Coleoptera, Hemiptera, Hymenoptera, and Lepidoptera (21, 22, 37–39). Also, the promoter region of Tsf1 genes from several insects is enriched in putative NF-κB binding sites, supporting the immune role of Tsf1 in these animals (37). Indeed, purified iron-free transferrins from *Sarcophaga bullata, Bombyx mori*, and *Manduca sexta* were shown to have antibacterial activity in vitro, which was dependent on the transferrin's ability to sequester iron (21, 22, 40).

However, the in vivo role of insect transferrins in host defense at the organismal level has never been addressed. In this study, we used *D. melanogaster* as a genetically tractable model to investigate the role of iron and Tsf1 in insect host defense.

Results

***D. melanogaster* Exhibits Infection-Induced Hypoferremia.** Infections in mammals induce a transient depletion of plasma iron (8), motivating us to investigate whether infection-induced hypoferremia also happens in *Drosophila*. To this end, we infected flies by pricking with a range of pathogens, including the Gram-positive bacterium *Micrococcus luteus*, the Gram-negative bacteria *Pectobacterium carotovorum* (*Ecc15*) and *Pseudomonas entomophila*, and the yeast *Candida albicans*. We measured iron content in the extracted hemolymph from unchallenged and infected flies using inductively coupled plasma optical emission spectrometry (ICP-OES). Compared to uninfected flies, there was a significant decrease in hemolymph iron levels in all tested infections (Fig. 1*A*). Importantly, pricking with heat-killed bacteria triggered the same drop in hemolymph iron level as infection with live bacteria (Fig. 1*B*). This suggests that iron withdrawal from hemolymph is a host-mediated process, and does not result from bacterial consumption. To track where hemolymphatic iron might be redistributed to upon infection, we monitored iron levels in other major tissues. The decrease of hemolymph iron after *M. luteus* infection was correlated with a concomitant increase in iron level in the fat body, while other tissues were not affected (Fig. 1*C*). This result suggests that iron was relocated from the hemolymph to the fat body after infection.

Infection-Mediated Hemolymph Iron Depletion Requires the Toll and Imd Pathways. We next explored whether the Toll and Imd immune pathways contribute to the depletion of hemolymphatic iron upon infection. We found that, in contrast to wild-type flies, Toll pathway-deficient mutants, including spz^{rm7}, $GNBP1^{osi}$, $PGRP\text{-}SA^{Seml}$, and $ModSP^{1}$ flies, were unable to remove iron from the hemolymph after *M. luteus* infection, a challenge known to predominantly activate the Toll pathway (27, 33). In fact, iron amount after infection stayed at the same level as in uninfected flies (Fig. 1*D*). Similarly, Imd pathway-deficient mutants $PGRP\text{-}SD^{sk1}$, $PGRP\text{-}LC^{E12}$, and $Relish^{E20}$ were impaired in iron removal from the hemolymph after pricking with *Ecc15* heat-killed bacteria (Fig. 1*E*), which potently activates Imd pathway but does not kill mutant flies (41). Interestingly, in $PGRP\text{-}SD^{sk1}$ mutant, we observed significant decrease in hemolymph iron, but not as strong as in wild-type flies. This result is explained by the fact that $PGRP\text{-}SD^{sk1}$ mutants have only partial reduction in Imd pathway activity, and therefore they have partial hypoferremic response (41, 42). Thus, the Imd and Toll pathways appear to be required for iron withdrawal from hemolymph after infections that activate these pathways.

Transferrin 1 Is Required for Iron Relocation from Hemolymph to Fat Body after Infection. The fact that the Toll and Imd pathways are necessary for iron removal from hemolymph after infection suggests that potential immune effectors downstream of these pathways can transport iron from hemolymph to fat body. A good candidate was the iron transporter transferrin 1 (Tsf1), as transcriptomic studies have shown that this gene is induced upon infection (33, 34). Using RT-qPCR, we showed that this gene is strongly induced by *M. luteus* in a Toll pathway-dependent manner, and by *Ecc15* in an Imd pathway-dependent manner (Fig. 2 *A* and *B*). Importantly, *Tsf1* up-regulation upon infection was tissue-specific and was restricted to the fat body (*SI Appendix*, Fig. S1*A*). Also, *Tsf1* is the only infection-responsive transferrin in *Drosophila*, since none of the other two transferrins was induced by *M. luteus* or *Ecc15* (*SI Appendix*, Fig. S1 *B* and *C*). Using an endogenously GFP-tagged *Tsf1* transgenic line, we additionally confirmed that Tsf1 protein abundance is strongly increased in the hemolymph after both *M. luteus* and *Ecc15* infections (Fig. 2*C*). To further explore the role of Tsf1 in infection-induced iron transport, we generated a *Tsf1* mutant ($Tsf1^{JP94}$) using CRISPR-Cas9. The mutant has two nucleotide substitutions and a single nucleotide deletion, which leads to a frameshift with a premature stop codon at position 19 (Fig. 2*D*). Using qPCR, we showed that there was no *Tsf1* transcript in the $Tsf1^{JP94}$ mutant in contrast to wild-type flies after *M. luteus* infection (*SI Appendix*, Fig. S1*D*). $Tsf1^{JP94}$ mutants were viable and did not show any obvious morphological defects under standard laboratory conditions. Also, both Toll and Imd pathways were induced properly in this mutant, as illustrated by the level of *Drs* and *Dpt* expression after *M. luteus* and *Ecc15* infections, respectively (*SI Appendix*, Fig. S1 *E* and *F*). Next, we compared iron distribution between wild-type and $Tsf1^{JP94}$ mutant tissues after *M. luteus* infection. There was no difference in iron content between uninfected wild-type and $Tsf1^{JP94}$ mutant in the hemolymph and fly tissues. Strikingly, after *M. luteus* infection, $Tsf1^{JP94}$ mutant contained significantly more iron in the hemolymph and significantly less in the fat body compared to wild-type flies (Fig. 2*E*). Overexpression of a wild-type copy of *Tsf1* in the $Tsf1^{JP94}$ mutant background rescued the phenotype (Fig. 2*F*). This result suggests that Tsf1 contributes to the iron relocalization from hemolymph to fat body after infection. To confirm this result and identify the source of Tsf1, we performed tissue-specific RNA interference (RNAi)-mediated *Tsf1* knockdown. Similar to $Tsf1^{JP94}$, flies with ubiquitous *Tsf1* knockdown retain high iron load in the hemolymph after infection (Fig. 2*F*). Fat body-specific, but not gut- or hemocyte-specific, *Tsf1* knockdown recapitulated this phenotype, indicating that the fat body is the major source of Tsf1 (*SI Appendix*, Fig. S1*G*). This result is consistent with a recent study (32) that showed Tsf1 is produced by the fat body and is secreted into the hemolymph (Fig. 2*C*), where it binds to iron and transports it to the fat body. DmTsf1 is homologous to human plasma Transferrin that has been functionally and structurally well characterized. Structure–function analysis has shown that five amino acid residues of hTsf are required for iron binding (11, 12). Sequence homology analysis showed that three out of these five residues are conserved in *Drosophila* Tsf1 (*SI Appendix*, Fig. S2). We substituted these three residues with alanine and generated a fly line that overexpresses this mutated form of *Tsf1* ($UAS\text{-}Tsf1^{Fe\ mut}$) that should

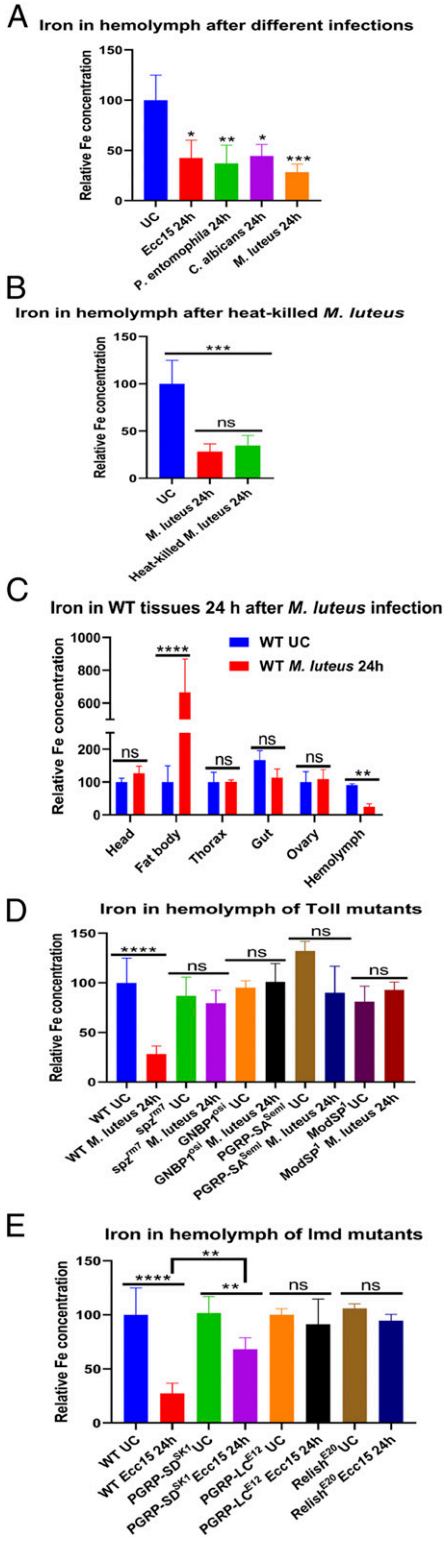

Fig. 1. *D. melanogaster* exhibits infection-induced hypoferremia. (*A* and *B*) Iron content of flies' hemolymph after indicated infections. Asterisks indicate statistical significance relative to unchallenged (UC) (one-way ANOVA) ($n = 50$ flies per treatment). (*C*) Iron content in indicated tissues of wild-type (WT) flies 24 h after *M. luteus* infection compared to uninfected controls ($n = 20$ organs per group, $n = 50$ flies for hemolymph). (*D* and *E*) Hemolymph iron content (*D*) in Toll pathway mutants (spz^{rm7}, $GNBP1^{osi}$, $PGRP\text{-}SA^{Seml}$, and $ModSP^{1}$) 24 h after *M. luteus* infection and (*E*) in Imd pathway mutants ($PGRP\text{-}SD^{sk1}$, $PGRP\text{-}LC^{E12}$, and $Relish^{E20}$) 24 h after heat-killed *Ecc15* injection ($n = 50$ flies per group). For all graphs, iron content in uninfected wild-type flies was set to 100, and all other values were expressed as a percentage of this value. The mean and SD of three independent experiments are shown. $^{*}P \le 0.05$; $^{**}P \le 0.01$; $^{***}P \le 0.001$; $^{****}P \le 0.0001$; ns, nonsignificant, $P > 0.05$.

not bind iron. Overexpression of this mutated version of *Tsf1* did not rescue the $Tsf1^{JP94}$ mutant (Fig. 2*F*). This reinforces our conclusion that the ability of Tsf1 to bind iron is necessary to relocate the metal from hemolymph to fat body.

***Transferrin 1* Mutants Are Susceptible to *Pseudomonas* and Mucorales Fungal Infections.** Having shown that Tsf1 mediates the transport of iron upon infection, we investigated the relevance of this immune process in host survival to various pathogens. As shown in *SI Appendix*, Fig. S3, $Tsf1^{JP94}$ mutants exhibited wild-type levels of survival after systemic infection with *Ecc15*, *Enterobacter cloacae*, *Listeria monocytogenes*, *Streptococcus pyogenes*, *S. aureus, Enterococcus faecalis*, *C. albicans*, and *B. bassiana* (natural infection). We next explored $Tsf1^{JP94}$ mutants' susceptibility to fungi of the order Mucorales and *Pseudomonas* bacteria, the virulence of which is known to be strongly modulated by iron availability (43–45). Interestingly, we observed an increased susceptibility of $Tsf1^{JP94}$ mutant and *Tsf1 RNAi* flies to systemic infection with *Cunninghamella bertholletiae*, a representative Mucorales that infects humans (Fig. 3 *A* and *B*). Notably, Toll pathway activation by *C. bertholletiae* was not affected in the $Tsf1^{JP94}$ mutant (Fig. 3*C*). Overexpression of wild-type *Tsf1*, but not the iron binding sites mutated form of *Tsf1*, rescued the increased susceptibility of $Tsf1^{JP94}$ mutants to this fungus (Fig. 3*D*). We could almost completely rescue the susceptibility of $Tsf1^{JP94}$ mutants to *C. bertholletiae* by injection of the iron chelator bathophenanthrolinedisulfonic acid disodium (BPS) (46), suggesting that excessive iron in the hemolymph of $Tsf1^{JP94}$ mutants contributes to their increased susceptibility to *C. bertholletiae* (Fig. 3*E*). BPS injection also has a protective effect in wild-type flies, although not as significant as in $Tsf1^{JP94}$ mutants. We obtained similar increased sensitivity of $Tsf1^{JP94}$ mutants to another Mucorales representative, *Rhizopus oryzae* (Fig. 3*F*), suggesting that transferrins are important for the defense against this group of fungi.

We also observed an increased susceptibility of $Tsf1^{JP94}$ and *Tsf1* RNAi flies to systemic infections with two *Pseudomonas* species, *Pseudomonas aeruginosa* (Fig. 4 *A* and *B*) and *P. entomophila* (*SI Appendix*, Fig. S4 *A* and *B*). Consistent with the impaired resistance of $Tsf1^{JP94}$ flies, *P. aeruginosa* (Fig. 4*C*) and *P. entomophila* (*SI Appendix*, Fig. S4*C*) reached significantly higher loads in $Tsf1^{JP94}$ mutants. As $Tsf1^{JP94}$ mutants showed wild-type levels of Imd pathway activation after *P. entomophila* and *P. aeruginosa* infections (*SI Appendix*, Fig. S4*D*), the increased susceptibility of these mutants was not due a general immune deficiency but rather due to their inability to sequester iron away from the hemolymph. Consistent with this, injection of the iron chelator BPS into the hemolymph significantly improves survival of $Tsf1^{JP94}$ mutants upon *P. aeruginosa* infection (Fig. 4*D*). Ubiquitous overexpression of wild-type but not the mutated *Tsf1* form was sufficient to rescue the enhanced susceptibility of $Tsf1^{JP94}$ mutants to *P. aeruginosa* (Fig. 4*E*) and *P. entomophila* (*SI Appendix*, Fig. S4*E*). Similarly, *P. aeruginosa* elevated load in $Tsf1^{JP94}$ mutants was not observed when wild type but not the mutated *Tsf1* form was ubiquitously overexpressed (Fig. 4*F*). Interestingly, overexpression of wild type but not the mutated *Tsf1* form in wild-type background led to a significant reduction in *P. aeruginosa* load (Fig. 4*F*), which correlated with improved survival of the flies (Fig. 4*B*). This protective effect of Tsf1 overexpression is comparable to the effect of BPS injection (Fig. 4*D*), indicating that Tsf1 may function as endogenous iron chelator.

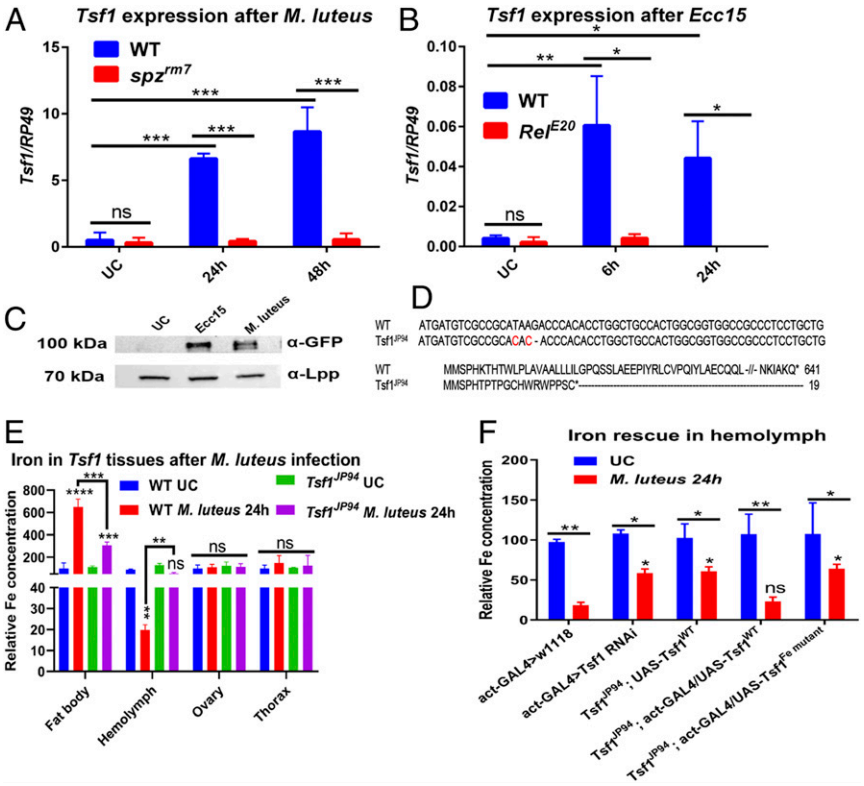

Fig. 2. Contribution of Tsf1 to infection-induced hypoferremia. (*A* and *B*) *Tsf1* expression (*A*) in wild type and spz^{rm7} mutants after *M. luteus* infection and (*B*) in wild type and Rel^{E20} mutants after *Ecc15* infection, measured by RT-qPCR ($n = 10$ flies per group). (*C*) Western blot of Tsf1-GFP hemolymph extracted 24 h after *M. luteus* or *Ecc15* infection showing Tsf1 induction after these infections. Lipophorin (α-Lpp) was used as a loading control. A representative Western blot out of three independent experiments is shown ($n = 30$ flies per group). (*D*) Nucleotide and amino acid sequence alignment of wild-type and $Tsf1^{JP94}$ transferrin. (*E*) Iron content in indicated tissues of wild-type and $Tsf1^{JP94}$ flies 24 h after *M. luteus* infection compared to uninfected controls ($n = 20$ organs per group, $n = 50$ flies for hemolymph). Asterisks above the red bars indicate significance relative to wild-type UC. (*F*) Hemolymph iron content of indicated fly genotypes 24 h after *M. luteus* infection compared to uninfected controls ($n = 50$ flies per group). Asterisks above bars indicate significance relative to *act-GAL4 > w1118 M. luteus*-infected. Iron content in uninfected wild-type or *act-GAL4 > w1118* flies was set to 100, and all other values were expressed as a percentage of this value. The mean and SD of three independent experiments are shown unless otherwise stated. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P \leq 0.0001$; ns, nonsignificant, $P > 0.05$.

Pyoverdine-Mediated Iron Acquisition Is Essential for *P. aeruginosa* Infection in *Drosophila*. Siderophore production by pathogens is a key mechanism for scavenging iron from a variety of host iron sources. Siderophores are small ferric iron chelators capable of binding iron with high affinity, and can therefore effectively outcompete host transferrin (1). *P. aeruginosa* produces large amounts of pyoverdine and pyochelin siderophores that scavenge iron and deliver it to the bacteria (47). We next compared the susceptibility of wild-type and $Tsf1^{JP94}$ mutant flies to wild-type *P. aeruginosa* PA14 and to its transposon insertion derivatives *pchE* and *pvdP* that lack the siderophores pyochelin and pyoverdine, respectively (48). As shown in Fig. 5*A*, the *pchE* mutant was as efficient as wild-type *P. aeruginosa* at killing both wild-type and $Tsf1^{JP94}$ mutant flies, indicating that pyochelin is not required for *P. aeruginosa* virulence in *Drosophila*. Interestingly, the virulence of *pvdP* mutant was attenuated compared to wild-type *P. aeruginosa* in wild-type flies, indicating that pyoverdine contributes to *Pseudomonas* pathogenicity. In contrast, the pathogenicity of *pvdP* was similar to that of wild-type *P. aeruginosa* when assayed in $Tsf1^{JP94}$ mutant background (Fig. 4*B*). In line with this, *pvdP* colony-forming unit (cfus) were the same as for wild-type *P. aeruginosa* in $Tsf1^{JP94}$ mutant but were significantly lower in wild-type flies (Fig. 5*C*). To further reinforce the role of pyoverdine, we assessed the survival of flies preinjected prior to infection with flucytosine, a known repressor of pyoverdine (49). We observed that flucytosine was protective in wild-type flies but had no effect in $Tsf1^{JP94}$ mutant (Fig. 5*D*), which is similar to what we found with the genetic disruption of pyoverdine (Fig. 5*B*). Using another *P. aeruginosa* strain (PAO1) and its derived pyochelin and pyoverdine mutants (50), we confirmed that pyoverdine is essential for virulence in wild-type flies but not in $Tsf1^{JP94}$ mutant (Fig. 5*E*). This result suggests that 1) pyoverdine is necessary for *P. aeruginosa* to acquire iron from wild-type *Drosophila*, and 2) pyoverdine becomes dispensable in $Tsf1^{JP94}$ mutant due to excessive iron present in the hemolymph. Additionally, we assessed the virulence of *P. aeruginosa* PAO1 *tonB1* mutants that are defective for siderophore-mediated iron uptake (51). These mutants were severely attenuated in both wild-type and $Tsf1^{JP94}$ mutant flies. Nevertheless, they still killed $Tsf1^{JP94}$ mutants faster than wild-type flies, likely due to high iron levels in the hemolymph of $Tsf1^{JP94}$ mutants (Fig. 5*F*). Thus, using bacterial and fly genetics, we could show that Tsf1 is required for the *Drosophila* defense against certain pathogens, by sequestering iron from hemolymph and limiting pathogen access to this essential element.

Transferrin 1 Plays a Role in Intestinal Immunity. Considering that *Tsf1* is induced by *Ecc15* and *P. entomophila* oral infections in the gut in an Imd pathway-dependent manner (Fig. 6 *A* and *B*) (52), we explored whether this iron transporter is also implicated in intestinal immunity. As shown in Fig. 6*C*, $Tsf1^{JP94}$ mutants succumbed faster to *P. entomophila* oral infection compared to wild-type flies. Enterocyte-specific *Tsf1* knockdown by RNAi also resulted in increased sensitivity to *P. entomophila* oral infection (Fig. 6*D*). This increased susceptibility was not due to an impaired Imd pathway activity (*SI Appendix*, Fig. S5*A*) or

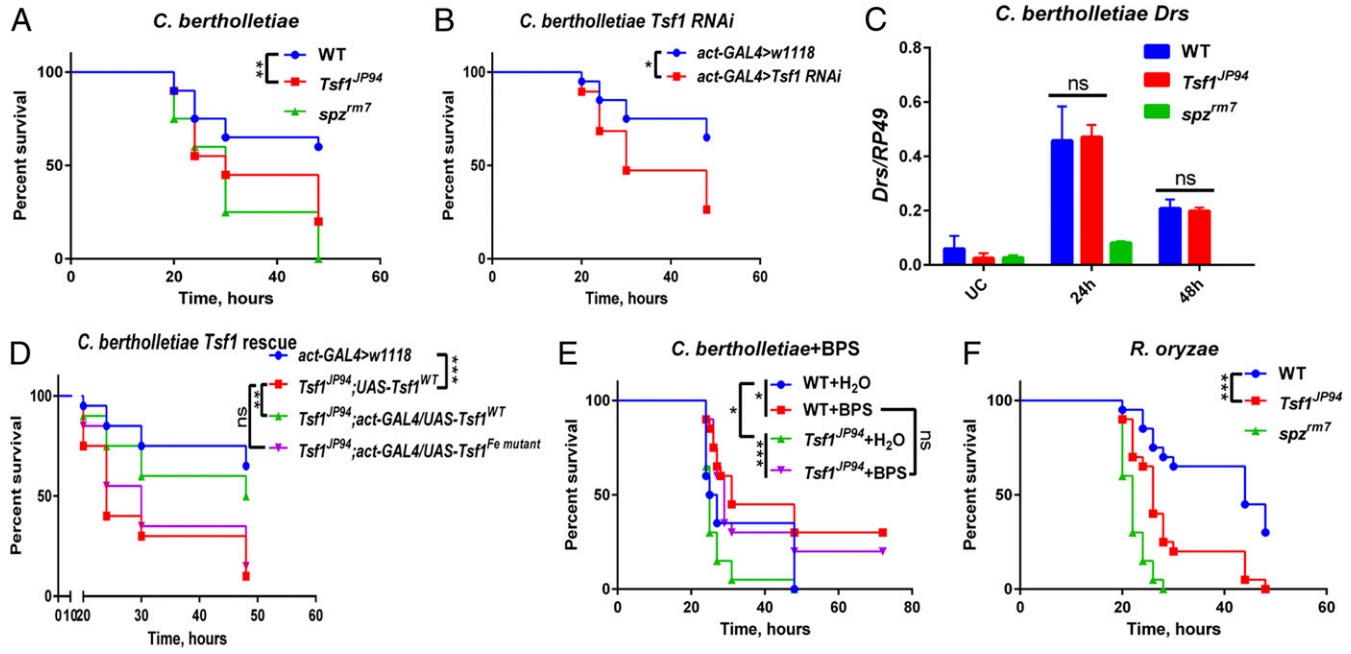

Fig. 3. Tsf1 is required for the defense against Mucorales. (*A*) Survival rates of wild-type, *spz*^{rm7}, *and Tsf1*^{JP94} flies infected with *C. bertholletiae* (10^6 spores per ml). (*B*) Survival rates of flies with ubiquitous knockdown of *Tsf1* is significantly reduced compared to wild-type flies after infection with *C. bertholletiae*. (*C*) *Drs* expression in wild-type, *spz*^{rm7}, *and Tsf1*^{JP94} flies after *C. bertholletiae* infection measured by RT-qPCR (n = 10 flies per group). The mean and SD of three independent experiments are shown. (*D*) Increased susceptibility of *Tsf1*^{JP94} mutant flies to *C. bertholletiae* infection is rescued by the ubiquitous overexpression of the wild-type (*UAS-Tsf1*^{WT}) but not mutated form of *Tsf1* (*UAS-Tsf1*$^{Fe\ mutant}$). (*E*) Survival rates of wild-type and *Tsf1*^{JP94} flies preinjected with 13.4 nL of H_2O (control) or with 13.4 nL of 200 μM iron chelator BPS prior to infection with *C. bertholletiae*. (*F*) Survival rates of wild-type, *spz*^{rm7}, *and Tsf1*^{JP94} flies infected with *R. oryzae* (10^6 spores per mL). Survival graphs show one representative experiment out of three independent experiments with similar results with two or three cohorts of 20 male flies per treatment. *$P \le 0.05$; **$P \le 0.01$; ***$P \le 0.001$; ****$P \le 0.0001$; ns, nonsignificant, $P > 0.05$.

compromised gut repair, as PH3 staining revealed that *Tsf1*^{JP94} mutants had the same number of proliferating stem cells after *Ecc15* infection as wild-type flies (*SI Appendix*, Fig. S5*B*). In line with this, *upd3*, a ligand of the JAK-STAT pathway playing a crucial role in epithelial renewal (52), was expressed in *Tsf1*^{JP94} mutants at wild-type levels after both *Ecc15* and *P. entomophila* infections (*SI Appendix*, Fig. S5*C*). Importantly, we found that the susceptibility of *Tsf1*^{JP94} mutants to *P. entomophila* oral infection could be rescued by gut-specific overexpression of wild-type but not iron binding-defective *Tsf1* (Fig. 6*E*), indicating that the ability of Tsf1 to bind iron is necessary for defense against *P. entomophila* intestinal infection, similar to its effect in systemic infection. Therefore, Tsf1 plays a similar role in the gut as in the hemolymph. To further reinforce this conclusion, we performed oral infection with *P. aeruginosa* PA14 and observed that *Tsf1*^{JP94} mutants were also more susceptible to this pathogen (Fig. 6*F*). Consistent with systemic infections, pyoverdine-deficient *pvdP* mutant virulence was attenuated in wild-type but not in *Tsf1*^{JP94} mutant flies, while pyochelin-deficient mutant virulence was comparable to wild-type *P. aeruginosa* (Fig. 6*F*). The fact that pyoverdine is unnecessary for virulence in *Tsf1*^{JP94} mutant suggests that there is enough available iron in the guts of these flies. Indeed, we could detect significantly more iron in *Tsf1*^{JP94} mutant guts compared to wild-type unchallenged guts (*SI Appendix*, Fig. S5*D*). We conclude that iron sequestration by transferrin is also a potent mechanism contributing to intestinal immunity.

Discussion

Despite the well-established role of iron and iron-binding proteins in mammalian immunity, their role in insect immunity remains understudied. Using *D. melanogaster* as a model, we found that 1) flies trigger a hypoferremic response after infection to limit iron availability to invading microbes, and 2) the iron transporter Tsf1 mediates nutritional immunity by sequestering iron from invading pathogens (see model in *SI Appendix*, Fig. S6).

In mammals, hypoferremia of infection has been known since the 1940s and is characterized by iron withdrawal from the serum and accumulation in storage organs, like the liver (8). Consistent with mammalian studies, we discovered that flies trigger a hypoferremic response upon challenge with a variety of pathogens. During this response, iron was relocated from the hemolymph to the fat body, which is the equivalent of mammalian liver. Given that the same response was also triggered by heat-killed bacteria, depleting hemolymph iron appears to be a host-mediated process. Notably, this mechanism requires the *Drosophila* Toll and Imd pathways, since mutants for these pathways were not able to induce a hypoferremic response to infection. Thus, beyond regulating antimicrobial effector-mediated immunity, the Toll and Imd pathways also mediate nutritional immunity in flies.

We hypothesized that Tsf1 might play a major role in *Drosophila* nutritional immunity downstream of Toll and Imd pathways. Out of the three *Drosophila* transferrins, Tsf1 is consistently induced by a variety of immune challenges (33, 34, 36). Using a *Transferrin* null mutant, we indeed found that Tsf1 is required for iron trafficking from the hemolymph to the fat body after infection. Therefore, our study agrees with a recently published work that Tsf1 is indeed an iron transporter (32). However, in contrast to Xiao et al. (32), who used a *Tsf1 RNAi*, we did not observe any lethality or developmental defects in *Tsf1*^{JP94} mutants and *Tsf1 RNAi*. This discrepancy could be due to the fact that we used different RNAi lines targeting different parts of the transcript or because, in contrast to Xiao et al., we used conditional knockdown specifically during adult stage.

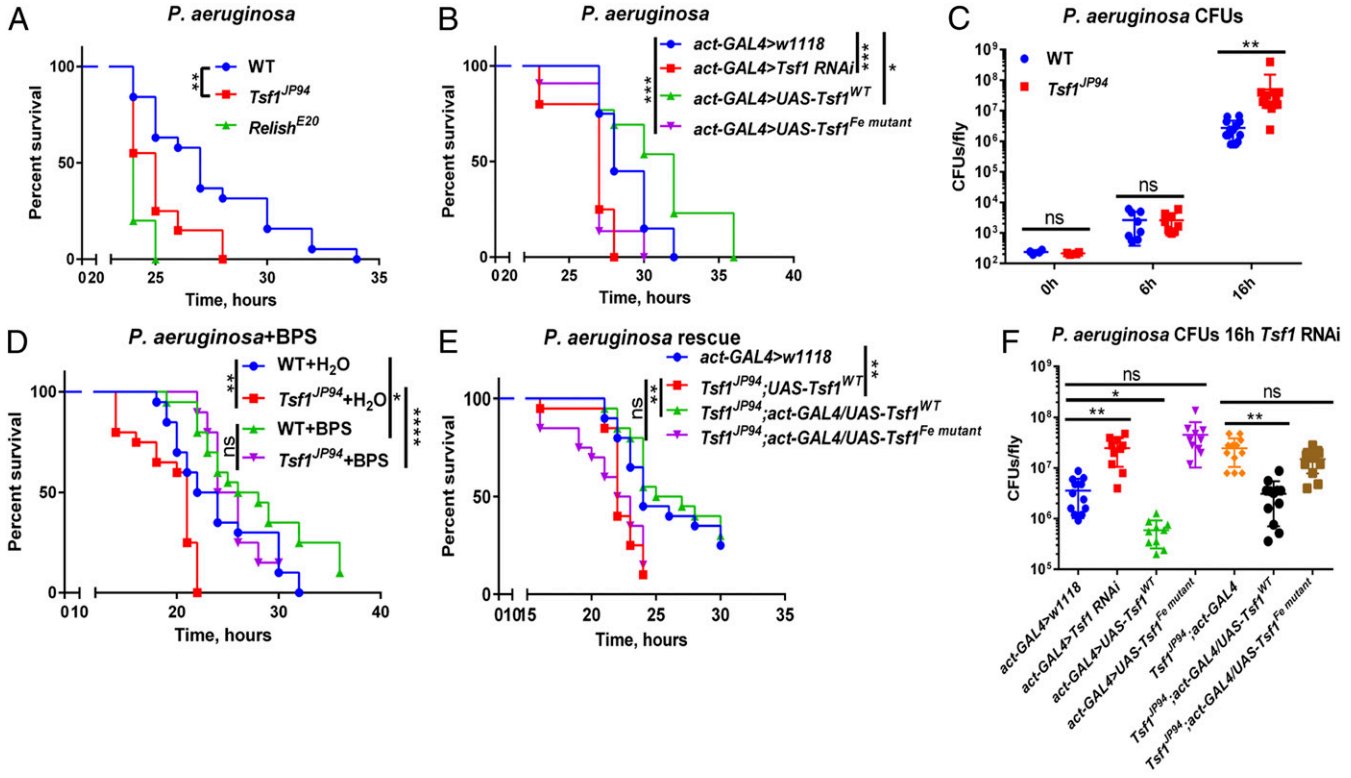

Fig. 4. Tsf1-mediated iron sequestration protects against *P. aeruginosa* infection. (*A*) Survival rates of wild-type, *Rel*^{E20}, *and Tsf1*^{JP94} flies infected with *P. aeruginosa*. (*B*) Survival rates of flies with ubiquitous knockdown of *Tsf1*, and overexpression of either wild-type (*UAS-Tsf1*^{WT}) or mutated *Tsf1* after *P. aeruginosa* infection. (*C*) Measurement of *P. aeruginosa* burden at different time points after infection of wild type and *Tsf1*^{JP94} mutant. (*D*) Survival rates of wild-type and *Tsf1*^{JP94} flies preinjected with 13.4 nL of H_2O (control) or with 13.4 nL of 200 μM iron chelator BPS prior to infection with *P. aeruginosa*. (*E*) Increased susceptibility of *Tsf1*^{JP94} mutant flies to *P. aeruginosa* infection is rescued upon ubiquitous overexpression of wild-type (*UAS-Tsf1*^{WT}) but not mutated form of *Tsf1* (*UAS-Tsf1*$^{Fe\ mutant}$). (*F*) *Pseudomonas aeruginosa* load 16 h after infection of flies with indicated genotypes. For cfu counts, each dot represents cfus from a pool of five animals, calculated per fly. The mean and SD are shown. Survival graphs show one representative experiment out of three independent experiments with similar results with two or three cohorts of 20 male flies per treatment. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$; ns, nonsignificant, $P > 0.05$.

Our study raises an intriguing question regarding how Tsf1 relocates iron specifically to the fat body and not to other tissues. One possibility could be that fat body expresses transferrin receptor that directs iron transport by Tsf1. To date, no transferrin receptor homolog has been identified in *Drosophila* (and other insects). Finding this receptor and mechanism of iron uptake by the fat body during infection would be an interesting future research avenue.

Despite the elevated level of iron in the hemolymph, *Tsf1*^{JP94} mutants did not show any increased susceptibility to the majority of pathogens that we tested, including several Gram-positive and Gram-negative bacteria, fungi, and yeast. However, we observed increased susceptibility of *Tsf1*^{JP94} mutants to Mucorales fungi and to *Pseudomonas* bacteria. This increased susceptibility was linked to the ability of Tsf1 to bind iron, as we could not rescue *Tsf1*^{JP94} mutants' susceptibility with a form of *Tsf1* mutated in iron binding sites. Given that Mucorales virulence is known to be enhanced by increased iron supply (43), it is reasonable to assume that *Tsf1*^{JP94} flies are more susceptible to these infections because of elevated hemolymph iron levels. *P. aeruginosa* virulence is also known to be strongly regulated by iron. The importance of iron to *P. aeruginosa* is exemplified by the fact that 6% of its transcribed genes are iron responsive (44, 45). Not surprisingly, these bacteria evolved a diversity of mechanisms to scavenge iron from a variety of host iron sources. Siderophore production is one such mechanism. Pyoverdine and pyochelin are two major siderophores produced by *P. aeruginosa* (47), and pyoverdine is essential for *P. aeruginosa* pathogenesis in various mammalian and invertebrate host models (48, 53–55). A recent study showed that *P. aeruginosa* mutants for the algR regulator are deficient for pyoverdine production, and virulence is attenuated in the algR mutant in a *Drosophila* oral infection model (56). In line with this, we showed that the *P. aeruginosa* pyoverdine mutant is less pathogenic compared to its wild-type counterpart during both systemic and oral infections. Importantly, *Tsf1*^{JP94} mutant flies were killed by the *P. aeruginosa* pyoverdine mutant as efficiently as by wild-type bacteria. This suggests that 1) pyoverdine is necessary for iron acquisition by *P. aeruginosa* during *Drosophila* infection and, 2) in the absence of transferrin, pyoverdine becomes unessential, as there is an excess of free iron. The extreme dependence of *P. aeruginosa* on iron makes these bacteria vulnerable to iron chelation therapy by transferrin, which has been proposed as a novel antimicrobial therapy (24). Efficacy of such therapy is also supported by our results showing that Tsf1 overexpression is sufficient to increase the survival of flies to *Pseudomonas* infections.

Why Tsf1 flies are not sensitive to the majority of pathogens is an intriguing question that our work raises. A likely explanation for this result is that, beyond iron sequestration, the host relies on other arms of defense, like phagocytosis or production of antimicrobial peptides, to combat pathogens. Those additional arms of defense might be sufficient to eliminate most pathogens at the infectious doses we used, even if iron sequestration is impaired. There is accumulating evidence that some elements of the immune system are specifically required against certain pathogens. For instance, from *Drosophila* studies, it is known that melanization is important to survive *S. aureus* infection (57),

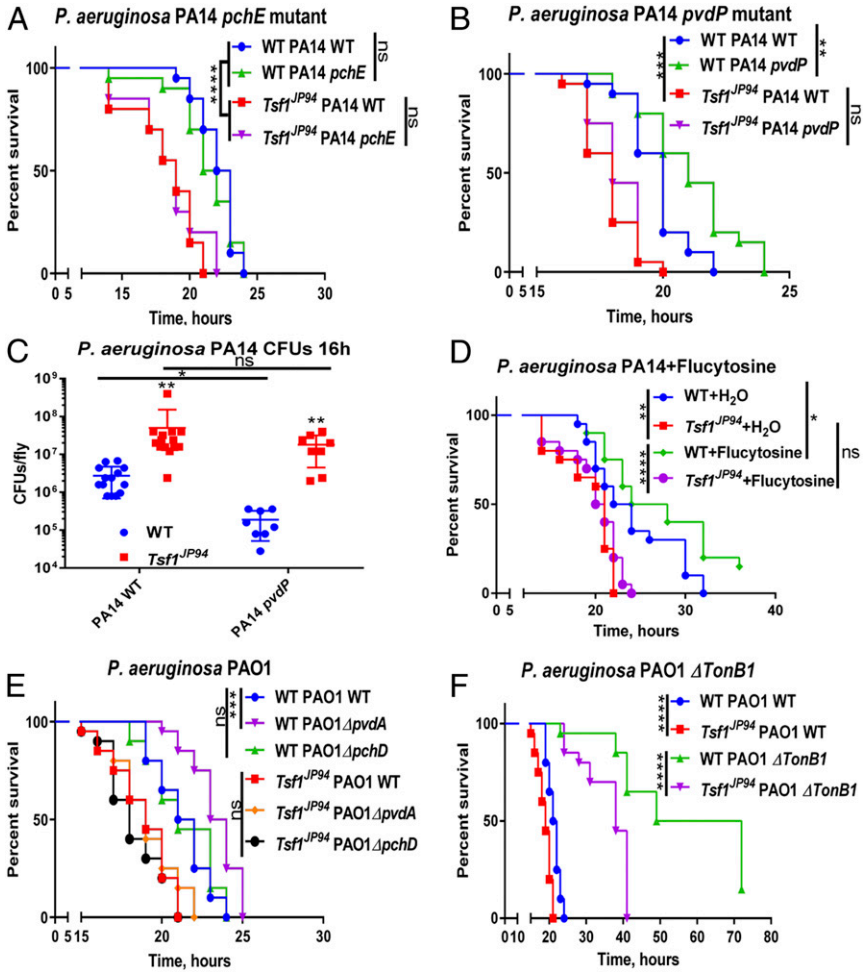

Fig. 5. Pyoverdine is required for *P. aeruginosa* virulence against wild-type but not $Tsf1^{JP94}$ flies. (*A* and *B*) Survival rates of wild-type and $Tsf1^{JP94}$ mutant flies infected with (*A*) wild-type, pyochelin-deficient *pchE* or (*B*) pyoverdine-deficient *pvdP P. aeruginosa* PA14. (*C*) Measurement of wild-type and *pvdP PA14* load 16 h after infection of wild-type and $Tsf1^{JP94}$ flies. Each dot represents cfus from a pool of five animals, calculated per fly. The mean and SD are shown. (*D*) Survival rates of wild-type and $Tsf1^{JP94}$ flies preinjected with 13.4 nL of H_2O (control) or with 13.4 nL of 100 μM repressor of pyoverdine flucytosine prior to infection with *P. aeruginosa*. (*E* and *F*) Survival rates of wild-type and $Tsf1^{JP94}$ flies infected with wild-type, (*E*) *ΔpchD*, *ΔpvdA*, and (*F*) *ΔTonB1 P. aeruginosa* PAO1. Survival graphs show one representative experiment out of three independent experiments with similar results with two or three cohorts of 20 male flies per treatment. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P \leq 0.0001$; ns, nonsignificant, $P > 0.05$.

phagocytosis [*S. aureus* and *Salmonella typhimurium* (58, 59)], the antimicrobial peptide Diptericin [*Providencia rettgeri* (60, 61)], and Drosocin [*E. cloacae* (61)]. Our study suggests that iron sequestration is an important defense mechanism against Mucorales and *Pseudomonas*, while, in the case of other pathogens, other arms of defense might play a more prominent role. It will be an interesting avenue for future research to explore functional redundancy between different arms of the host defense against specific pathogens.

Taken together, our results reveal that nutritional immunity is an important arm of innate immune defense in *Drosophila*. Using fly and bacterial genetics, we showed that the iron transporter Tsf1 mediates nutritional immunity by sequestering iron from the pathogens in vivo on the whole-organism level. So far, two studies have identified immune-related phenotypes resulting from RNAi-mediated knockdown of transferrin: increased prevalence of trypanosome infections in *Glossina morsitans*, and increased mortality of *Bacillus thuringiensis*-infected *Plutella xylostella* (62, 63). Those in vivo and accumulating in vitro studies support the immune role of insect transferrins against infections via an iron withholding strategy. Considering the multifactorial function of iron beyond immunity, our work opens avenues for future research addressing the role of transferrins in the host physiology.

Materials and Methods

Pathogen Strains and Survival Experiments. The bacterial strains used and their respective optical densities (OD) at 600 nm were, unless otherwise stated, the Gram-negative bacteria *P. carotovorum* (*Ecc15*, OD 200), *E. cloacae β12* (OD 200), *P. entomophila* (OD 1), *P. aeruginosa* PA14 (OD 1), *P. aeruginosa* PA14 *pvdP* (OD 1), *P. aeruginosa* PA14 *pchE* (OD 1), *P. aeruginosa* PAO1 (OD 1), *P. aeruginosa* PAO1 *ΔpvdA* (OD 1), *P. aeruginosa* PAO1 *ΔpchD* (OD 1), and *P. aeruginosa* PAO1 *ΔtonB1* (OD 1); the DAP-type peptidoglycan-containing Gram-positive bacteria *L. monocytogenes BUG2377* (, OD 40); the Lys-type peptidoglycan containing Gram-positive bacteria *M. luteus* (OD 200), *S. aureus* (OD 0.5), *S. pyogenes* ATCC19615 (OD 200), and *E. faecalis* OG1RF (OD 15); and the yeast *C. albicans* (OD 200). Microbes were cultured in Brain-Heart Infusion Broth (*L. monocytogenes* and *E. faecalis*), Yeast extract-Peptone-Glucose Broth (*C. albicans*), or Luria Broth (all others) at 29 °C (*E. carotovora*, *M. luteus*, *C. albicans*, and *P. entomophila*) or 37 °C (all others). To compare the virulence of *P. aeruginosa* wild type and siderophore mutants, bacteria were grown in M9 minimal media at 37 °C to stimulate siderophore production. *P. aeruginosa* PAO1 *ΔtonB1* mutant was grown in media supplemented with 100 μM $FeSO_4$. The *pvdP* and *pchE P. aeruginosa* PA14 mutants were grown in the presence of 15 μg/mL gentamicin. Spores of the entomopathogenic fungus *B. bassiana 802* and

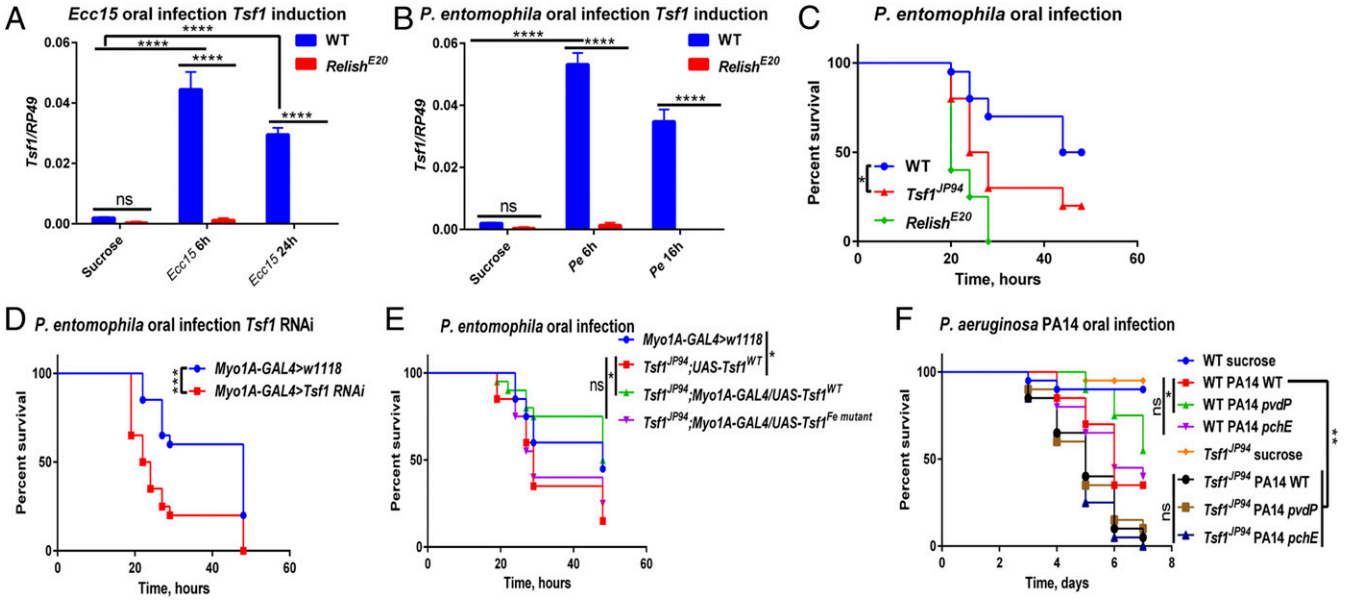

Fig. 6. Tsf1 contributes to intestinal immunity. (*A* and *B*) *Tsf1* expression in wild-type and Rel^{E20} guts after (*A*) *Ecc15* and (*B*) *Pe* infection, measured by RT-qPCR ($n = 20$ guts per group). The mean and SD of three independent experiments are shown. (*C*) Survival rates of wild-type, Rel^{E20}, and $Tsf1^{JP94}$ flies orally infected with *P. entomophila*. (*D*) Survival rates of flies with gut-specific (*Myo1A*) knockdown of *Tsf1* are significantly reduced compared to wild-type flies after *Pe* oral infection. (*E*) Increased susceptibility of $Tsf1^{JP94}$ mutant flies to *Pe* oral infection is rescued by gut-specific overexpression of the wild-type ($UAS\text{-}Tsf1^{WT}$) but not mutated form of *Tsf1* ($UAS\text{-}Tsf1^{Fe\ mutant}$). (*F*) Survival rates of wild-type and $Tsf1^{JP94}$ flies orally infected with wild-type, *pchE*, and *pvdP P. aeruginosa* PA14. Survival graphs show one representative experiment out of three independent experiments with similar results with two to three cohorts of 20 flies per treatment. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P \leq 0.0001$; ns, nonsignificant, $P > 0.05$.

Mucorales *C. bertholletiae* 506313 and *R. oryzae* 557969 were grown on malt agar plates at 29 °C for ~3 wk until sporulation. Natural infections were performed by shaking anesthetized flies in a Petri dish containing a sporulating culture of *B. bassiana*. Systemic infections (septic injury) were performed by pricking adult flies (2 d to 5 d old) in the thorax with a thin needle previously dipped into a concentrated pellet of a bacterial culture or in a suspension of *fungal* (*C. bertholletiae* and *R. oryzae*) spores. Infected flies were subsequently maintained at 29 °C (most of the infections) or at 25 °C (*E. faecalis*, *S. aureus*, *S. pyogenes*, and *P. aeruginosa*). In some experiments, flies were injected prior to infection with 13.4 nL of 200 μM BPS (iron chelator) or with 13.4 nL of 100 μM Flucytosine using a Nanoject apparatus (Drummond). Oral infections were performed as described previously (42, 52, 64). At least two vials of 20 flies were used for survival experiments, and survivals were repeated at least three times.

Iron Measurement Using ICP-OES. Flies were infected with different pathogens as described above. Right before hemolymph collection, 50 flies were pricked in the thorax to breach the cuticle and increase hemolymph yield. These flies were placed on a 10-μm filter of an empty mobicol spin column (MoBiTec), covered with glass beads, and centrifuged for 5 min at 4 °C, 5,000 rpm. Then 5 μL of hemolymph per each sample were digested with 0.5 mL of 32% ultrapure hydrochloric acid (VWR Chemicals) under heating conditions (60 °C) for 2 h; 9.5 mL of nitric acid was added to each sample, and the total iron concentration was measured using ICP-OES (Perkin-Elmer Optima 8300 ICP-OES). To measure iron content of tissues, tissues of interest were dissected in phosphate-buffered saline and digested in 0.5 mL of 32% ultrapure hydrochloric acid at 60 °C for 2 h. The samples were filtered to remove impurities and any undigested material. Protein concentration in digested samples was determined using the Pierce BCA protein assay kit. Iron concentration in each sample was normalized to the total protein amount to standardize sample size differences.

RT-qPCR. For quantification of messenger RNA, whole flies ($n = 10$) or dissected tissues ($n = 20$) were collected at indicated time points. Total RNA was isolated using TRIzol reagent and dissolved in RNase-free water. Five hundred nanograms of total RNA was then reverse-transcribed in 10-μL reactions using PrimeScript RT (Takara) and random hexamer primers. The qPCR was performed on a LightCycler 480 (Roche) in 96-well plates using the LightCycler 480 *SYBR Green* I Master Mix. RP49 was used as a housekeeping gene for normalization.

All data are available in the manuscript and *SI Appendix*.

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1. J. E. Cassat, E. P. Skaar, Iron in infection and immunity. *Cell Host Microbe* **13**, 509–519 (2013).
2. M. I. Hood, E. P. Skaar, Nutritional immunity: Transition metals at the pathogen-host interface. *Nat. Rev. Microbiol.* **10**, 525–537 (2012).
3. E. E. Johnson, M. Wessling-Resnick, Iron metabolism and the innate immune response to infection. *Microbes Infect.* **14**, 207–216 (2012).
4. C. A. Lopez, E. P. Skaar, The impact of dietary transition metals on host-bacterial interactions. *Cell Host Microbe* **23**, 737–748 (2018).
5. S. T. Ong, J. Z. Ho, B. Ho, J. L. Ding, Iron-withholding strategy in innate immunity. *Immunobiology* **211**, 295–314 (2006).
6. U. E. Schaible, S. H. E. Kaufmann, Iron and microbial infection. *Nat. Rev. Microbiol.* **2**, 946–953 (2004).
7. M. P. Soares, G. Weiss, The iron age of host-microbe interactions. *EMBO Rep.* **16**, 1482–1500 (2015).
8. G. E. Cartwright *et al.*, The anemia associated with chronic infection. *Science* **103**, 72 (1946).
9. F. A. Khan, M. A. Fisher, R. A. Khakoo, Association of hemochromatosis with infectious diseases: Expanding spectrum. *Int. J. Infect. Dis.* **11**, 482–487 (2007).
10. A. Bezkorovainy, "Antimicrobial properties of iron-binding proteins" in *Diet and Resistance to Disease*, M. Phillips, A. Baetz, Eds. (Springer, 1981), pp. 139–154.
11. L. A. Lambert, H. Perri, P. J. Halbrooks, A. B. Mason, Evolution of the transferrin family: Conservation of residues associated with iron and anion binding. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **142**, 129–141 (2005).
12. L. A. Lambert, Molecular evolution of the transferrin family and associated receptors. *Biochim. Biophys. Acta* **1820**, 244–255 (2012).
13. K. Gkouvatsos, G. Papanikolaou, K. Pantopoulos, Regulation of iron transport and the role of transferrin. *Biochim. Biophys. Acta* **1820**, 188–202 (2012).
14. S. Farnaud, R. W. Evans, Lactoferrin—A multifunctional protein with antimicrobial properties. *Mol. Immunol.* **40**, 395–405 (2003).

15. H. Jenssen, R. E. W. Hancock, Antimicrobial properties of lactoferrin. *Biochimie* **91**, 19–29 (2009).
16. P. P. Ward, M. Mendoza-Meneses, P. W. Park, O. M. Conneely, Stimulus-dependent impairment of the neutrophil oxidative burst response in lactoferrin-deficient mice. *Am. J. Pathol.* **172**, 1019–1029 (2008).
17. A. L. Schade, L. Caroline, An iron-binding component in human blood plasma. *Science* **104**, 340–341 (1946).
18. R. R. Arnold, M. Brewer, J. J. Gauthier, Bactericidal activity of human lactoferrin: Sensitivity of a variety of microorganisms. *Infect. Immun.* **28**, 893–898 (1980).
19. S. H. M. Rooijakkers *et al*., Human transferrin confers serum resistance against *Bacillus anthracis*. *J. Biol. Chem.* **285**, 27609–27613 (2010).
20. P. P. Ward, O. M. Conneely, Lactoferrin: Role in iron homeostasis and host defense against microbial infection. *Biometals* **17**, 203–208 (2004).
21. E.-Y. Yun *et al*., *Bombyx mori* transferrin: Genomic structure, expression and antimicrobial activity of recombinant protein. *Dev. Comp. Immunol.* **33**, 1064–1069 (2009).
22. L. M. Brummett, M. R. Kanost, M. J. Gorman, The immune properties of *Manduca sexta* transferrin. *Insect Biochem. Mol. Biol.* **81**, 1–9 (2017).
23. K. W. Bruhn, B. Spellberg, Transferrin-mediated iron sequestration as a novel therapy for bacterial and fungal infections. *Curr. Opin. Microbiol.* **27**, 57–61 (2015).
24. L. Lin *et al*., Transferrin iron starvation therapy for lethal bacterial and fungal infections. *J. Infect. Dis.* **210**, 254–264 (2014).
25. J. T. Bu, T. B. Bartnikas, The use of hypotransferrinemic mice in studies of iron biology. *Biometals* **28**, 473–480 (2015).
26. B. Lemaitre, J. Hoffmann, The host defense of *Drosophila melanogaster*. *Annu. Rev. Immunol.* **25**, 697–743 (2007).
27. N. Buchon, N. Silverman, S. Cherry, Immunity in *Drosophila melanogaster*—From microbial recognition to whole-organism physiology. *Nat. Rev. Immunol.* **14**, 796–810 (2014).
28. D. Ferrandon, J.-L. Imler, C. Hetru, J. A. Hoffmann, The *Drosophila* systemic immune response: Sensing and signalling during bacterial and fungal infections. *Nat. Rev. Immunol.* **7**, 862–874 (2007).
29. T. Tanji, Y. T. Ip, Regulators of the toll and Imd pathways in the *Drosophila* innate immune response. *Trends Immunol.* **26**, 193–198 (2005).
30. K. Tiklová, K.-A. Senti, S. Wang, A. Gräslund, C. Samakovlis, Epithelial septate junction assembly relies on melanotransferrin iron binding and endocytosis in *Drosophila*. *Nat. Cell Biol.* **12**, 1071–1077 (2010).
31. K. Mandilaras, F. Missirlis, Genes for iron metabolism influence circadian rhythms in *Drosophila melanogaster*. *Metallomics* **4**, 928–936 (2012).
32. G. Xiao, Z.-H. Liu, M. Zhao, H.-L. Wang, B. Zhou, Transferrin 1 functions in iron trafficking and genetically interacts with ferritin in *Drosophila melanogaster*. *Cell Rep.* **26**, 748–758.e5 (2019).
33. E. De Gregorio, P. T. Spellman, G. M. Rubin, B. Lemaitre, Genome-wide analysis of the *Drosophila* immune response by using oligonucleotide microarrays. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 12590–12595 (2001).
34. K. Troha, J. H. Im, J. Revah, B. P. Lazzaro, N. Buchon, Comparative transcriptomics reveals CrebA as a novel regulator of infection tolerance in *D. melanogaster*. *PLoS Pathog.* **14**, e1006847 (2018).
35. T. Yoshiga *et al*., *Drosophila melanogaster* transferrin. Cloning, deduced protein sequence, expression during the life cycle, gene localization and up-regulation on bacterial infection. *Eur. J. Biochem.* **260**, 414–420 (1999).
36. F. Levy, P. Bulet, L. Ehret-Sabatier, Proteomic analysis of the systemic immune response of *Drosophila*. *Mol. Cell. Proteomics* **3**, 156–166 (2004).
37. D. L. Geiser, J. J. Winzerling, Insect transferrins: Multifunctional proteins. *Biochim. Biophys. Acta* **1820**, 437–451 (2012).
38. T. Yoshiga, V. P. Hernandez, A. M. Fallon, J. H. Law, Mosquito transferrin, an acute-phase protein that is up-regulated upon infection. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 12337–12342 (1997).
39. K. Mandilaras, T. Pathmanathan, F. Missirlis, Iron absorption in *Drosophila melanogaster*. *Nutrients* **5**, 1622–1647 (2013).
40. A. Ciencialová *et al*., Mapping the peptide and protein immune response in the larvae of the fleshfly *Sarcophaga bullata*. *J. Pept. Sci.* **14**, 670–682 (2008).
41. I. Iatsenko, S. Kondo, D. Mengin-Lecreulx, B. Lemaitre, PGRP-SD, an extracellular pattern-recognition receptor, enhances peptidoglycan-mediated activation of the Drosophila Imd pathway. *Immunity* **45**, 1013–1023 (2016).
42. I. Iatsenko, J.-P. Boquete, B. Lemaitre, Microbiota-derived lactate activates production of reactive oxygen species by the intestinal NADPH oxidase nox and shortens Drosophila lifespan. *Immunity* **49**, 929–942.e5 (2018).
43. G. Chamilos *et al*., *Drosophila melanogaster* as a model host to dissect the immunopathogenesis of zygomycosis. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 9367–9372 (2008).
44. A. A. Reinhart, A. G. Oglesby-Sherrouse, Regulation of *Pseudomonas aeruginosa* virulence by distinct iron sources. *Genes (Basel)* **7**, E126 (2016).
45. M. L. Vasil, U. A. Ochsner, The response of *Pseudomonas aeruginosa* to iron: Genetics, biochemistry and virulence. *Mol. Microbiol.* **34**, 399–413 (1999).
46. R. E. Cowart, F. L. Singleton, J. S. Hind, A comparison of bathophenanthrolinedisulfonic acid and ferrozine as chelators of iron(II) in reduction reactions. *Anal. Biochem.* **211**, 151–155 (1993).
47. P. Visca, F. Imperi, I. L. Lamont, Pyoverdine siderophores: From biogenesis to biosignificance. *Trends Microbiol.* **15**, 22–30 (2007).
48. N. V. Kirienko *et al*., *Pseudomonas aeruginosa* disrupts *Caenorhabditis elegans* iron homeostasis, causing a hypoxic response and death. *Cell Host Microbe* **13**, 406–416 (2013).
49. F. Imperi *et al*., Repurposing the antimycotic drug flucytosine for suppression of *Pseudomonas aeruginosa* pathogenicity. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 7458–7463 (2013).
50. F. Minandri *et al*., Role of iron uptake systems in *Pseudomonas aeruginosa* virulence and airway infection. *Infect. Immun.* **84**, 2324–2335 (2016).
51. H. Takase, H. Nitanai, K. Hoshino, T. Otani, Requirement of the *Pseudomonas aeruginosa tonB* gene for high-affinity iron acquisition and infection. *Infect. Immun.* **68**, 4498–4504 (2000).
52. N. Buchon, N. A. Broderick, M. Poidevin, S. Pradervand, B. Lemaitre, *Drosophila* intestinal response to bacterial infection: Activation of host defense and stem cell proliferation. *Cell Host Microbe* **5**, 200–211 (2009).
53. J. M. Meyer, A. Neely, A. Stintzi, C. Georges, I. A. Holder, Pyoverdin is essential for virulence of *Pseudomonas aeruginosa*. *Infect. Immun.* **64**, 518–523 (1996).
54. N. V. Kirienko, F. M. Ausubel, G. Ruvkun, Mitophagy confers resistance to siderophore-mediated killing by *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* **112**, 1821–1826 (2015).
55. A. Zaborin *et al*., Red death in *Caenorhabditis elegans* caused by *Pseudomonas aeruginosa* PAO1. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 6327–6332 (2009).
56. A. S. Little *et al*., *Pseudomonas aeruginosa* AlgR phosphorylation status differentially regulates pyocyanin and pyoverdine production. *MBio* **9**, e02318-17 (2018).
57. J. P. Dudzic, M. A. Hanson, I. Iatsenko, S. Kondo, B. Lemaitre, More than black or white: Melanization and toll share regulatory serine proteases in *Drosophila*. *Cell Rep.* **27**, 1050–1061.e3 (2019).
58. A. Defaye *et al*., Genetic ablation of *Drosophila* phagocytes reveals their contribution to both development and resistance to bacterial infection. *J. Innate Immun.* **1**, 322–334 (2009).
59. B. Charroux, J. Royet, Elimination of plasmatocytes by targeted apoptosis reveals their role in multiple aspects of the *Drosophila* immune response. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 9797–9802 (2009).
60. R. L. Unckless, S. M. Rottschaefer, B. P. Lazzaro, The complex contributions of genetics and nutrition to immunity in *Drosophila melanogaster*. *PLoS Genet.* **11**, e1005030 (2015).
61. M. A. Hanson *et al*., Synergy and remarkable specificity of antimicrobial peptides in vivo using a systematic knockout approach. *eLife* **8**, e44341 (2019).
62. J. Kim, Y. Kim, A viral histone H4 suppresses expression of a transferrin that plays a role in the immune response of the diamondback moth, *Plutella xylostella*. *Insect Mol. Biol.* **19**, 567–574 (2010).
63. M. J. Lehane, W. Gibson, S. M. Lehane, Differential expression of fat body genes in *Glossina morsitans morsitans* following infection with *Trypanosoma brucei brucei*. *Int. J. Parasitol.* **38**, 93–101 (2008).
64. N. Vodovar *et al*., *Drosophila* host defense after oral infection by an entomopathogenic *Pseudomonas* species. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 11414–11419 (2005).