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Genetic Ablation of *Drosophila* Phagocytes Reveals Their Contribution to Both Development and Resistance to Bacterial Infection

Arnaud Defaye[a, d] Iwan Evans[b] Michèle Crozatier[c] Will Wood[b]
Bruno Lemaitre[a, e] François Leulier[a]

[a]Centre de Génétique Moléculaire, CNRS FRE 3144, Gif sur Yvette, France; [b]Department of Biology and Biochemistry, University of Bath, Bath, UK; [c]Centre de Biologie du Développement, UMR 5547 and IFR 109, CNRS and Université Paul Sabatier, Toulouse III, Toulouse, and [d]Institut de Biologie du Développement de Marseille-Luminy, CNRS UMR 6216, Université de la Méditerranée Aix-Marseille II, Marseille, France; [e]Global Health Institute, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

Key Words
Haemocytes · Phagocytes · *Drosophila* · Invertebrate immunity

Abstract
Drosophila phagocytes participate in development and immune responses through their abilities to perform phagocytosis and/or secrete extra-cellular matrix components, antimicrobial peptides, clotting factors and signalling molecules. However, our knowledge of their functional impact on development and host resistance to infection is limited. To address this, we have used a genetic cell ablation strategy to generate *Drosophila* individuals lacking functional phagocytes. Our results highlight the essential contribution of phagocytes to embryonic development including central nervous system morphogenesis. Phagocytes also ensure optimal viability during post-embryonic development through immune functions. The use of phagocyte-depleted flies reveals the contribution of phagocytes in the resistance of *Drosophila* adults upon systemic infections with specific bacteria. Phagocytes were not involved in the expression of antimicrobial peptides by the fat body indicating a clear separation between cellular and humoral immune responses at this stage. Finally, we confirm that phagocytosis is a critical effector mechanism of the cellular arm by demonstrating that phagocytosis contributes to resistance to infection with *Staphylococcus aureus* in adults. Our results highlight the power of this cell ablation strategy to reveal the contribution of phagocytes to specific biological processes. We now provide a blueprint of phagocyte importance during both development and innate immune responses in *Drosophila*.

Introduction

In mammals, phagocytes of the blood cell lineage are essential components of the innate and adaptive immune systems and play a role in tissue homeostasis and embryogenesis [1]. Recent findings have revealed an important degree of conservation in the signalling pathways involved in the development and function of blood cells in vertebrates and various invertebrate phyla, including *Drosophila melanogaster* [2, 3]. *Drosophila* haemocytes, the insect blood cells, are now widely used to analyse in vivo cell migration and phagocytic processes [4, 5]. However, our knowledge of their functional impact on development and host resistance to infection is limited.

As in vertebrates, *Drosophila* blood cells development occurs in two phases. A first haematopoietic wave takes place during embryogenesis when a population of hae-

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Dr. François Leulier
Centre de Génétique Moléculaire
CNRS FRE 3144, Bâtiment 26
Avenue de la terrasse, FR–91190 Gif sur Yvette (France)
Tel. +33 1 6982 3200, Fax +33 1 6982 4386, E-Mail leulier@cgm.cnrs-gif.fr

mocytes originate from the procephalic mesoderm, which will differentiate into either plasmatocytes or crystal cells [6–9]. Plasmatocytes, the *Drosophila* professional phagocytes, make up to 95% of all embryonic haemocytes. They are extremely long-lived and persist through the larval stages, some being still detected in adults [10]. These cells are highly migratory and ingest microorganisms as well as 'altered-self' particles such as apoptotic cell corpses by phagocytosis [4, 5]. Therefore, they are referred to as phagocytes hereafter. A second population of haemocytes, the crystal cells, comprises up to 5% of embryonic haemocytes, persisting until the onset of metamorphosis and contribute to larval melanization reactions [11, 12]. A second wave of haematopoiesis occurs later, at the larval stages, in the lymph gland. The precursors of the lymph gland derive from the dorsal thoracic mesoderm and coalesce during embryogenesis to form the first paired lobes of the organ, which subsequently grow by cell proliferation during the first and second larval instars. By the late third instar larval stage, the lymph gland is the main site of haemocyte production in the animal, and the primary lobes can be divided into 3 structurally different zones: an outer cortical zone containing proliferating mature phagocytes and crystal cells, an inner medullary zone containing quiescent immature haemocytes, and a posterior signalling centre that acts as a niche [13–15]. The lymph gland is also the production site of a third type of haemocyte found only in larvae, the lamellocytes. Lamellocytes are large, adhesive cells devoted to the encapsulation of foreign bodies too large to be phagocytosed; these cells differentiate from the immature haemocyte pool only in response to specific conditions, such as parasitization of larvae by Hymenoptera [11]. Under normal conditions, the lymph gland phagocytes remain in the lymph gland throughout larval stages and enter the circulation only at the onset of metamorphosis and ingest doomed larval structures. At later stages, no haematopoietic organ is found. Consequently, all phagocytes present in the larval haemocoel are of embryonic origin while adult phagocytes, the only circulating cells at this stage, consist of a mixture of embryonic and lymph gland-derived phagocytes [10, 11].

Previous studies have revealed that *Drosophila* phagocytes participate in both development and immune responses [4, 16]. Indeed, they contribute to central nervous system (CNS) morphogenesis during embryonic development through clearance of apoptotic corpses and deposition of extracellular matrix components [17–19]. They also execute at least four immune tasks: (1) they secrete antimicrobial peptides [20, 21] and clotting factors [22, 23]; (2) they engulf and digest micro-organisms through phagocytosis [5]; (3) they contribute to local and systemic tissue damage responses in larvae [24] and adults [25], and (4) they contribute, in larvae, to the systemic production of antimicrobial peptides by the fat body, the functional equivalent of the mammalian liver [26–28]. However, our knowledge of their functional impact on development and host resistance to infection is limited.

To test the functional importance of *Drosophila* phagocytes, we have generated animals depleted of phagocytes using a specific genetic cell ablation strategy and studied their development and capacity to resist infection. Our results strengthen the notion that *Drosophila* phagocytes contribute to development. They also reveal that phagocytes significantly contribute to host resistance to several bacterial infections in adults while being dispensable for humoral immune responses at this stage. Furthermore, we confirm that phagocytosis is an important effector mechanism of the cellular arm of *Drosophila* immune responses by demonstrating that phagocytosis contributes to host resistance to systemic *Staphylococcus aureus* infection in adults. Altogether, our analysis reveals the important contribution of *Drosophila* phagocytes to both development and immunity.

Experimental Procedures

Fly Stocks

Fly stocks and crosses were maintained on polenta-agar medium. To obtain *Haemo*^{less} embryos (embryos depleted of haemocytes), Bax was specifically expressed in all embryonic haemocytes by crossing UAS-*bax*/CyO-*actin*-GFP [29] males with *serpent*(hemo)-GAL4,UAS-GMA [30] virgin females at 29°C. GMA encodes the actin-binding domain of moesin fused to GFP. *Phago*^{less} larvae (*hml*(delta)GAL4, UAS-eGFP/UAS-*bax* larvae) and adults were obtained following crossing during 24 h at 25°C of virgin flies *hml*(delta)-GAL4,UAS-eGFP [31] with males UAS-*bax*/CyO-*actin*-GFP progenies were then switched to 29°C. Parental control UAS-*bax*/CyO-*actin*-GFP and *serpent*(hemo)-GAL4,UAS-GMA or sibling control *hml*(delta)-GAL4,UAS-eGFP/CyO-*actin*-GFP and *serpent*(hemo)-GAL4,UAS-GMA/CyO-*actin*-GFP were used as appropriate. Conditional *Phago*^{less} animals were obtained by crossing virgin females *hml*(delta)-GAL4,UAS-eGFP with males UAS-*bax*,*Tub*-GAL80^{ts}/CyO-*actin*-GFP. The activity of the GAL4 system was controlled by placing the progenies either at restrictive temperature (29°C, GAL80 off, GAL4 system on) or at permissive temperature (18°C, GAL80 on, GAL4 system off). Germ-free *Phago*^{less} animals were obtained by crossing germ-free virgin females *hml*(delta)-GAL4, UAS-eGFP with germ-free males UAS-*bax*/CyO-*actin*-GFP on autoclaved standard medium. Germ-free parental stocks were generated by bleaching embryos and cultivating them on

autoclaved polenta-agar medium. *Relish* ^{E20} *(Rel*^{E20}*), spätzle*^{rm7} *(spz*^{rm7}*), Df(3R)D605* and *Df(3R)Tl-I* fly strains were described previously [32, 33]. The *Tub*-GAL80^{ts} line was obtained from Bloomington Stock Center and recombined with UAS-*bax*/CyO-*actin*-GFP flies. The UAS-*GFP*-IR was provided by the National Institute of Genetics (Mishima, Japan) stock centre. The fly lines UAS-*Eater*-IR#1 (obtained from the Vienna RNAi stock centre, transformant ID 4301) targets the region 345–575 of the *Eater* ORF while the RNAi line UAS-Eater-IR#2 (obtained from the National Institute of Genetics) targets the region 27–525. No off-targets of these RNAi constructs were detected using a web-based search tool (http://www.dkfz.de/signaling/tools.php). 4- to 7-day-old adults flies were used in all experiments.

Viability Studies

Stage 14–17 *serpent*(hemo)-GAL4,UAS-GMA/UAS-*bax (Haemo*^{less}*)* or *serpent*(hemo)-GAL4,UAS-GMA (wild type) embryos laid and raised at 29°C on apple juice agar plates supplemented with yeast paste were counted and separated under a fluorescent dissection microscope and allowed to develop further at 29°C. Viability was scored by counting the number of larvae that hatched after 24 h and again at 48 h. Post-embryonic viability was assayed by comparing the number of *Phago*^{less} adults [*hml*(delta)-GAL4,UAS-eGFP/UAS-*bax*] and wild-type siblings [*hml*(delta)-GAL4,UAS-eGFP/CyO-*actin*-GFP] derived from crosses between *hml*(delta)-GAL4,UAS-eGFP females and UAS-*bax*/CyO-*actin*-GFP males. Crosses were performed at 25°C and transferred at 29°C after 24 h. A 100% being arbitrary attributed to the number of emerging wild-type sibling controls.

Haemocyte Observation and Counts

hml(delta)-GAL4,UAS-eGFP/CyO-*actin*-GFP (wild type) and *hml*(delta)-GAL4,UAS-eGFP/UAS-*bax (Phago*^{less}*)* larvae or adults were anaesthetized and viewed under epifluorescent illumination (excitation filter 480 nm, dichroic filter 505 nm, emission filter 510 nm) with a Leica MZFLIII (Heerburg, Switzerland) dissecting microscope. Images were recorded with a charge-coupled device camera (Sony). For haemolymph observations, wild-type and *Phago*^{less} larvae were rinsed in sterile PBS, opened using forceps at the level of the posterior segment. Haemolymph samples were observed directly under a microscope (Leica DMRB) with either differential interference contrast or epifluorescent illumination. Circulating cells in wild-type and *Phago*^{less} larvae were counted per optical field using a haemocytometer under differential interference contrast illumination. Circulating cells expressing weak or strong GFP were counted in the same optical field under epifluorescent illumination. Acridine orange (AO) staining was used to reveal apoptotic corpses and acidic cell compartments. Haemocytes collected from wild-type and *Phago*^{less} larvae were incubated with 10 μl of 1.6 μM AO in PBS and observed under epifluorescent illumination.

Antibody Staining

serpent(hemo)-GAL4,UAS-GMA/UAS-*bax (Haemo*^{less}*)* and *serpent*(hemo)-GAL4,UAS-GMA/CyO-*actin*-GFP (wild type) embryos laid and raised at 29°C were fixed and co-immunostained. Rabbit anti-GFP (1/1,000; Abcam Inc.) was used to detect the presence or absence of the fluorescent balancer in combination with mouse anti-fascin (sn7c concentrate, diluted 1/100; Developmental Studies Hybridoma Bank, DSHB) to detect embryonic haemocytes (I. Evans and W. Wood, unpubl. data); BP102 mouse anti-CNS (supernatant diluted 1/100; DSHB) or mouse anti-fasciclin 2 (1D4 supernatant diluted 1/5; DSHB) were used in combination with anti-GFP to show CNS morphology. Primary antibodies were detected with FITC-conjugated goat anti-mouse (1/200; Jackson ImmunoResearch Laboratories) and Alexa Fluor 594-conjugated goat anti-rabbit (1/200; Molecular Probes). Immunostained embryos were visualized using a Leica LSM510 confocal microscope, and Z-stack projections were assembled using ImageJ.

Lymph glands from *hml*(delta)-GAL4,UAS-eGFP/CyO-*actin*-GFP (wild type) animals were dissected, fixed for 5 min in 4% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.3), stained with primary rabbit polyclonal anti-GFP (1/1,000; Torrey Pines Biolabs) and TOPRO3 (Molecular Probes) to reveal nuclei, mounted in Vectashield medium (Vector Laboratories, Burlingame, Calif., US) and observed under confocal microscopy on a Zeiss LSM 510 microscope (Zeiss, Oberkochen, Germany).

Bacterial Strains and Infection Experiments

Injuries were performed by pricking 4- to 7-day-old adult females in the lateral thoracic region with a thin needle either clean (washed and rinsed in 70% ethanol) or previously dipped into a concentrated pellet of the following bacteria. Flies were incubated at the indicated temperatures. *Erwinia carotovora carotovora 15 (Ecc15):* optical density (OD) at 600 nm = 170, 29°C; *Escherichia coli:* OD 160, 25°C; *Enterococcus faecalis:* OD 5, 25°C; *Bacillus subtilis:* OD 35, 29°C; *Staphylococcus saprophyticus:* OD 30, 25°C; *Streptococcus agalactiae:* OD 10, 25°C; *Enterobacter cloacae:* OD 100, 29°C; *Salmonella enterica* serovar *typhimurium*: OD 200, 29°C; *S. aureus:* OD 10, 25°C, or a mixture of *Micrococcus luteus* and *E. coli:* OD 100, 25°C. Peptidoglycan (PGN) preparations and injections were performed as previously described [32]. Survival analyses were performed on 20 adult females and repeated at least 3 times.

Quantitative Real-Time PCR

SYBR Green quantitative real-time PCR analysis was performed as previously described [32]. Primer information can be obtained upon request. The amount of mRNA detected was normalized to control *rp49* mRNA values. Normalized data were used to quantify the relative levels of a given mRNA according to cycling threshold analysis (ΔCt). Relative $\Delta Ct^{gene}/\Delta Ct^{rp49}$ ratios of unchallenged wild-type controls were anchored in 1 to indicate fold induction. Graphs represent the mean and SD of relative ratios detected in 3 biological repetitions of a pool of 10 females.

Results

Drosophila *Haemocytes Are Required for Embryonic Development*

In order to reveal the functional importance of *Drosophila* haemocytes, we have used a genetic system to perform cell-specific ablation by promoting targeted ectopic cell death. This relies on the use of the yeast UAS/GAL4 binary system [34] to direct the expression of the murine

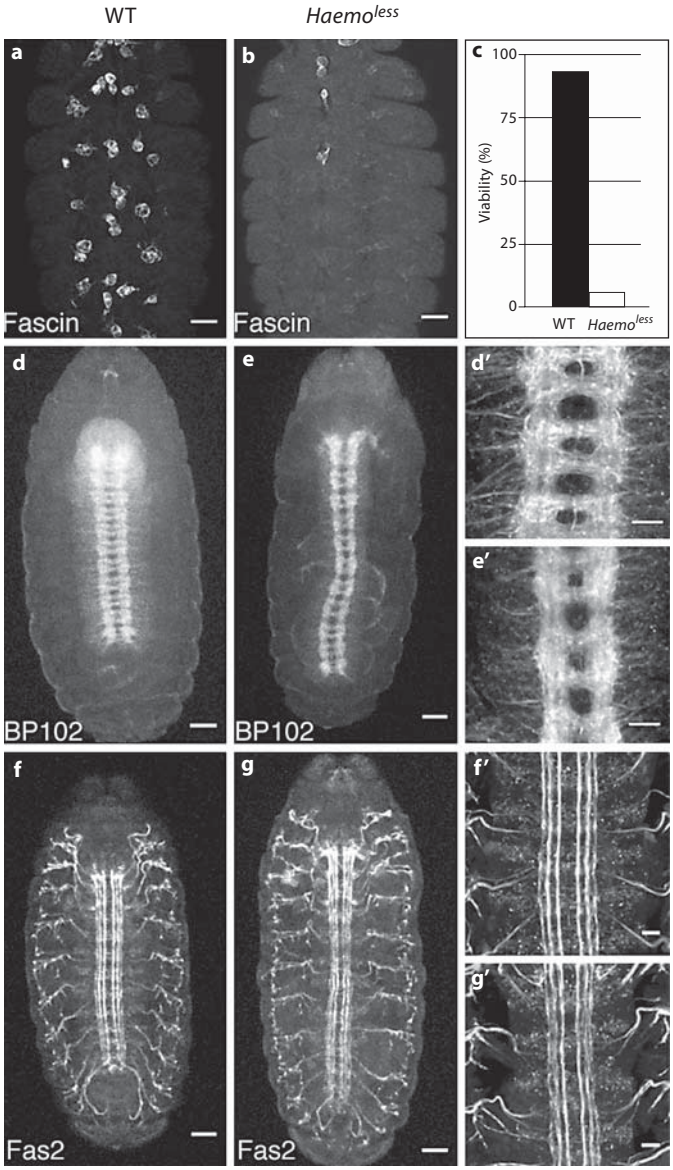


Fig. 1. *Drosophila* haemocytes contribute to embryonic development. **a**, **b** Anti-fascin staining of stage 14 wild-type (WT) and *Haemo*^{less} embryos reveals near complete ablation of embryonic haemocytes in *Haemo*^{less} embryos. Anti-fascin staining allows visualization of haemocytes. Scale bars = 20 μm. **c** Developmental viability of wild-type (WT, n = 150) versus *Haemo*^{less} (n = 162) embryos expressed as the percentage of first instar larvae emerging from laid eggs. **d–g** BP102 staining (**d**, **e**) and fasciclin 2 (Fas2; **f**, **g**) staining of stage 17 wild-type and *Haemo*^{less} embryos reveal CNS condensation failure but otherwise grossly normal CNS architecture in *Haemo*^{less} embryos. Scale bars = 30 μm. BP102 stains all CNS axons while fasciclin 2 stains longitudinal CNS axon tracts. **d′–g′** Enlargements of panels **d–g**. The anterior is up in all images. Genotypes: wild type: *serpent*(hemo)-GAL4,UAS-GMA/CyO-*actin*-GFP (**a**, **d**, **f**) or *serpent*(hemo)-GAL4,UAS-GMA (**c**); *Haemo*^{less}*: serpent*(hemo)-GAL4,UAS-GMA/UAS-*bax* (**b**, **e**, **g**).

bax gene. Bax is a potent pro-apoptotic member of the Bcl-2 family, which promotes mitochondrial permeabilization [35] and efficiently triggers *Drosophila* apoptotic pathways [29]. Embryonic haemocyte identity is specified by the expression of the GATA factor *serpent*. *serpent* is required for specification of the haemocyte primordium within the head mesoderm at an early embryonic stage and, later, for adequate modification of the gene expression profile during haemocyte maturation [7]. To promote the ectopic death of most embryonic haemocytes, we drove the expression of a UAS-*bax* transgene using the *serpent*(hemo)-GAL4 driver. This driver is specific to all embryonic blood cell types and expresses from early haemocyte differentiation onwards [30]. *serpent*-(hemo)-GAL4-mediated Bax expression led to almost complete deletion of embryonic haemocytes at stage 14 (fig. 1a, b). The lack of haemocytes was clearly associated with a strong lethality with only 6% of *Haemo*^{less} embryos (n = 162) reaching the first instar larval stage (fig. 1c). This strongly suggests that haemocytes are essential for normal embryonic development.

Previous studies had revealed that embryonic haemocytes promote CNS morphogenesis through clearance of apoptotic corpses and deposition of extracellular matrix components [17–19]. Therefore, we wondered if CNS morphogenesis was affected in *Haemo*^{less} embryos. During *Drosophila* embryogenesis, the ventral nerve cord (VNC) axons establish a precise pattern reiterated in each segment forming two longitudinal tracts that run the length of the embryo on either side of the midline, with a subset of these axons crossing the midline (fig. 1d, d′). During stage 16 and 17 of embryogenesis, the VNC undergoes a condensation phase along the antero-posterior axis. We observed that the VNC of *Haemo*^{less} embryos at stage 17 was longer and narrower than that of wild-type embryos (compare fig. 1e, g with fig. 1d, f). This indicates a failure of CNS condensation in these animals. Although CNS architecture was normal in *Haemo*^{less} embryos, the precise ladder-like axonal scaffold seen in wild-type embryos was disrupted leading to a rounding of the spaces between anterior and posterior commissures (fig. 1e, e′). Despite differences in axon scaffold shape, no inappropriate midline crossing of axons was detected (compare fig. 1f with fig. 1g). In wild-type animals, 3 major tracts of fasciclin-2-positive axons are observed near the dorsal surface of the CNS on either side of the midline (fig. 1f, f′). Three tracts of fasciclin-2-positive axons were also observed in *Haemo*^{less} embryos (fig. 1g, g′). Although these tracts were relatively normal, they did show very mild defasciculation in some segments (fig. 1g′). Alto-

gether, these results indicate that embryonic haemocytes are required for CNS morphogenesis.

Drosophila *Phagocytes Are Essential for Optimal Viability during Post-Embryonic Development*

Using the same strategy, we next addressed the functional impact of phagocytes during post-embryonic development. To overcome the essential requirement of haemocytes during embryonic development, we generated flies expressing Bax under the control of the *hml*(delta)-GAL4 driver. This transgene exclusively drives strong GAL4 expression in mature phagocytes from early first instar larval stage to adulthood [31] (fig. 2a, b, c′, g, i, k, and data not shown). Of note, this driver expresses in both pools of phagocytes those of embryonic origin, which are sessile or in circulation in larvae (fig. 2a, c′), and those in the cortical zone of the lymph gland, which will populate adults (fig. 2b). *hml*(delta)-GAL4-mediated expression of Bax led to a massive disruption of the lymph gland during early larval stages (data not shown). The haemolymph of *hml*(delta)GAL4, UAS-eGFP/UAS-*bax* larvae (*Phago*^{less}) showed a large amount of apoptotic corpses, revealed by AO staining (fig. 2f, f′) and a significantly reduced amount of circulating cells with an average of 74% decrease compared with wild-type larvae

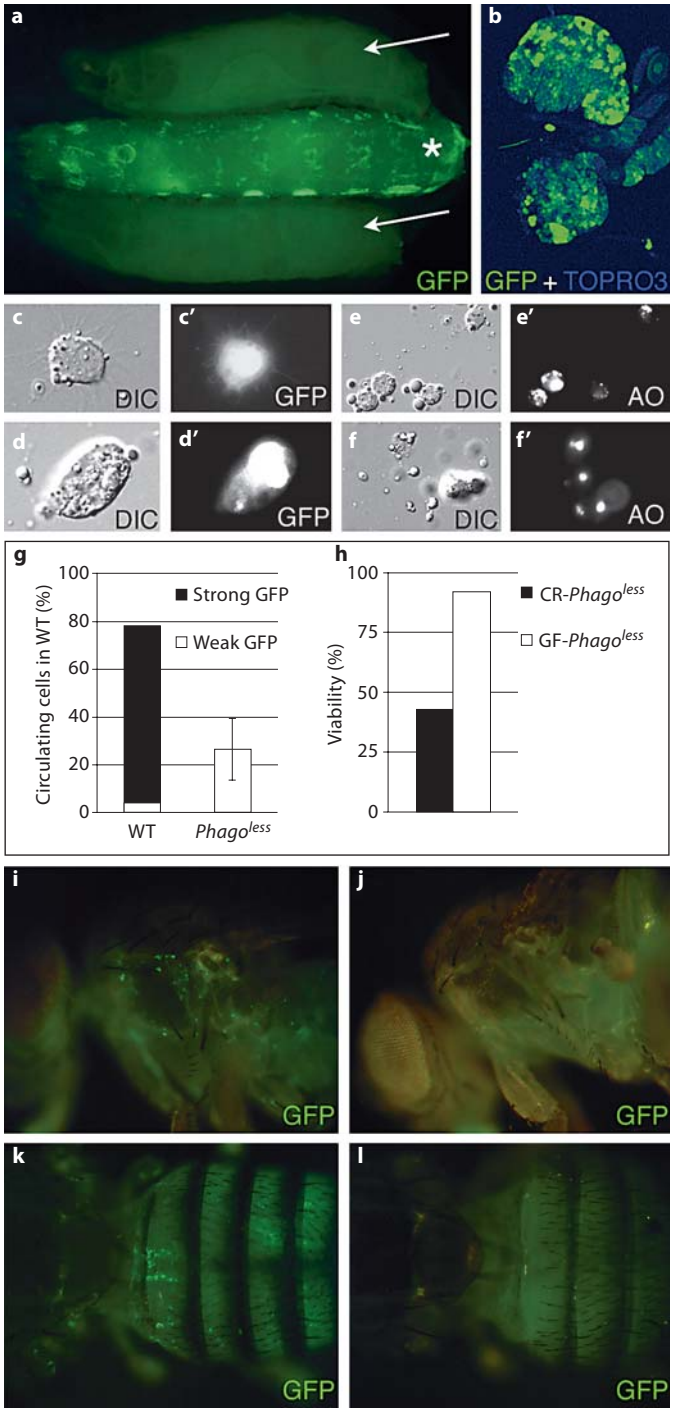


Fig. 2. *Drosophila* phagocytes are partially redundant for post-embryonic development. **a** Wild-type (asterisk) and *Phago*^{less} (arrows) wandering third instar larvae observed under epifluorescence illumination. Sessile and circulating larval phagocytes express the eGFP under the control of the *hml*(delta)-GAL4 driver. **b** Dissected lymph gland from *hml*(delta)-GAL4,UAS-eGFP/CyO-*actin*-GFP (wild type) third instar larvae stained with anti-GFP and TOPRO3 to reveal nuclei. *hml*(delta)-GAL4-positive phagocytes are observed in the cortical zone of the primary lobes. **c–f**, **c′–f′** Circulating haemocytes from wild-type (**c**, **c′** and **e**, **e′**) or *Phago*^{less} (**d**, **d′** and **f**, **f′**) larval haemolymph, non-treated (**c**, **c′** and **d**, **d′**) or stained with AO to reveal apoptotic corpses and acidic cellular compartments (**e**, **e′** and **f**, **f′**), observed under differential interference contrast (**c–f**) or epifluorescent illumination (**c′–f′**). ×40 objective (**c**, **d**); ×20 objective (**e**, **f**); 400 ms exposure (**c′**); 800 ms exposure (**d′**); 200 ms exposure (**e′**, **f′**). **g** Quantifications of circulating cells per optical field showing weak (as in **d′**) or strong (as in **c′**) fluorescent signal under epifluorescence illumination in wild-type (WT) and *Phago*^{less} larvae expressed as the percentage of circulating cell number observed in wild-type larvae under differential interference contrast (DIC) illumination. **h** Relative developmental viability of *Phago*^{less} individuals raised under conventional (CR, n = 295) or germ-free (GF, n = 191) conditions at 29°C. Shown are the percentage of emerging adults compared with their respective wild-type siblings [*hml*-(delta)-GAL4,UAS-eGFP/CyO-*actin*-GFP] anchored to 100%. **i–l** *Phago*^{less} adults (**j**, **l**) and sibling controls (**i**, **k**) observed under epifluorescence illumination. Lateral view of the thoracic region (**i**, **j**); dorsal view of the abdomen (**k**, **l**). Note the accumulation of sessile GFP-positive phagocytes on adult tissues (**i**) or circulating GFP-positive phagocytes along the dorsal vessel (**k**). Genotypes: wild type: *hml*(delta)-GAL4,UAS-eGFP/CyO-*actin*-GFP; *Phago*^{less}: *hml*(delta)-GAL4,UAS-eGFP/UAS-*bax;* siblings controls: *hml*(delta)-GAL4,UAS-eGFP/CyO-*actin*-GFP.

(fig. 2g). The remaining circulating cells appeared enlarged with an unusual morphology and lacked filipodia in sharp contrast to wild-type phagocytes (compare fig. 2c, e with fig. 2d, f). These enlarged cells weakly expressed the UAS-*gfp* transgene driven by the *hml*(delta)-GAL4 driver (fig. 2d′, exposition time twice that of fig. 2c′) and were unable to phagocytose AO-positive apoptotic corpses (fig. 2f′). In addition, phagosomal acidification, a key feature of phagosome maturation, which can be revealed by AO staining, did not occur in contrast to wild-type circulating phagocytes (fig. 2e′, f′). Although the remaining circulating cells observed in *Phago*^{less} larvae may correspond to the previously identified population of undifferentiated circulating cells that are normally present in *Drosophila* larvae [11], we hypothesize that they rather correspond to a population of non-functional phagocytes at an early step of the apoptotic process or to abnormal lamellocytes released during the early rupture of the lymph gland. Nevertheless, these results indicate that efficient phagocyte ablation occurred during larval stages of developing *Phago*^{less} animals.

Remarkably, *Phago*^{less} adults were recovered and no *hml*(delta)-GAL4-positive circulating or sessile cells were observed through the cuticle (fig. 2j, l) or attached to dissected tissues (data not shown). In collected haemolymph, no circulating cell could be identified indicating that complete depletion of adult phagocytes occurred (data not shown). The recovery of viable and fertile adults deprived of phagocytes suggests that they are partially redundant for post-embryonic development to proceed. Indeed, they presented no obvious morphological defects apart from small melanotic masses found occasionally in the abdomen. However, phagocyte depletion still had a profound impact on developmental viability since, compared with wild-type siblings, 43% of the *Phago*^{less} animals reached adulthood when conventionally reared (n = 295; fig. 2h). This reduced viability may stem from a developmental function of phagocytes as suggested by previous reports showing that during metamorphosis, phagocytes ingest doomed larval tissues, in particular muscle cells [11]. Alternatively, phagocytes may ensure essential immune functions during larval stages to protect the host against opportunistic infections. To challenge these hypotheses, we generated germ-free *Phago*^{less} individuals and reared them under sterile condition. In this context, the developmental lethality of *Phago*^{less} animals was almost completely rescued to wild-type levels (n = 191; fig. 2h). Altogether, these results indicate that *Drosophila* phagocytes are dispensable for normal post-embryonic development to proceed. However they are required for optimal viability during post-embryonic development probably through immune functions protecting the host to infection by environmental micro-organisms.

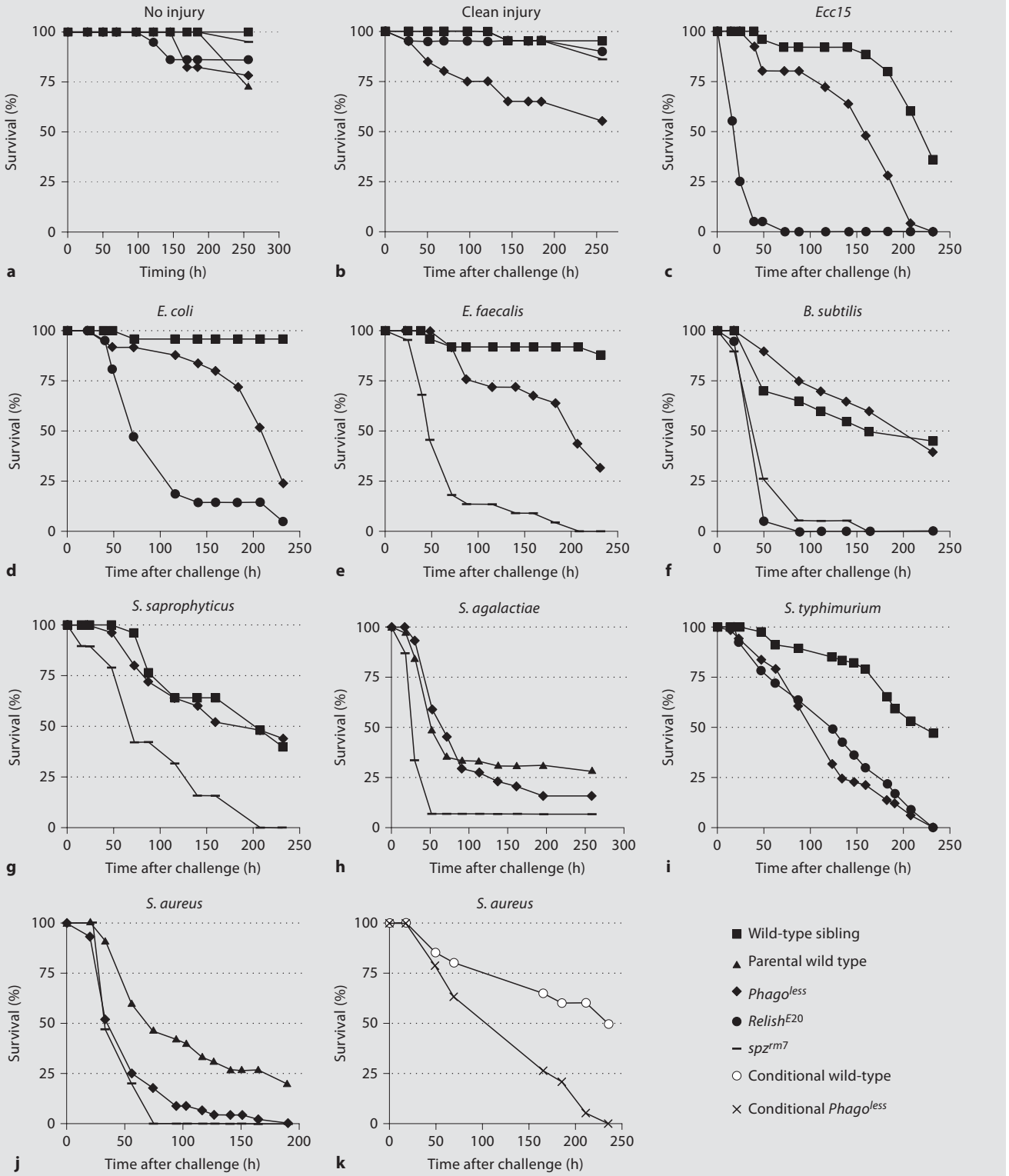
Reduced Resistance of Phago^{less} *Adults to Bacterial Infections*

Using *Phago*^{less} adults, we next addressed the functional impact of *Drosophila* phagocytes in adult fly resistance to bacterial infection. To this end, we used an established model of systemic bacterial infection by septic injury, which consists of wounding the adult thoracic cuticle with a needle previously dipped into a concentrated bacterial solution. In the past, this infection model had been successfully used to reveal the essential contribution of Toll and immune deficiency (Imd) signalling pathways in host resistance to systemic bacterial infection. Upon infection by different classes of micro-organisms, Toll and Imd pathways control the expression of particular sets of immune effector genes, including genes encoding antimicrobial peptides in the fat body [16]. As a consequence, Toll pathway mutants such as those affecting the *spz* gene present a striking susceptibility to systemic infection by several Gram-positive cocci while Imd pathway mutants such as those affecting the *Rel* gene show a dramatic susceptibility to systemic infection by several Gram-negative rods [16]. Therefore, we compared the resistance of *Phago*^{less} flies to wild-type flies and *spz* or *Rel* mutants after septic injury using various bacterial strains.

Phagocyte-depleted adults showed a slight reduction in resistance to clean wounding compared with wild-type flies or *Rel* and *spz* mutants (fig. 3b) while they behaved as wild-type, *spz* and *Rel* flies in absence of challenge during the course of our survival analysis (fig. 3a).

(For figure see next page.)

Fig. 3. Reduced resistance of *Phago*^{less} adults to bacterial infections. Survival analysis of *Phago*^{less}, wild-type siblings, wild-type parental control, conditional wild-type, conditional *Phago*^{less}, *Relish*^{E20} and *spz*^{rm7} flies in absence of injury (**a**), upon clean injury (**b**) or *Ecc15* (**c**), *E. coli* (**d**), *E. faecalis* (**e**), *B. subtilis* (**f**), *S. saprophyticus* (**g**), *S. agalactiae* (**h**), *S. typhimurium* (**i**) or *S. aureus* (**j**, **k**) septic injury. Twenty adult females were used per experiment, and a representative experiment out of a minimum of 3 repetitions is shown. Genotypes: *Phago*^{less}: *hml*(delta)-GAL4,UAS-eGFP/UAS-*bax;* wild-type sibling: *hml*(delta)-GAL4,UAS-eGFP/CyO-*actin*-GFP; parental wild type: UAS-*bax*/CyO-*actin*-GFP; conditional wild type: UAS-*bax,Tub*-GAL80^{ts}/CyO-*actin*-GFP; conditional *Phago*^{less}: *hml*(delta)-GAL4,UAS-eGFP/UAS-*bax, Tub*-GAL80^{ts}.



This observation correlates with a reported role for circulating cells in the fat body response to injury in adults [25] and their production of clotting factor [22, 23]. This observation suggests that adult phagocytes may also contribute to local responses to wounding, as observed in embryos [36, 37] and larvae [38, 39], probably by engulfing wound debris at the wound site and contributing to clot formation.

Compared with wild-type flies, *Phago*^{less} flies showed a late and mild increase in their susceptibility to systemic infections with the Gram-negative rods *Ecc15* (fig. 3c) and *E. coli* (fig. 3d) or the Gram-positive coccus *E. faecalis* (fig. 3e). This is in sharp contrast with *Rel* and *spz* mutants, which showed an acute and strong susceptibility to infection with these Gram-negative rods or Gram-positive cocci, respectively (fig. 3c–e). *Phago*^{less} adults resisted as wild-type animals to infection by the Gram-positive bacillus *B. subtilis* (fig. 3f), the Gram-positive cocci *S. saprophyticus* (fig. 3g) and *S. agalactiae* (fig. 3h) or the Gram-negative rod *E. cloacae* (data not shown). These results contrast with the strong susceptibility of Toll and/or Imd pathway mutant flies (fig. 3f–h, data not shown). In contrast, *Phago*^{less} animals were severely affected, compared with wild-type animals, in their resistance to systemic infection by the Gram-negative rod *S. typhimurium* or the Gram-positive coccus *S. aureus* (fig. 3i, j). Of note, upon infection by those germs, *Phago*^{less} flies exhibited the same level of susceptibility than Toll and Imd pathway mutants, respectively.

To test if the observed susceptibility to bacterial infection was directly associated with phagocyte ablation, we generated conditional *Phago*^{less} individuals using an additional ubiquitously expressed transgene encoding a thermosensitive version of the GAL4 inhibitor, GAL80 [40]. When reared at permissive temperature (18°C, GAL80 on, GAL4 system off) during the entire development, conditional *Phago*^{less} emerging adults had phagocytes and their developmental viability was similar to their wild-type siblings (95%, n = 55; data not shown). We then cultured them at restrictive temperature (29°C, GAL80 off, GAL4 system on) for 7 days. As observed with *Phago*^{less} escapers, conditional *Phago*^{less} adults were depleted of phagocytes (data not shown) and showed an increased susceptibility to *S. aureus* infection (fig. 3k). These results demonstrate that the susceptibility observed is indeed associated with adult phagocyte depletion and indicate that adult phagocytes contribute to the host survival to several bacterial infections and are important to resist *S. aureus* and *S. typhimurium* systemic infections.

The Humoral Antimicrobial Responses of Phago^{less} *Animals Are Not Affected*

Previous reports have suggested that the activation of Imd-mediated synthesis of antimicrobial peptides by the fat body of *Drosophila* larvae relies, at least partially, on the activity of haemocytes upon ingestion of the Gram-negative rod *Ecc15* [26, 27] or upon *E. coli* injection into the body cavity [28]. However, other reports suggest that phagocytes are not essential for this process [41, 42]. We wondered if the activation of the Toll or Imd pathway relies on phagocytes at the adult stage. To address this question, we monitored the expression of specific immune genes regulated either by the Toll pathway *(IM1),* the Imd pathway *(Diptericin)* or both pathways *(Defensin)* upon *S. aureus* and *S. typhimurium* infections in *Phago*^{less} adults. As observed in wild-type flies, both *IM1* and *Defensin* genes were significantly induced in *Phago*^{less} individuals upon *S. aureus* infection (fig. 4a, b). Similarly, both *Diptericin* and *Defensin* genes were strongly induced in *Phago*^{less} individuals as observed in wild-type animals upon *S. typhimurium* infection (fig. 4c, d). These results suggest that both Toll and Imd pathway activities upon infection with *S. aureus* and *S. typhimurium* are not affected by the absence of phagocytes. To further test this hypothesis, we assayed *Diptericin* and *IM1* expression in *Phago*^{less} individuals upon injection of purified DAP-type PGN (DAP-PGN) from Gram-negative rods *(E. coli)* or purified Lys-type PGN (Lys-PGN) from Gram-positive cocci *(E. faecalis)* that are potent elicitors of the Imd and Toll pathways, respectively [32]. Interestingly, both *Diptericin* and *IM1* genes were induced as in wild-type or even higher in *Phago*^{less} animals upon DAP-PGN and Lys-PGN injection, respectively (fig. 4e, f). Similar results were obtained when a mixture of *E. coli* and *M. luteus* bacteria was injected in wild-type and *Phago*^{less} adults (data not shown). Altogether, these results demonstrate that Imd and Toll pathway activities are normal in *Phago*^{less} animals and indicate that the increased susceptibility of *Phago*^{less} flies to *S. aureus* or *S. typhimurium* infections is not associated with a defect in the activation of Toll or Imd-dependent antimicrobial responses. Collectively, these results demonstrate that *Drosophila* adult humoral responses controlled by Toll and Imd signalling pathways are not significantly affected in the absence of phagocytes and support the notion that activation of the humoral arm of the *Drosophila* adult antimicrobial response does not rely on phagocyte activities.

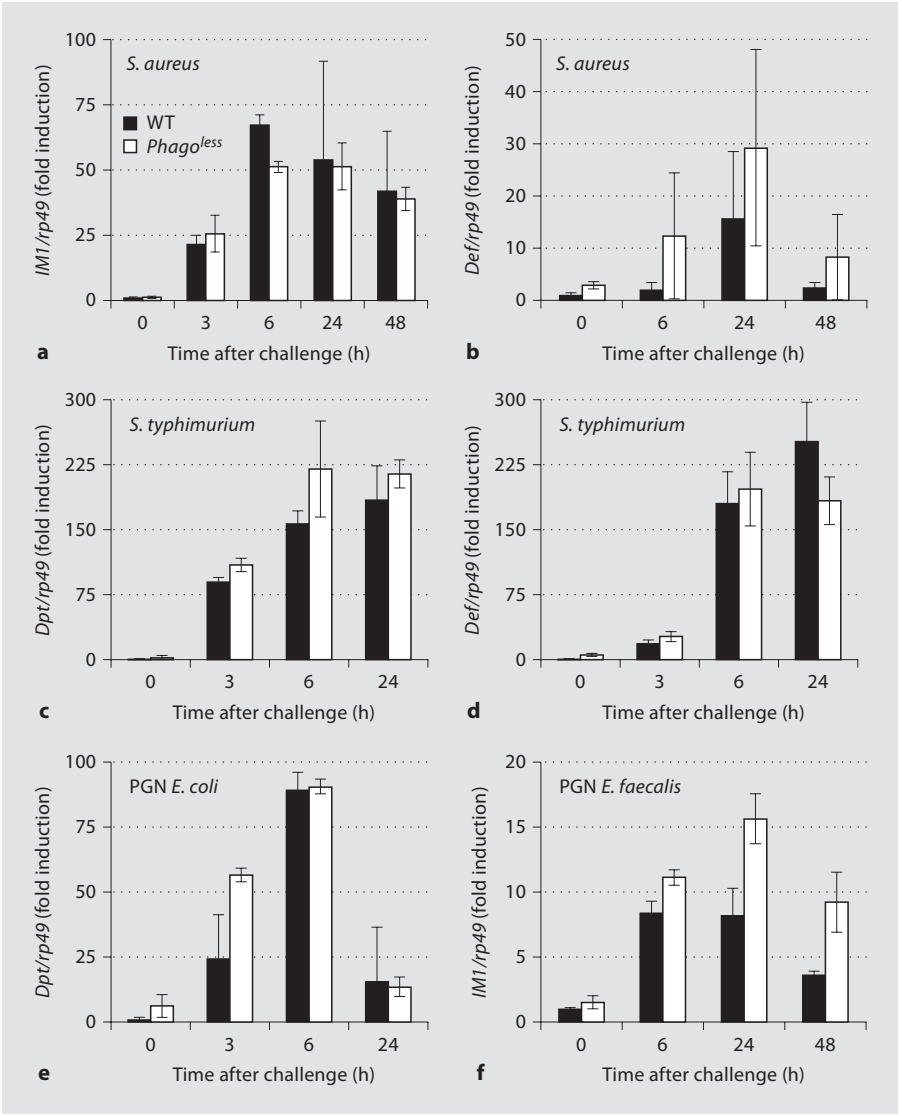


Fig. 4. The humoral antimicrobial response of *Phago*^{less} animals is not affected. **a**, **b** Quantitative RT-qPCR analysis of *IM1* (**a**) and *Defensin* (*Def;* **b**) induction after *S. aureus* septic injury in wild-type and *Phago*^{less} adults. **c**, **d** Quantitative RT-qPCR analysis of *Diptericin* (*Dpt;* **c**) and *Defensin* (*Def;* **d**) induction after *S. typhimurium* septic injury in wild-type and *Phago*^{less} adults. **e**, **f** Quantitative RT-qPCR analysis of *Diptericin* (*Dpt;* **e**) and *IM1* (**f**) upon injection of purified DAP-PGN from *E. coli* or purified Lys-PGN from *E. faecalis* in wild-type and *Phago*^{less} adults. *rp49* was used as the experimental expression standard. Relative $\Delta Ct^{gene}/\Delta Ct^{rp49}$ ratios of unchallenged wild-type controls were anchored in 1 to indicate fold induction. Graphs represent the mean and SD of relative ratios detected in 3 biological repetitions of a pool of 10 females. Genotypes: wild type: *hml*(delta)-GAL4, UAS-eGFP/CyO-*actin*-GFP; *Phago*^{less}: *hml*(delta)-GAL4,UAS-eGFP/UAS-*bax*.

Phagocytosis Contributes to Drosophila *Resistance to Bacterial Infection*

In absence of any obvious defect in the activation of humoral immune responses, we wondered why *Phago*^{less} animals are less resistant than wild-type flies to *S. aureus* systemic infections. Of note, *S. aureus* is efficiently engulfed and digested by *Drosophila* phagocytes either ex vivo or in vivo [33]. Therefore, we hypothesized that phagocytosis may be an important resistance mechanism to *S. aureus* systemic infections in *Drosophila*. To test this hypothesis, we analysed the resistance to *S. aureus* infection of a *Drosophila* null mutant for *Eater,* a gene encoding a phagocytic receptor essential to mediate phagocytosis of *S. aureus* in vivo [33]. In addition, using the inducible in vivo RNAi technique, we selectively knocked down *Eater* expression in phagocytes using two independent UAS-*Eater*-RNAi transgenes driven by the *hml*(delta)-GAL4 driver and assayed the resistance of these *Eater*-RNAi flies to *S. aureus* infection. Figure 5 shows that both *Eater* null mutant flies and flies where *Eater* expression was selectively knocked down in phagocytes presented an increased susceptibility to *S. aureus* infection compared with wild-type flies. Of note, silencing of *Eater* expression led to the same increase in susceptibility as in *Phago*^{less} animals. Therefore, these results demonstrate that phagocytosis is a predominant defence mechanism employed by *Drosophila* phagocytes to fight *S. aureus* systemic infections and suggest that the

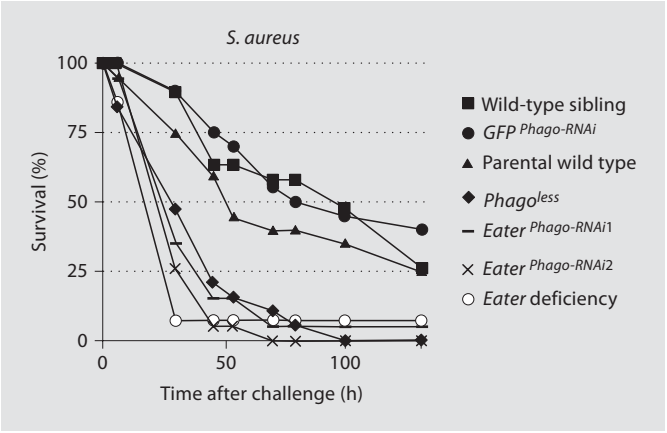


Fig. 5. Phagocytosis contributes to *Drosophila* resistance to systemic bacterial infection. Survival analysis of $Phago^{less}$, wild-type siblings, wild-type parental control, $GFP^{Phago\text{-}RNAi}$, $Eater^{Phago\text{-}RNAi1}$, $Eater^{Phago\text{-}RNAi2}$ and *Eater* deficiency flies upon *S. aureus* septic injury. Genotypes: $Phago^{less}$: *hml*(delta)-GAL4, UAS-eGFP/UAS-*bax;* wild-type sibling: *hml*(delta)-GAL4,UAS-eGFP/CyO-*actin*-GFP; parental wild type: UAS-*bax*/CyO-*actin*-GFP; $GFP^{Phago\text{-}RNAi}$: *hml*(delta)-GAL4,UAS-eGFP/UAS-*GFP-IR;* $Eater^{Phago\text{-}RNAi1}$: *hml*(delta)-GAL4,UAS-eGFP/UAS-*Eater-IR#1;* $Eater^{Phago\text{-}RNAi2}$: *hml*(delta)-GAL4,UAS-eGFP/UAS-*Eater-IR#2;* *Eater* deficiency: *Df(3R)D605/Df(3R)Tl-I.*

enhanced susceptibility of $Phago^{less}$ animals to *S. aureus* infection likely relies on the lack of this cellular defence mechanism.

Discussion

Based on their multiple developmental and immune functions, it has been inferred that *Drosophila* phagocytes play important roles during both development and immune responses. However, to our knowledge, no cell ablation experiments have ever been reported to confirm this. Here, we have challenged these ideas by generating *Drosophila* individuals lacking functional phagocytes. We now provide several lines of evidence supporting the essential requirement of haemocytes for embryonic development, the important contribution of larval phagocytes in host resistance to opportunistic infections during post-embryonic development and the critical role of adult phagocytes in the immune defence to several systemic bacterial infections.

Indeed, we have shown that specific ablation of *Drosophila* haemocytes leads to near complete lethality of the developing embryos indicating that haemocytes are essential for embryonic viability. In addition, we confirm that haemocytes contribute to CNS morphogenesis, in particular to VNC condensation. Interestingly, the abnormal CNS morphogenesis phenotypes of $Haemo^{less}$ embryos are reminiscent of those observed in mutants that disrupt either haemocyte production *(serpent)* or migration *(pvr)* [17, 18]. Similar phenotypes were also observed in embryos injected with *croquemort* dsRNA that impairs engulfment of apoptotic corpses by haemocytes [17] or when haemocytes express dsRNA of *SPARC,* a gene encoding a collagen binding extracellular matrix glycoprotein [19]. Collectively, our results and these observations demonstrate that haemocytes are essential for embryonic development most likely through their function of apoptotic corpse clearance and deposition of extracellular matrix components.

We also report that individuals depleted of phagocytes from early larval stages present a reduced developmental viability indicating that phagocytes ensure optimal survival during post-embryonic development. However, the emergence of viable and fertile $Phago^{less}$ adults in proportion equivalent to wild-type siblings when reared in sterile conditions suggests that larval phagocytes are dispensable for normal post-embryonic development to proceed. Moreover, emerging germ-free $Phago^{less}$ adults presented no obvious developmental delay or morphological defects apart from small melanotic masses found occasionally in the abdomen. Melanotic masses were also observed in animals reared under conventional condition and probably result from the encapsulation of incompletely eliminated larval tissues during metamorphosis. Indeed, it has been reported that *Drosophila* phagocytic cells participate in metamorphosis by ingesting doomed larval tissues, in particular muscle cells [11]. The fact that $Phago^{less}$ larvae and pupae were perfectly viable when raised in axenic conditions indicates that larval phagocytes ensure important immune functions during post-embryonic development rather than performing developmental functions as observed in embryos. This is supported by the previous observation by Matova and Anderson [42], who reported that double mutants lacking the *Drosophila* Rel proteins Dif and Dorsal are often infected by opportunistic microbes and die from infection during larval stages. Specific expression of either Dif or Dorsal in the larval blood cell lineage was sufficient to clear microbes and allow partial survival to the adult stage. In addition, Braun et al. [41] observed that *domino* and *l(3)hem* mutant, which are devoid of functional blood cells contain numerous live micro-organisms in their haemocoele in contrast to wild-type larvae. Hence, our

results and these observations demonstrate that phagocytes significantly contribute to *Drosophila* larval viability in their standard laboratory environment, probably through clearance of opportunistic micro-organisms in the haemocoel.

Using adults depleted of phagocytes, we now demonstrate that these cells contribute to host resistance to systemic infections with specific bacteria. We observed that *Phago*^{less} individuals are as resistant as wild-type animals to *B. subtilis, S. saprophyticus, S. agalactiae* and *E. cloacae* infections while Toll or Imd pathway mutants present a striking enhanced susceptibility. This indicates that the cellular arm of *Drosophila* adult immune responses has either no role or is largely redundant against these germs. In contrast, Toll and Imd pathway-mediated immune responses play a key role. Nevertheless, the enhanced susceptibility of *Phago*^{less} animals to infections with *S. aureus* and *S. typhimurium* indicate that *Drosophila* phagocytes are critical players in the host resistance to these specific bacterial infections. The notion that phagocytes contribute to host resistance is further corroborated by the enhanced susceptibility of *Phago*^{less} flies to *Ecc15, E. coli* or *E. faecalis* infections compared with wild-type flies. In addition, the striking differences between the survival profiles of *Phago*^{less} animals and mutants affecting Toll and Imd pathways upon a large range of bacterial systemic infections indicate that the cellular and humoral antimicrobial defences have distinct and complementary functions in *Drosophila* adults. Recently, a report has suggested that the induction of the anti-bacterial peptide gene *Defensin* in the larval fat body during systemic infection requires blood cell contributions [28]. This points to a possible role of phagocytes in the activation of the antimicrobial peptide genes during systemic infection. This observation was controversial since previous studies did not reveal a role for phagocytes in this process at the larval stage [41, 42]. Here, we clearly show that in adults, phagocytes are dispensable for the activation of Toll and Imd-dependent antimicrobial responses upon systemic infection. This contrasts with oral infection models, where larval blood cells contribute at least partially to Imd pathway activation in the fat body upon ingestion of the Gram-negative bacteria *Erwinia carotovora* [26, 27] while adult phagocytes actually dampen Imd pathway activation in the fat body upon ingestion of the entomopathogenic bacterium *Serratia marcescens* [43].

Our results also indicate that phagocytosis is probably the main mechanism by which phagocytes contribute to resistance to systemic bacterial infection. Indeed, *Drosophila* null mutants for *Eater,* a gene encoding a phagocytic receptor essential to mediate phagocytosis of *S. aureus* in vivo [33], presented an increased susceptibility to *S. aureus* infection similar to *Phago*^{less} animals. This was also observed in flies where *Eater* expression was selectively knocked down by RNA interference in phagocytes. Interestingly, Toll and Imd pathway activities are not affected in *Eater* mutant flies upon systemic bacterial infection [33]. These observations demonstrate that phagocytosis contributes to resistance to systemic bacterial infection and acts as a complementary defence mechanism to antimicrobial humoral responses. This complementary role was suggested by a previous study that revealed the enhanced susceptibility to *E. coli* systemic infection of *Imd* mutant flies injected with polystyrene beads to saturate the phagocytic ability of adult phagocytes [44]. Finally, the importance of phagocytes upon systemic bacterial infection correlates with their critical role in host defence against oral infections by the entomopathogenic bacterium *S. marcescens* [33, 43].

Taken together, our observations indicate that the immune system of *Drosophila* adults relies on both the cellular and humoral defence mechanisms to resist systemic bacterial infections. However, the relative contribution of the cellular versus the humoral arm of its antimicrobial defence mechanisms appears to be highly dependent on the bacterial species. The contribution of phagocytes to host survival seems dependent on the specific virulence strategies employed by the infectious bacteria. Indeed, a clear contribution of phagocytes to host survival has so far only been revealed either when the humoral response was affected [44] or upon infection with pathogenic bacteria *(S. marcescens, S. aureus, S. typhimurium)* [this study and 33]. In contrast, the prevalence of Toll and/or Imd-mediated humoral responses for the host survival has been revealed upon all tested systemic bacterial infection, indicating a broader contribution of these humoral immune responses. Our study suggests that systemic infection by *S. typhimurium* or *S. aureus* are good infection models to study the molecular function of phagocytes in *Drosophila*.

In conclusion, we have now established a novel strategy to efficiently and selectively ablate phagocytes allowing the in vivo analysis of their contribution to specific biological processes in an invertebrate animal model, *D. melanogaster.* This completes previous phagocyte depletion strategies developed in vertebrates, which include suicide gene expression by macrophages in transgenic mice [45, 46] or the targeted disruption of the myeloid transcription factor *pu.1* expression in mouse or zebrafish [47, 48]. These strategies have been instrumental for

the appreciation of phagocyte functions in vertebrates but remained limited given the partial ablation generated or the developmental lethality induced [49, 50]. Using a selective and efficient phagocyte ablation strategy in *Drosophila,* we now provide a blueprint of phagocyte importance during both development and innate immune responses. Given the technical potential of the *Drosophila* model, our results pave the way to tissue-specific in vivo systematic genome-wide RNA interference screens to identify all genes important for phagocyte activities.

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