A secreted factor NimrodB4 promotes the elimination of apoptotic corpses by phagocytes in Drosophila

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Abstract

Programmed cell death plays a fundamental role in development and tissue homeostasis. Professional and non-professional phagocytes achieve the proper recognition, uptake, and degradation of apoptotic cells, a process called efferocytosis. Failure in efferocytosis leads to autoimmune and neurodegenerative diseases. In Drosophila, two transmembrane proteins of the Nimrod family, Draper and SIMU, mediate the recognition and internalization of apoptotic corpses. Beyond this early step, little is known about how apoptotic cell degradation is regulated. Here, we study the function of a secreted member of the Nimrod family, NimB4, and reveal its crucial role in the clearance of apoptotic cells. We show that NimB4 is expressed by macrophages and glial cells, the two main types of phagocytes in Drosophila. Similar to draper mutants, NimB4 mutants accumulate apoptotic corpses during embryogenesis and in the larval brain. Our study points to the role of NimB4 in phagosome maturation, more specifically in the fusion between the phagosome and lysosomes. We propose that similar to bridging molecules, NimB4 binds to apoptotic corpses to engage a phagosome maturation program dedicated to efferocytosis.

Keywords apoptotic cell; bridging molecule; Drosophila; Nimrod; phagocytosis

Subject Categories Autophagy & Cell Death; Development; Membranes & Trafficking

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Introduction

The clearance of apoptotic cells by phagocytes is an essential process during development and for the maintenance of tissue homeostasis (Arandjelovic & Ravichandran, 2015). Defective apoptotic cell clearance affects organ function and leads to the release of cytotoxic molecules and the development and progression of inflammatory and immune responses (Elliott & Ravichandran, 2010; Nagata et al., 2010; Poon et al., 2014). Recent studies also point to the importance of apoptotic cell clearance in the brain, as excessive or reduced phagocytosis can lead to neurodegeneration (Etkin et al., 2016; Purice et al., 2016; Hakim-Mishnaevski et al., 2019; Hilu-Dadia & Kurant, 2020). Efferocytosis, the phagocytosis of apoptotic cells, is a multi-step process often mediated by professional phagocytes such as macrophages and glia. It begins with the identification of the target cells by receptors that recognize specific "eat-me" signals that are exposed at the surface of apoptotic cells and distinguish them from living cells (Elliott & Ravichandran, 2016; Boada-Romero et al., 2020). One of the best characterized "eat-me" signals is phosphatidylserine (PS), a membrane phospholipid species usually found in the inner leaflet of the plasma membrane, but exposed on the surface of cells undergoing apoptosis (Segawa & Nagata, 2015; Nagata et al., 2016). Upon ligand recognition, phagocytic receptors engage downstream signaling pathways that initiate the uptake of the particle through an active and dynamic remodeling of the plasma membrane, which is mainly guided by the actin cytoskeleton (Pearson et al., 2003; Stuart & Ezekowitz, 2005; Melcarne et al., 2019a). Newly formed phagosomes undergo a maturation process to acquire digestive activity. The phagosome matures through fusion with endosomes and lysosomes, a process involving small GTPases of the Rab family (Kinchin & Ravichandran, 2010). Rab5 regulates the initial fusion events, by tethering early endosomes to the newly formed phagosome (Alvarez-Dominguez et al., 1996; Duclos et al., 2000; Kinchen et al., 2008). As the phagosome matures, Rab5 is replaced by Rab7 in a process called Rab conversion (Kinchin & Ravichandran, 2010; Poteryaev et al., 2010; Yousefian et al., 2013). Once recruited, Rab7 is responsible for inducing fusion of the phagosome and the lysosome, initiating acidification...
and digestion of the corpse. A characteristic protein present on the phagolysosome membrane is lysosomal-associated membrane protein 1 (Lamp1), which is needed for lysosome fusion with the phagosome (Huynh et al., 2007). Less is known about the final stage of phagosome resolution and how the phagosome is resorbed to allow the phagocyte to return to homeostasis. However, studies have shown that bacteria-containing phagolysosomes in macrophages undergo fragmentation through vesicle budding and constriction (Levin et al., 2016; Botelho et al., 2020; preprint: Lancaster et al., 2020).

Recent studies in mammals have highlighted the importance of bridging molecules in the regulation of efferocytosis. Bridging molecules are secreted proteins that bind to the “eat-me” signal to provide a link between apoptotic cells and phagocytic receptors and enhancing recognition and phagocytosis of apoptotic cells (Savill & Fadok, 2000; Ravichandran, 2003, 2011). In mammals, several bridging molecules have been identified, including milk fat globule-epidermal growth factor 8 (MFG-E8) and growth arrest-specific 6 (Gas6) (Wu et al., 2006). Studies have shown that MFG-E8 binds in a Ca<sup>2+</sup>-dependent manner to PS exposed at the surface of apoptotic cells, engaging their uptake by integrins αβ3/5 (Hanayama et al., 2002; Borisenko et al., 2004; Kusunoki et al., 2012). Moreover, the absence of MFG-E8 in murine models causes a lupus-like autoimmune disease due to defective clearance of apoptotic cells (Huang et al., 2017). In Caenorhabditis elegans (C. elegans), the secreted protein TTR-52 mediates recognition of dying cells by linking PS with the phagocyte receptor CED-1 (Wang et al., 2010). While these studies have highlighted the relevance of bridging molecules in efferocytosis, the precise function of these molecules remains poorly understood. Some studies point to a role during the early phase of phagocytosis, facilitating apoptotic cell uptake, while others suggest that bridging molecules orient the maturation of the phagosome (Peng & Elkon, 2011; Galvan et al., 2012). The mechanism underlying efferocytosis has been well characterized in Drosophila (Serizier & McCall, 2017; Davidson & Wood, 2020). In this insect, three phagocytic receptors, the α-PS3/βv integrin heterodimer, Draper, and SIMU, have been identified for their role in the uptake of apoptotic cells (Freeman et al., 2003; Manaka et al., 2004a; Kurant et al., 2008; Nagaosa et al., 2011; Nonaka et al., 2013; Roddie et al., 2019). Studies have shown that the phagocytic ability of embryonic glia to engulf and degrade apoptotic neurons is determined by both SIMU and Draper. SIMU is required for recognition and engulfment of apoptotic neurons by glia, whereas Draper is mostly needed for their degradation (Kurant et al., 2008; Shklyar et al., 2014). Flies deficient for the two main receptors for apoptotic cells, Draper and SIMU, are viable but their lifespan is reduced and their central nervous system accumulates apoptotic bodies (Draper et al., 2014; Etchegaray et al., 2016). Although several engulfment receptors in Drosophila have been identified, little is known about their mechanism of action. So far, no bridging molecules have been identified in Drosophila.

In this study, we find that NimB4, a secreted protein of the Nimrod family, binds to apoptotic corpses in a PS-dependent manner to promote phagocytosis of apoptotic cells by glia and macrophages (also called plasmatocytes). Macrophages from NimB4 mutants show defective phagocytosis of apoptotic cells ex vivo. Similar to draper mutants, NimB4 mutants accumulate apoptotic cells in both embryonic and larval phagocytes, and exhibit motor defect in larvae and reduced lifespan of adults. Further functional studies reveal the role of NimB4 in phagosome maturation, notably in the formation of the phagolysosome. Collectively, this study identifies the first secreted factor involved in the phagocytosis of apoptotic cells in Drosophila and suggests an evolutionarily conserved role of bridging molecules in efferocytosis.

**Results**

**NimB4 is a secreted protein enriched in phagocytes**

To identify potential bridging molecules, we searched for secreted proteins expressed in phagocytes that could promote the phagocytosis of apoptotic cells. We focused our attention on secreted members of the Nimrod superfamily with characteristic EGF-like repeats (Bork et al., 1996; Callebaut et al., 2003; Kurucz et al., 2007), transmembrane Nimrod proteins have already been implicated in phagocytosis of bacteria (NimC1, Eater) or apoptotic corpses (SIMU, Draper), and in macrophages sessility and adhesion (Eater). In addition to these receptors, there are five secreted proteins of the Nimrod family called NimB1, B2, B3, B4, and B5 (Ju et al., 2006; Kurucz et al., 2007; Somogyi et al., 2008). Of these, only NimB5 has been characterized and has been shown to regulate peripheral hematopoiesis (Ramond et al., 2020). As bridging molecules are usually secreted by phagocytes, we monitored the expression of these five NimBs in macrophages of third-instar larvae. Consistent with a recent transcriptome analysis (Ramond et al., 2020), RT-qPCR analysis showed that NimB4 was the most highly expressed NimB member in macrophages (Fig 1A). To track the expression of NimB4 at the subcellular level, we generated a transgenic fly line carrying a V5-sGFP-tagged NimB4 fusion under its own regulatory sequences, derived from the Dresden pFlyFos collection (Sarov et al., 2016). We used this reporter gene to follow NimB4 during embryonic and larval stages with a focus on glia and macrophages. At the embryonic stage, NimB4-sGFP appeared to be strongly enriched exclusively in macrophages (Fig 1B), whereas at the larval stage NimB4-sGFP was expressed in both macrophages and glial cells (Figs 1C and D, and EV1A). Live and fixed imaging of third-instar larval macrophages showed that NimB4-sGFP is present intracellularly. Live confocal imaging showed that NimB4-sGFP localizes in small dots throughout the cytoplasm, suggesting a localization in the endosomal compartment (Fig 1C).

To investigate whether expression of NimB4 was modulated upon tissue damage, we collected larvae after clean injury with a thin needle in the anterior dorsal cuticle and monitored NimB4 expression in the two major immune-responsive tissues, the fat body, and hemocytes. We observed that clean injury induces NimB4 expression in hemocytes with an acute phase profile 1 h following challenge (Fig EV1B). In contrast, the transcription of NimB4 in the fat body remained low and unchanged (Fig EV1C).

**NimB4 binds to apoptotic cells**

NimB4 is predicted to be a secreted protein due to the presence of a signal peptide (Somogyi et al., 2008). We generated a transgenic fly lines carrying a UAS-NimB4-RFP insertion and overexpressed NimB4-RFP in the fat body using the fat body-specific Gal4 driver...
Western blot analysis of larval hemolymph extracts confirmed that NimB4-RFP protein was enriched in the hemolymph, as expected for a secreted protein (Fig EV1D). Furthermore, a strong NimB4-RFP signal was detected in the nephrocytes (a filtrating organ involved in the removal of hemolymph proteins) when NimB4-RFP was expressed either from the fat body or the hemocytes (Fig EV1E and F) (Ivy et al., 2015; Troha et al., 2019).

MFG-E8 binds PS exposed at the surface of apoptotic cells in a Ca²⁺-dependent manner, engaging their uptake by the integrin αvβ3/5 phagocytic receptor (Borisenko et al., 2004). To explore if NimB4 could...
similarly bind to apoptotic cells, we incubated hemolymph containing NimB4-RFP with healthy S2 cells, or with S2 cells undergoing apoptosis. We observed that NimB4-RFP protein colocalized exclusively with the apoptotic cells, stained with carboxyfluorescein succinimidyl ester (CFSE; Figs 2B and D, and EV2A). The binding of NimB4 to apoptotic cells was specific as no binding was observed when labeled apoptotic corpses were incubated with a control secreted protein composed of the RFP protein fused to the signal peptide of the Viking protein (SP\(^{vkg}\)-RFP) (Liu et al., 2017) (Fig 2A and D).

We then repeated the same experiment, but pre-treated apoptotic cells with Annexin V, a protein that coats apoptotic cells by binding to PS (Engeland et al., 1998; Janko et al., 2013). Pre-incubation of apoptotic corpses with Annexin V strongly reduced the binding of NimB4-RFP to the apoptotic cells, supporting the notion that NimB4 binds to apoptotic corpses in a PS-dependent manner (Fig 2C and D).

Next, we investigated if NimB4 can bind to apoptotic cells in vivo. For this, we induced apoptosis by expressing the apoptosis activator reaper in the imaginal wing disk using the driver Apterous-Gal4. After induction of apoptosis, we observed the binding of NimB4-sGFP to the region of the wing disk that expressed Apterous-Gal4 (Fig EV2B). Additionally, we observed the binding of NimB4 to apoptotic cells released in the hemolymph after clean injury. No binding was observed between apoptotic corpses and the secreted protein SP\(^{vkg}\)-RFP (Fig EV2C and D). Collectively, our studies revealed that NimB4 has key features of a bridging molecule in that it is secreted and binds to apoptotic corpses in a PS-dependent manner.

### NimB4-deficient animals accumulate apoptotic corpses during development

To investigate the role of NimB4 in vivo, we generated a null mutation in the NimB4 gene by CRISPR-Cas9, referred to as NimB4\(^{sk2}\). The NimB4\(^{sk2}\) mutant has a 14bp deletion in the second exon, inducing a stop codon at amino acid 113 (Fig EV3A). This finding suggests that NimB4 is required for the initial phase of uptake. To this end, we incubated macrophages and apoptotic cells on ice to inhibit the engulfment process, without altering binding to the phagocytic cell (Pearson et al., 2016). We observed that NimB4\(^{sk2}\) deficient macrophages had an increased level of Dcp-1 staining (Fig EV3B and C). Conversely, the number of apoptotic corpses bound to the cell membrane of Draper\(^{55}\) macrophages was reduced. Scanning electron microscopy experiments confirmed that apoptotic corpses bind to NimB4\(^{sk2}\) but not Draper\(^{55}\) macrophages (Fig 4C). This indicates that, unlike the Draper receptor, NimB4 is not involved in the initial phagocytic step of binding.

We then investigated whether NimB4 is required for the subsequent step of phagocytosis, that is the engulfment of bound apoptotic corpses. For this, we tested the ability of NimB4\(^{sk2}\) larval macrophages to internalize fluorescently labeled apoptotic bodies using an ex vivo phagocytic assay. Macrophages from wild-type, NimB4\(^{sk2}\), Draper\(^{55}\), and the double mutant NimC1\(^{5}\), eater\(^{1}\) were incubated for 30 min, 1 h, or 2 h with Alexa555 fluorescent apoptotic bodies, and flow cytometry was used to measure their phagocytic index. We found that, at 30 min, only the double mutant NimC1\(^{5}\), eater\(^{1}\) exhibited a reduced phagocytosis. At 1 h, we observed reduced phagocytosis in both NimC1\(^{5}\), eater\(^{1}\), and Draper\(^{55}\) mutants. Interestingly, the NimB4\(^{sk2}\) mutant exhibited reduced phagocytosis of apoptotic corpses at a later time point (2 h).
compared to the wild type (Fig 4D). Use of a genomic fragment encompassing the NimB4 gene rescued this decreased phagocytosis of the NimB4sk2 mutant at 2 h (Fig 4D). Of note, NimC11; eater1 mutants were unable to phagocytose apoptotic cells at all time points, indicating that the role of these two receptors is not restricted to bacterial phagocytosis.

To test if the role of NimB4 is specific to efferocytosis, we performed the same experiment with fluorescent AlexFluor488

**Figure 2. NimB4 binds to apoptotic cells ex vivo.**

A–C Representative confocal imaging of CFSE-stained apoptotic bodies (green) incubated with secreted NimB4-RFP (B, C) or SPVkg-RFP (A). Apoptotic bodies were pre-incubated in absence (A, B) or presence (C) of Annexin V (25 μg/ml). Scale bar = 10 μm.

D Quantification of the colocalization of NimB4-RFP or SPVkg-RFP with the apoptotic bodies in presence or absence of Annexin V, as measured by Pearson’s correlation coefficient. Values from at least four independent experiments are represented as mean ± SD (**P < 0.01 by ANOVA test followed by post hoc Dunnett’s multiple comparison tests. ns: not significant).
Staphylococcus aureus Bioparticles. Interestingly, NimB4Δ2 macrophages retained the ability to phagocytose S. aureus bacteria at 2 h similar to the wild-type macrophages (Fig 4E). We included macrophages from NimC1Δ and eaterΔ larvae that show impaired phagocytosis of bacteria as a positive control (Melcarne et al, 2019b). We conclude that NimB4 is not specifically required for the binding of apoptotic corpses but enhances phagocytosis of apoptotic cells.

Figure 3. The NimB4Δ2 mutant is defective in cell corpse clearance.
A Representative confocal imaging of wild-type (w1118), NimB4Δ2, and apoptosis-null NimB4Δ2; Df(3L) H99 embryonic macrophages at stage 16 of development; ventral and lateral view; Tissues were co-stained with Dcp-1 (red, corresponding to apoptotic corpses) and anti-SIMU (green, corresponding to macrophages) antibodies. The arrows show the presence of apoptotic cells inside embryonic macrophages. Scale bars = 20 μm.

B Quantification of caspase-positive particles (anti-Dcp-1, red) within macrophages (anti-SIMU, green). Values from at least five independent experiments are represented as mean ± SD total volume of caspase-positive particles (***P < 0.01, by Mann-Whitney test).

C Representative projections from confocal stacks of entire third-instar wild-type (w1118) and NimB4Δ2 larval brains stained with anti-Draper (green) and anti-Dcp-1 (red). The arrows show the presence of apoptotic cells in the central area of NimB4Δ2 larval brain. Scale bars = 100 μm.

D Quantification of caspase-positive particles in the central brain area of larval brain. Values from at least five independent experiments are represented as mean ± SD total volume of caspase-positive particles (***P < 0.01, by Mann-Whitney test).
Figure 4. Phagocytosis of apoptotic cells is reduced in NimB4 sk2 mutant.
A Representative confocal imaging of wild-type (w1118), NimB4 sk2, and draperΔ5 macrophages incubated with fluorescently labeled apoptotic cells (red, CellTrace™ Red) on ice for 1 h and stained with Alexa Fluor™ 488 phalloidin (green). Scale bar: 10 μm.
B Quantification of the number of apoptotic cells (red) binding to the macrophages (green). Values from three independent experiments are represented as mean ± SD (***P < 0.001 by ANOVA test followed by post hoc Dunnett’s multiple comparison tests. ns: not significant).
C Representative Scanning Electron Microscopy images of spread macrophages extracted from wild-type (w1118), NimB4 sk2, and draperΔ5 L3 wandering larvae and incubated 30 min with apoptotic cells (artificially colored in red) at room temperature. The arrows show the binding of apoptotic cells to the macrophages. Scale bar: 1 μm.
D Ex vivo phagocytosis assay using Alexa555 fluorescent apoptotic bodies. Wild-type, NimB4 sk2, NimB4 genomic rescue (NimB4 sk2, [NimB4]), draperΔ5 and NimC11, eaterΔ macrophages from L3 wandering were incubated with Alexa555 fluorescent apoptotic bodies for 30, 60, or 120 min at room temperature. Phagocytosis was quantified by flow cytometry. Data are represented as mean ± SD from three independent experiments (*P < 0.05, **P < 0.01, ****P < 0.0001 by ANOVA test followed by post hoc Dunnett’s multiple comparison tests. ns: not significant).
E Ex vivo phagocytosis assay using AlexFluor488 Staphylococcus aureus Bioparticles. Wild-type, NimB4 sk2, and NimC11, eaterΔ macrophages from L3 wandering were incubated with bioparticles for 30, 60, or 120 min at room temperature. Phagocytosis was quantified by flow cytometry. Data are represented as mean ± SD from three independent experiments (****P < 0.0001 by ANOVA test followed by post hoc Dunnett’s multiple comparison tests. ns: not significant).
Loss of NimB4 inhibits phagosome maturation

The accumulation of apoptotic corpses in embryonic NimB4Δ22 macrophages in vivo indicates impaired phagocytosis in these cells, while a reduction in phagocytosis only at late time points suggests a defect in phagosome maturation. We therefore explored if the loss of NimB4 alters the phagosome maturation process. In these experiments, we included as controls draperΔ5 and croquemortΔ mutants previously shown to have defective phagosome maturation (Kurant et al, 2008; Han et al, 2014). The intracellular vesicles were analyzed using the fluorochrome LysoTracker Red, which fluoresces in acidic compartments. We observed that NimB4Δ22, draperΔ5, and croquemortΔ but not eater1 or NimC1Δ22 macrophages contained numerous and enlarged acidic vesicles compared to wild-type macrophages (Figs 5A and B, and EV4A). To study possible NimB4 interactions with phagocytic receptors, we measured LysoTracker signal in macrophages double mutants for NimB4Δ22 and draperΔ5, croquemortΔ, eater1, or NimC1Δ (Fig EV4A). We observed an increased LysoTracker signal in the NimB4Δ22; draperΔ5 mutant suggesting that Draper and NimB4 additively contribute to phagosome maturation. The NimB4Δ5, croquemort Δ double mutants did not show any increased LysoTracker signal compared to the single mutants. This result suggests that Croquemort and NimB4 might work together in the phagosome maturation process.

The accumulation of intracellular vesicles in NimB4Δ22 macrophages was rescued by complementing the mutant with a transgene containing a wild-type copy of NimB4 gene (Fig EV4B and C). In addition, silencing NimB4 using RNAi with the Hml-Gal4 macrophage driver reproduces this phenotype, confirming that the accumulation of intracellular vesicles was indeed caused by the inactivation of NimB4 in macrophages (Fig EV4D and E). We next analyzed the subcellular morphology of NimB4Δ22 intracellular vesicles by transmission electron microscopy. NimB4Δ22 but not wild-type macrophages were filled with large intracellular vesicles that occupied most of the cell volume. These vesicles were surrounded by a single lipid bilayer and had a clear lumen (Fig 5C and D). A similar vesicle accumulation was also observed upon silencing of NimB4 by RNAi (Fig EV4F and G). To confirm that this phenotype was not linked to a defect due to loss of NimB4 during development, we used a temperature-inducible macrophage driver (HmlΔ22) to express NimB4-RNAi after the second-instar larval (L2) stage. LysoTracker staining showed that hemocytes in which NimB4 was inactivated after the L2 stage also showed accumulation of acidic vesicles (Fig EV4H and I). Therefore, we conclude that the accumulation of acidic vesicles in NimB4 macrophages is not caused by a defect at the early stages of development.

NimB4 plays a crucial role in phagosome–lysosomes fusion

Phagosome maturation culminates in the fusion of the phagosome with lysosomes, leading to the formation of an acidic phagolysosome. The formation of phagolysosomes is a necessary step to ensure efficient digestion of apoptotic corpses (Zhou & Yu, 2008). During phagosome maturation, the transition from early phagosome to late phagosome is marked by the conversion of Rab5 to Rab7. In addition, the phagosome acquires Lamp1, which is required for phagolysosome fusion (Huynh et al, 2007). Our observation that accumulated vesicles in NimB4Δ22 macrophages are LysoTracker-positive and have therefore initiated acidification suggests that NimB4 is required at a later stage of phagosome maturation. To determine more precisely the step at which maturation of NimB4Δ22 phagosomes is blocked, we tested whether Rab7, a marker of late phagosomes, was enriched on the vesicular membrane of NimB4Δ22 macrophages. We observed that the enlarged vesicles of NimB4Δ22 macrophages were positive for Rab7EYFP, confirming that phagosomes are blocked at a late stage of maturation (Fig 5E and F). To determine whether fusion between the phagosome and lysosomes requires NimB4, we analyzed the fate of wild-type, NimB4Δ22, and draperΔ5 macrophages stained with the phagosome marker LysoTracker and the lysosome marker Lamp1-mcherry. In wild-type macrophages, these markers colocalized, indicating correct formation of the phagolysosomes. In contrast, we found no colocalization of LysoTracker and Lamp1-mcherry in the NimB4Δ22 and draperΔ5 macrophages (Fig 6A and B). The Lamp1-mcherry lysosomal signals appeared as small punctate structures between the LysoTracker-positive vacuoles. This suggests that a loss of NimB4 or draper impairs the fusion rather than the clustering of phagosomes with lysosomes (Fig 6A and B). An increased Lamp1-mcherry fluorescent signal in NimB4Δ22 mutant macrophages indicated that the blockage of phagolysosome fusion was not due to a reduced number of lysosomes in the NimB4Δ22 mutant. Interestingly, we observed a similar increase in the number of Lamp1-mcherry-positive structures when wild-type macrophages were pre-treated with chloroquine, a drug that inhibits endosomal acidification, which is used here to block phagosome maturation (Fig EV5A and B).

The fusion of the phagosome with the lysosome allows the acidification of the phagosome. We therefore assessed the acidification of phagosomes in both NimB4Δ22, draperΔ5, and wild-type macrophages. For this, we used apoptotic cells labeled with pHrodo-succinimidyl ester (SE), a pH-sensitive fluorescent dye. We then assessed the acidification of the phagosome measuring the mean fluorescence intensity at 1 h after incubation as the NimB4Δ22 mutant did not show phagocytosis defect at this time point. We observed by flow cytometry a reduced fluorescence intensity in the NimB4Δ22 and the draperΔ5 mutant compared to wild type consistent with defective acidification of the phagosome (Fig 6C).

We repeated the same experiment with pHrodo bioparticles labeled with S. aureus as the NimB4Δ22 mutant previously showed no defect in the phagocytosis of S. aureus Alexa Fluor 488 BioParticles (Fig 6D). We observed by flow cytometry a reduced fluorescence intensity of pHrodo-labeled S. aureus bioparticles in the NimB4Δ22 mutant macrophages compared to wild type (Fig 6D) consistent with defective acidification of the phagosome. It has been shown that overexpression of Rab7 efficiently promotes the phagosome-lysosome fusion (Harrison et al, 2003). We therefore investigated whether increased expression of Rab7 could rescue the phagosome-lysosome fusion defect of NimB4Δ22 macrophages. Overexpression of Rab7, but not Rab5, strongly reduced the number of accumulated vacuoles in NimB4Δ22 macrophages, showing that promoting the phagosome-lysosome fusion is sufficient to rescue the NimB4Δ22 cells (Fig 7A–C). In this experiment, we found that the overexpression of Rab5 in wild-type macrophage has a pronounced defect on its own as previously described (Bucci et al, 1992). We conclude that NimB4 is required for the late stage of phagosome maturation and more specifically, for the fusion of the phagosome and lysosome.
Figure 5. *NimB4*<sup>△sk</sup> mutant macrophages have an increased number of vesicles.

A Representative fluorescence microscopy images of wild-type (w<sup>1118</sup>), *NimB4*<sup>△sk</sup>, *draper<sup>▵5</sup>* and *croquemort<sup>▵</sup>* third-instar larvae macrophages stained with LysoTracker Red (live imaging). Overlay of fluorescence and differential interference contrast microscopy (DIC). Scale bar = 10 μm.

B Mean fluorescence intensity after staining with LysoTracker Red (live confocal imaging). Values from at least three independent experiments are represented as mean ± SD (**P < 0.01, ****P < 0.0001 by ANOVA test followed by post hoc Dunnett’s multiple comparison tests).

C Representative transmission electron micrographs of macrophages from wild-type (top, w<sup>1118</sup>) and *NimB4*<sup>△sk</sup> (bottom) L3 wandering larvae. Scale bar: 2 μm.

D Quantification of the number of vesicles per macrophages in the electron micrographs. Values from at least three independent experiments are represented as mean ± SD (**PP < 0.0001 by Mann–Whitney test).

E Representative confocal imaging of Rab<sup>7</sup>EYFP immunostaining in wild-type (w<sup>1118</sup>) and *NimB4*<sup>△sk</sup> macrophages. Tissues were stained with anti-GFP (red, Rab7), counterstained with phalloidin (gray) and DAPI (blue). The arrows indicate the enlarged vesicles are decorated with Rab<sup>7</sup>EYFP in the *NimB4*<sup>△sk</sup> macrophages. Scale bar = 10 μm.

F Quantification of the diameter of the vesicles. Values from at least three independent experiments are represented as mean ± SD (****P < 0.0001 by Mann–Whitney test).
Figure 6. Loss of NimB4 blocks the phagosome maturation process by impairing the phagosome-lysosome fusion.

A Representative confocal imaging of localization of Lamp1-mcherry and LysoTracker Green in wild-type (w1118), NimB4 sk2, and draperΔ5 macrophages (live imaging). The arrows indicate the colocalization (w1118) or the clustering (NimB4 sk2 and draperΔ5) of Lamp1-mcherry and LysoTracker Green signals. Scale bar: 10 μm.

B Quantification of the colocalization of Lamp1-mcherry with LysoTracker Green, as measured by Pearson’s correlation coefficient between the two signals. Values from at least five independent experiments are represented as mean ± SD (*P < 0.05 by ANOVA test followed by post hoc Dunnett’s multiple comparison tests).

C Ex vivo phagocytosis assay using apoptotic cells labeled with pHrodo™ Red. Wild-type (w1118), NimB4 sk2 and draperΔ5 macrophages from L3 wandering larvae were incubated with pHrodo™ Red apoptotic cells for 60 min at room temperature. Phagocytosis was quantified by flow cytometry. Data are represented as mean ± SD from three independent experiments (*P < 0.05 by ANOVA test followed by post hoc Dunnett’s multiple comparison tests. ns: not significant).

D Ex vivo phagocytosis assay using pHrodo™ Red Staphylococcus aureus Bioparticles™ conjugates. Wild-type, NimB4 sk2 and NimC1Δ, eaterΔ HmlΔ-Gal4 > UAS-CFP macrophages from L3 wandering larvae were incubated with pHrodo™ Red S. aureus Bioparticles™ for 30, 60, or 120 min at room temperature. Phagocytosis was quantified by flow cytometry. Data are represented as mean ± SD from three independent experiments (*P < 0.05 by ANOVA test followed by post hoc Dunnett’s multiple comparison tests. ns: not significant).
Discussion

The clearance of apoptotic cells by phagocytes is a critical event during the development of all multicellular organisms. Failure in this process can lead to autoimmune or neurodegenerative diseases. In this study we identified a secreted protein of the Nimrod family, NimB4, that binds to apoptotic corpses in a PS-dependent manner to promote efferocytosis. Similar to mutants of the phagocytic receptor Draper, the NimB4$^{sk2}$ mutant accumulates apoptotic cells in both embryonic and larval macrophages. Further functional studies reveal its role in phagosome maturation, notably the formation of the phagolysosome. Our study identifies the first secreted factor involved in the phagocytosis of apoptotic cells in Drosophila, resembling in many respects the bridging molecules found in mammals.

NimB4 is a secreted factor that promotes the phagocytosis of apoptotic cells

NimB4 was initially identified as a secreted member of the Nimrod family of unknown function. Our results show that NimB4 is expressed in macrophages and glial cells, and that its expression is induced upon injury, a stimulus associated with increased apoptosis (Steller, 2008). In mammals, the expression of the gene encoding the bridging molecule MFG-E8 is regulated by a “find me factor” released by apoptotic cells (Miksa et al., 2007). Further study should identify the signaling pathway regulating NimB4 upon injury and test whether Drosophila “find me factor” are involved in this process (Ravichandran, 2010, 2011).

We also observed that NimB4 is secreted by macrophages and that it is likely associated with the endosomal compartments intracellularly. NimB4 mutants display a shorter lifespan and a locomotor defect that can be causally linked to defective apoptotic cell clearance during development and aging. These phenotypes are very similar to those previously described for draper mutants, albeit slightly weaker (Kurant et al., 2008; Draper et al., 2014; Etchegaray et al., 2016).

Our data also indicate that NimB4 binds to apoptotic corpses in a PS-dependent manner. As a member of the Nimrod family, NimB4 shares homology with SIMU and Draper, two phagocytic receptors

Figure 7. The increased expression of RAB7 rescues the phagosomes accumulation in NimB4$^{sk2}$ macrophages.

A-B Representative fluorescence microscopy images of wild-type (A) or NimB4$^{sk2}$ (B) macrophages expressing Rab5 (middle panel) or Rab7 (right panel) driven by HmlΔ-Gal4 and stained with the LysoTracker Red (live imaging). Overlay of fluorescence and DIC. Scale bar = 10 μm.

C Quantification of the mean fluorescence intensity of LysoTracker in macrophages from larvae (confocal live imaging). Values from at least three independent experiments are represented as mean ± SD (***P < 0.01, ****P < 0.0001 by ANOVA test followed by post hoc Dunnett’s multiple comparison tests. ns: not significant).
that also interact with PS (Shklyar et al., 2013a; Tung et al., 2013). This suggests that the common EGF/Nimrod domain found in Nimrod family members may contribute to binding PS. However, it is unclear whether NimB binds to apoptotic cells alone and interacts directly with receptors involved in efferocytosis, or whether other co-factors are required. It is worth noting that the Drosophila genome encodes several other secreted NimB proteins such as NimB1, NimB2, and NimB3 that are not yet characterized (Somogyi et al., 2008). It is tempting to speculate that some of these proteins could work synergistically with NimB4 in the process of efferocytosis. The similarities between the NimB4\(^k2\) and draper\(^{e5}\) mutant phenotypes suggest that both proteins function in a similar efferocytosis pathway. In this study, we did not find any evidence that NimB4 interacts with Draper. Future studies should address whether NimB4 establishes a direct bridge between apoptotic corpses and phagocytic receptors.

**NimB4 is specifically required for the phagocytosis of apoptotic cells**

NimB4 is required for the phagocytosis of apoptotic cells but not bacteria. This indicates that it is not a core component of the phagocytic machinery. This result is in line with many studies that show that the phagocytic process is multi-faceted and is tailored to the nature of the ingested particle. For instance, studies have shown that phagosomes containing apoptotic cells mature faster than those containing opsonized viable cells (Erwig et al., 2006). These observations indicate that phagocytic targets can differentially affect the maturation rate, perhaps through the recruitment of different phagocytic receptors. In contrast to NimB4, Draper contributes to the phagocytosis of both bacteria (S. aureus) and apoptotic cells (Manaka et al., 2004b; Shiratsuchi et al., 2012), pointing to more versatile functions. In this study, we incidentally showed that NimC1 and Eater, two immune receptors implicated in the phagocytosis of microbes, are also required for the efferocytosis process. In contrast, the role of SIMU seems to be restricted to apoptotic cell clearance during embryonic and early adult phase development (Kurant et al., 2008). These authors speculated that the presence of SIMU reinforces uptake of apoptotic cells at critical developmental stages characterized by massive apoptosis. We hypothesize that, like SIMU, NimB4 may have a more specific function related to a particular efferocytosis program.

**The secreted NimB4 participates in phagosome maturation**

Importantly, our study shows that secreted NimB4 binds to apoptotic cells and is an essential component of the machinery that promotes efferocytosis. Thus, as observed in mammals and C. elegans, our study reveals that secreted factors contribute to the apoptotic cell clearance in Drosophila. However, NimB4 is not required for macrophages binding to apoptotic bodies. Instead, apoptotic corpse uptake is reduced only at late time points. We hypothesize that this phenotype is a secondary consequence of a defect in phagosome maturation. The accumulation of immature phagosomes that are not properly eliminated would indirectly impair the uptake of new apoptotic corpses. Efferocytosis would then be impaired only at late time points when the accumulation of phagosomes reaches a threshold preventing further phagocytosis. Phagosome maturation is punctuated by two main events: the Rab5-Rab7 conversion and the fusion between the phagosome and the lysosome (Vieira et al., 2002; Kinchen & Ravichandran, 2008; Akbar et al., 2011; Pauwels et al., 2017; Pradhan et al., 2019). Interestingly, overexpression of Rab7 rescues the phagosome maturation blockage of NimB4 mutant. Our study suggests that NimB4 is required late in the process, at the step of phagosome-lysosome fusion. Interestingly, the receptors Draper and Croquemort, initially thought to be involved in the early phase of phagocytosis, were later shown to be critical for phagosome maturation (Kurant et al., 2008; Han et al., 2014). How those receptors and NimB4 contribute to the maturation of phagosome is currently unknown. We speculate that the binding of NimB4 to apoptotic cells could orient the maturation process; that is, apoptotic corpses may require specific “digest me” signals similar to the “eat-me” signals that are required for uptake. Several mechanisms could explain how a secreted factor can impact phagosome maturation. Binding of NimB4 to apoptotic cells could cluster phagocytic receptors or activate a specific phagocytic receptor that initiates a dedicated digestion program affecting the degradation speed of ingested apoptotic cells. Alternatively, NimB4 could promote phagocytosis and lysosome maturation independently by interacting with different partners in the phagocytic process.

**Is NimB4 a bridging molecule?**

Bridging molecules recognize “eat-me” signals on apoptotic cells facilitating their uptake by phagocytic receptors (Ravichandran, 2010). In mammals, several soluble bridging molecules such as MFG-E8, Gas6, and protein S mediate recognition of apoptotic cells by cross-linking the PS “eat-me” signal with specific phagocytic receptors. For instance, MFG-E8 binds to the integrin receptor of phagocytic cells (Hanayama et al., 2002; Akakura et al., 2004; Nandrot et al., 2007). In C. elegans, the protein TTR-52 bridges the surface-exposed PS on apoptotic cells and the CED-1 receptor on phagocytes, which mediates recognition and engulfment of apoptotic cells (Wang et al., 2010). While the initial concept viewed bridging molecules as opsonins dedicated to the uptake of apoptotic cells, recent studies suggest greater complexity. Similar to NimB4, subsequent studies showed that MFG-E8 is required in the maturation phase of phagocytosis as well as in the recognition step. While apoptotic cells ingested by wild-type dendritic cells rapidly fused with lysosomes, in dendritic cells deficient for MFG-E8 smaller fragments of apoptotic cells persisted in endosomes (Peng & Elkon, 2011). NimB4 shares key features of bridging molecules, in that it is secreted by phagocytic cells, binds to apoptotic cells in the PS-dependent manner, and is specifically required for efficient efferocytosis.

Although this study has not conclusively demonstrated that NimB4 interacts with phagocytic receptors as mammalian bridging molecules do (Boada-Romero et al., 2020), our results reveal the importance of secreted factors that enhance efferocytosis and reinforce the notion that “bridging molecules” could play a broader role than initially thought, in orienting phagosome maturation. Future studies should characterize how secreted factors promote and direct the process of efferocytosis, and Drosophila offers a powerful system to address these questions.
# Materials and Methods

## Reagents and Tools table

| Reagent/Resource | Reference or Source | Identifier or Catalog Number |
|------------------|---------------------|------------------------------|
| **Experimental models** | | |
| DrosDel w^{1118} isogenic (wild type) | Gift from Luis Teixeria (Pais, 2018) | N/A |
| w^{1118}; NimB4^{1b2} | This study | N/A |
| w^{406}, NimB4^{1b2} (isogenized in the DrosDel background) | This study | N/A |
| w; UAS-NimB4 IR | VDRC | 106392 |
| w; UAS-NimB4 | This study | N/A |
| w; UAS-NimB4-RFP | This study | N/A |
| w; UAS-NimB4-HA | This study | N/A |
| w; UAS-NimB4-RFP, UAS-NimB4-HA | This study | N/A |
| w; UAS-NimB4-HA | This study | N/A |
| w; UAS-NimB4-IR, UAS-NimB4-RFP | This study | N/A |
| Df(3L)H99, ken^{1b1} p^TM3, Sb^{1} | Gift from Yuh-Nung jang (Han et al, 2014) | N/A |
| Df(3L)H99, ken^{1b1} p^TM3, Sb^{1} | Gift from Yuh-Nung jang (Han et al, 2014) | N/A |
| tubGal80^{59},RepoGal4 | Hakim-Mishnaevski et al (2019) | N/A |
| Act5Gal4(cyGFP) | Bretscher et al (2015) | N/A |
| UAS-SP^{59}-RFP,3.1; [30 first amino acids of Viking protein followed by RFP] | Gift from Pastor-Parja I (Liu et al, 2017) | N/A |
| UAS-SP^{59}-RFP,3.1; [30 first amino acids of Viking protein followed by RFP] | Gift from Pastor-Parja I (Liu et al, 2017) | N/A |
| BDSC | 62546 |
| Bloomington | 9771 |
| Gift from Brian McCabe | N/A |
| Gift from Luis Teixeira | N/A |
| Reagent/Resource | Reference or Source   | Identifier or Catalog Number |
|------------------|----------------------|----------------------------|
| FM7, iso, iso    | Gift from Luis Teixeira | N/A                        |
| Iso, GlcP, iso   | Gift from Luis Teixeira | N/A                        |
| **Recombinant DNA** |                      |                            |
| pENTR/D-TOPO     | Invitrogen           | K240020                    |
| pTW              | DGRC                 | 1129                      |
| pTWR             | DGRC                 | 1136                      |
| pOT2-CG16873 Full length cDNA clone | DGRC | IP09831 |
| pCaeSpeR4        | Our collection       | N/A                       |
| BACR09N24        | BACPAC Resources Center | RP98-9N24                |
| **Antibodies**   |                      |                            |
| Chicken anti-GFP | Abcam                | Cat# ab13970               |
| Rabbit monoclonal anti-SIMU antibody | Shklyar et al (2013b) | N/A                       |
| Mouse monoclonal anti-Draper antibody | Kurant et al (2008) | N/A                       |
| Rabbit anti Dcp-1 antibody | Cell Signaling | Cat # 9578                 |
| Mouse monoclonal anti mCherry antibody | Invitrogen | Cat # M11217                |
| Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP | Jackson ImmunoResearch labs | Cat# 31430 |
| Goat anti-Chiken IgY (H+L) Secondary antibody, Alexa Fluor 488 | Thermo Fisher Scientific | Cat# A11039 |
| Goat anti-Mouse IgG (H+L) Highly Cross-adsorbed Secondary Antibody, Alexa Fluor 555 | Thermo Fisher Scientific | Cat# A21424 |
| Goat anti-Rabbit IgG (H+L) Highly Cross-adsorbed Secondary Antibody, Alexa Fluor 555 | Thermo Fisher Scientific | Cat# A32731 |
| **Oligonucleotides and sequence-based reagents** | | |
| NimB4 F deletion screen: | Microsynth | N/A |
| CGA TCT CTG TGC CTC CAC TC | | |
| NimB4 R deletion screen | Microsynth | N/A |
| CTT GCA ACA AAC CTC CAT CA | | |
| NimB4 deletion screen | Microsynth | N/A |
| CCC AAC GGT TTG TCC GGA ACA TC | | |
| NimB1 qRT F | Microsynth | N/A |
| CGG CCA AAG TGT CAG AGA TT | | |
| NimB1 qRT R | Microsynth | N/A |
| TAT CGT CAC ACC TTC CGT TC | | |
| NimB2 qRT F | Microsynth | N/A |
| GAG TGT CTG CCG AAG TGT GA | | |
| NimB2 qRT R | Microsynth | N/A |
| TCA CAT ATC CGG TCT TGC AG | | |
| NimB3 qRT F | Microsynth | N/A |
| TCC CAA CTC CAG AAA TCG TC | | |
| NimB3 qRT R | Microsynth | N/A |
| AGC AGT CCT CCG AGC AAA T | | |
| NimB4 qRT F | Microsynth | N/A |
| TGG TGC TCA ACT ACC GCA AC | | |
| NimB4 qRT R | Microsynth | N/A |
| CGT CCA GCT CCT ATC CCT TA | | |
| NimB5 qRT F | Microsynth | N/A |
| CGT AAC GAC AAG GGT GAC TG | | |
| NimB5 qRT R | Microsynth | N/A |
| GTC TGG TCC AGC TTG TAG CC | | |
| Rp32 qRT F | Microsynth | N/A |
| GAC GCT TCA AGG GAC AGT ATC TG | | |
Reagents and Tools table (continued)

| Reagent/Resource | Reference or Source | Identifier or Catalog Number |
|------------------|---------------------|-------------------------------|
| RpL32 qRT R      | Microsynth          | N/A                           |
| AAA GCC GGT TCT CCA TGA G |                 |                               |
| NimB4_gateway cloning F | Microsynth          | N/A                           |
| CAC CAT GTC AAC AAT ACT GCG A |                  |                               |
| NimB4_gateway cloning R | Microsynth          | N/A                           |
| CAA CCA CCA CAT ATC GCT GAG |                  |                               |

Chemicals, enzyme and other reagents

- **PrimeScript RT** TAKARA Cat# RR037B
- **CFSE** (5(6)-CFDA/SE Thermo Fisher Scientific Cat# C34554
- **CellTracker™ Red CMTPX dye** Thermo Fisher Scientific Cat# C34572
- **Triton-X-114** (10% solution) Sigma 1169748001
- **Complete protease inhibitor** Sigma Cat# D9542
- **4',6-Diamidino-2-phenylindole dihydrochloride (DAPI)** Sigma Cat# A2287
- **AlexaFluor 488 Phalloidin** Life Technologies Cat# A34055
- **AlexaFluor 555 Phalloidin** Life Technologies Cat# L7528
- **LysoTracker® Red DND-99** Thermo Fisher Scientific Cat# L7526
- **LysoTracker® Green DND-26** Thermo Fisher Scientific Cat# L7526
- **phenylmethylsulfonyl fluoride** Sigma P7626
- **AlexFluor™ 488 S. aureus Bioparticles™** Thermo Fisher Scientific Cat# s23371
- **pHrodo™ Red S. aureus Bioparticles™** Thermo Fisher Scientific Cat# A10010

Software

- **Prism 5** GraphPad Prism N/A
- **Fiji 2.1.0/1.53c** Image J N/A

Others

- **Tecan Infinite M200** Tecan N/A
- **Leica SP8 IN1** Leica N/A
- **The Zeiss Axiosmager Z1** Zeiss N/A

Methods and Protocols

**Drosophila rearing conditions**

All *Drosophila* stocks were maintained at 25°C on standard fly medium consisting of 6% cornmeal, 6% yeast, 0.6% agar, 0.1% fruit juice (consisting of 50% grape juice and 50% multifruit juice), supplemented with 10.6 g/l moldex and 4.9 ml/l propionic acid. Third-instar (L3) wandering larvae were selected at 110–120 h AEL.

**Mutant and transgenic lines generation**

*NimB4* flies were generated using the CRISPR/Cas9 technique as previously described (Kondo & Ueda, 2013). Briefly, a transgenic fly line expressing Cas9 protein using the germ-line-specific nanos promoter was crossed to a line expressing a custom guide RNA (gRNA). The cross-produces offspring with an active Cas9–gRNA complex specifically in germ cells, which cleaves and mutates the genomic target site. The following gRNA sequence was used: GGTGGTTCGCGCCCGGGAG. To avoid any background effects, we introgressed *NimB4* mutant into the w1118 DrosDel isogenic background for seven generations. For complementation studies, the genomic region of *NimB4* was amplified from genomic DNA including 2 kb upstream and 2 kb downstream of the *NimB4* coding sequence. Gibson assembly was used to clone the fragment into a pUASP-attB-GFP-V5-His backbone (Rauskolb et al., 2011). The *NimB5* and *NimB3* genes present in the upstream and downstream sequence of *NimB4*, respectively, were inactivated by directed mutagenesis. Mutagenic primers were designed to delete the G of the ATG start codon of *NimB3* and to add GA in position 44 of *NimB5* to generate non-sense coding sequences. The plasmid was then microinjected in the VK33 attP embryos. For overexpression studies, the genomic region from the 5'UTR to the stop codon of the intron-less *NimB4* gene was amplified from BACR09N24 and cloned into the pDONR207 Gateway vector (Invitrogen, Carlsbad, CA, USA) and subcloned in the pTW (Drosophila Genomics Resource Center plasmid) transgenesis vector and used to generate transgenic *UAS-NimB4* flies. For the *UAS-NimB4-RFP*, the NimB4 cDNA sequence without STOP codon was cloned into the entry vector pENTR/D-Topo (Invitrogen) and subsequently shuttled into the RFP expression vectors pTWR (C-terminal RFP tag), obtained from the DGRC Drosophila Gateway vector collection. Plasmids were injected either at the Fly facility platform of Clermont-Ferrand (France) or by BestGene Inc. (Chino Hills, CA, USA).
RT-qPCR experiments
For quantification of mRNA, whole third-instar larvae (n = 8) or dissected tissues (n = 20–40) were isolated by TRIzol reagent and dissolved in RNase-free water. 500ng total RNA was then reverse-transcribed in 10 ml reaction volume using PrimeScript RT (TAKARA) and a mixture of oligo-dT and random hexamer primers. Quantitative PCR was performed on cDNA samples on a LightCycler 480 (Roche) in 96-well plates using the LightCycler 480 SYBR Green I master mix (Roche Diagnostics, Basel, Switzerland). Expression values were normalized to that of RpL32.

Western blot
Hemolymph samples were collected as follows: Forty L3 larvae were bled on a glass slide on ice; hemolymph was recovered by dissection, mixed with 10 μl of PBS supplemented with complete protease inhibitor solution (Roche) and 1mM phenylmethylsulfonyl fluoride (Sigma) and N-Phenylthiourea (Sigma), and then centrifuged for 10 min at 1,000 g, 4°C. This was followed by a second centrifugation 5 min 10,000 g. Protein concentration of the samples was determined by BCA assay, and 40 μg of protein extract was separated on a 4–12% acrylamide precast Novex NuPage gel (Invitrogen) under reducing conditions and transferred to membranes (Invitrogen iBlot 2). After blocking in 5% non-fat dry milk in PBS containing 0.1% for 1 h, membranes were incubated at 4°C overnight with a mouse anti-RFP antibody (Abcam) in a 1:1,000 dilution. Anti-mouse-HRP secondary antibody was detected using ECL (GE Healthcare) according to the manufacturer’s instructions. Membranes were imaged on a ChemiDoc XRS+ (Bio-Rad).

Apoptotic cells preparation
The S2 cells were cultured in Schneider’s insect medium (Sigma-Aldrich) containing 10% FBS (Gibco®), penicillin (Sigma-Aldrich), and streptomycin (Sigma-Aldrich) at a concentration of 100 U/ml. To induce apoptosis, cycloheximide (CHX, Sigma-Aldrich) was added at a final concentration of 50 μg/ml. 24 h after the cycloheximide treatment, the cells were isolated and removed by pelleting with centrifugation at 400 g for 5 min at 4°C. In order to stain the apoptotic bodies, CFSE (5(6)-CFDA/SE, Molecular Probes®), or CellTracker™ Red CMTPX dye was added to the supernatant harvested from the cells at a final concentration of 5 μM and incubated 15 min at room temperature in the dark.

pHrodo staining of apoptotic cells
Apoptotic cells were labeled with the pH-sensitive stain pHrodo™ Red, succinimidyl ester (Invitrogen), which is nonfluorescent at neutral pH and emits a strong red fluorescence (532 nm) in an acidic environment (pH 4–6). After the induction of apoptosis by cycloheximide treatment, apoptotic cells were washed twice with PBS and resuspended in PBS at 10⁶ cells/ml. 1 μl of 1 mg/ml pHrodo-SE (stock solution in DMSO) was added to 50 ml of cell suspension. After incubation for 30 min at RT, cells were washed twice with PBS and resuspended in PBS.

Immunohistochemistry
For immunofluorescence, L3 larvae were dissected into 150 μl PBS pH 7.4, and macrophages were allowed to adhere on a glass slide for 40 min and fixed for 10 min in PBS containing 4% paraformaldehyde. Larval tissues were dissected in PBS and fixed for at least 1 h at room temperature in 4% paraformaldehyde in PBS. For immunostaining, fixed tissues were subsequently rinsed in PBS + 0.1% Triton X-100 (PBT), permeabilized, and blocked in PBT + 2% bovine serum albumin (BSA) for 1 h and incubated with primary antibodies in PBT + 2% BSA overnight at 4°C. After 1 h washing, secondary antibodies and DAPI were applied at room temperature for 2 h. Primary antibodies used are as follows: chicken anti-GFP (Abcam, 1:1,000), rabbit anti-SIMU 1:100 (Shklyar et al, 2013b), mouse anti-Draper (Developmental Studies Hybridoma Bank, 1:100), rabbit anti-Dcp-1(cell Signaling, 1:100), Mouse anti-mCherry (Invitrogen, 1:1,000), Alexa Fluor 488 and Alexa Fluor 555-conjugated secondary antibodies (Life technologies, 1:100) were used. For the chloroquine experiment, macrophages were dissected into 150 μl PBS pH 7.4, with 50 μg/ml of chloroquine and allowed to adhere on a glass slide in this solution for 40 min. Cells were then fixed with PFA 4% and immunostained as described above. Finally, cells were stained with 1/15,000 dilution of DAPI (Sigma-Aldrich) and mounted in Dako fluorescence media. Imaginal wing disks were dissected in PBS and then fixed for 15 min at room temperature in 4% PFA in PBS. Samples were rinsed twice with PBS, stained with 1/15,000 dilution of DAPI (Sigma-Aldrich), and mounted in Dako fluorescence media.

Macrophages LysoTracker red staining
Macrophages were allowed to adhere on slides for 45 min and then incubated with 1 μM LysoTracker® Red DND-99 (Invitrogen™, L7528) or LysoTracker® Green DND-26 (Invitrogen™, L7526) in PBS for 1 min at RT. The samples were washed twice in PBS and mounted for immediate observations under fluorescence or confocal microscope.

Scanning electron microscopy
Samples for SEM were prepared as follows. Six wandering third-instar larvae were bled into 50 μl of Schneider’s insect medium (Sigma-Aldrich) containing 1 μM phenylthiourea (PTU; Sigma-Aldrich). The collected hemolymph was incubated on a glass coverslip for 30 min with apoptotic cells, before being fixed for 1 h with 1.25% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Samples were then washed in cacodylate buffer (0.1 M, pH 7.4), fixed again in 0.2% osmium tetroxide in the same washing buffer, and then dehydrated in graded alcohol series. Samples underwent critical point drying and Au/Pd coating (4 nm). Scanning electron micrographs were taken with a field emission scanning electron microscope Merlin, (Zeiss, Oerzen, Embsen, Germany).

Transmission electron microscopy
Third-instar wandering larvae were bled in 50 μl of Schneider’s insect medium (Sigma-Aldrich) containing 1 μM phenylthiourea (PTU; Sigma-Aldrich). The collected hemolymph was incubated on a glass coverslip for 1 h before being fixed for 2 h with 2% paraformaldehyde + 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Samples were then washed in cacodylate buffer (0.1 M, pH 7.4), fixed again in 1% osmium tetroxide and potassium ferrocyanide 1.5% in cacodylate buffer. After several washes in distilled water, samples were stained in 1% uranyl acetate in water, washed again, and then dehydrated in graded alcohol series (50, 70, 90, 95, 100%). Embedding was performed first in 1: 1 Hard EPON
samples were incubated, respectively, with 2 low binding tubes (LoBind, Eppendorf, Hamburg, Germany). The macrophage suspension was then transferred to 1.5 ml (Sigma-Aldrich) containing 1 μl of M phenylthiourea (PTU; Sigma-Aldrich). The macrophage suspension was then transferred to 1.5 ml low binding tubes (LoBind, Eppendorf, Hamburg, Germany). The samples were incubated, respectively, with 2 × 10² Bioparticles™-Texas Red™ Conjugate from S. aureus Wood (Invitrogen), 1 × 10⁶ Red-labeled apoptotic cells or 10⁵ pHrodo™ Red S. aureus Bioparticles™ for 30, 60, or 120 min to enable phagocytosis, and then placed on ice in order to stop the reaction. Phagocytosis was quantified using a flow cytometer (BD Accuri C6 flow cytometer, Becton Dickinson biosciences, Franklin Lakes, NJ, USA) in order to measure the fraction of cells phagocytosing, and their fluorescent intensity. 75 μl volume was read in ultra-low attachment 96-well flat-bottom plates (Costar no. 3474, Corning, Midland, NY, USA) at medium speed (35 μl/min). In a first step, macrophages were identified using the HmlΔgal4, UAS-GFP live staining. The fluorescence intensity of single macrophages was measured in the green channel with 488nm laser and 530/30 standard filter. The Red signal of apoptotic cells, Alexa Fluor™ 488 S. aureus Bioparticles™ (Invitrogen), or pHrodo™ Red S. aureus Bioparticles™, indicative of macrophages with effective phagocytosis, was monitored with 488 nm laser and 585/40 standard filter. At least 2,000 cells per genotype and per assay were analyzed. Results are an average of three independent experiments.

The phagocytic index was calculated as follows:

\[
\begin{align*}
\text{Fraction of hemocytes phagocytosing (f)} = & \frac{\text{number of hemocytes in fluorescence positive gate}}{\text{total number of hemocytes}} \\
\text{Phagocytic index (PI)} = & \frac{\text{Mean fluorescence intensity of hemocytes in fluorescence positive gate}}{f} \times f.
\end{align*}
\]

**Image analysis and quantification**

All images used for quantification were captured with a Zeiss LSM780 microscope, and all analyses were performed using ImageJ. For quantification of the fluorescence signal intensity, the fluorescence images were first converted to 8-bit images, and the total intensity value with an identical threshold was captured and measured with ImageJ. The freehand selection tool in ImageJ was used to capture and measure the area of the macrophages. Colocalization analysis was done with the ImageJ plugin “Just another Colocalization Plugin” after channel splitting and background subtraction. Rr (Pearson’s correlation coefficient), Ch1:Ch2 ratios, M1 and M2 (Manders’ colocalization coefficient for channel 1 and 2) were tabulated for each image.

**Crawling assay**

For the crawling assay, wandering third-instar larvae were used. Each larva was taken out of the wall of the vial using a paint brush and placed onto a 10 cm Petri dish plate containing 1.5% agar. A transparent, 1.5-cm-wide plastic ring was placed on the outer rim of the agar to prevent the animal from crawling to the edge of the plate. The animal was then left on the plate for at least 1 min to acclimate to the media. Total larval movement was followed for 1 min at 25°C.

**Lifespan and behavioral assays**

Lifespan experiments were repeated independently at least three times using two cohorts of 20 male flies per genotype/treatment each time. Freshly emerged flies were allowed to mate for 2 days at room temperature and sorted according to sex and genotype. Experiments were performed at 25°C, and flies were flipped to fresh vials every other day using standard medium. For climbing assays, flies were gently tapped to the bottom of a tube and filmed with a digital camera. The percentage of flies climbing above 7 cm within 10 s was calculated.

**Statistical tests**

Each experiment was repeated independently a minimum of three times (unless otherwise indicated), error bars represent the standard error of the mean of replicate experiments. Data were analyzed using appropriate statistical tests as indicated in figure legends using the GraphPad Prism software. Significance tests were performed using the Mann–Whitney test. For experiments with more than two conditions, significance was tested using ANOVA test followed by post hoc Dunnett’s multiple comparison tests. P values of < 0.05 = *, < 0.01 = **, and < 0.001 = ***.

**Data availability**

No data were deposited in public databases.

**Expanded View** for this article is available online.

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Author contributions
BP, SR, and BL conceived and designed the experiments and wrote the paper. BP, SR, KH, FM, ER, RH, MP, SK performed the experiments. BP, SR, BL, EK analyzed the data.

Conflict of interest
The authors declare that they have no conflict of interest.

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