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ARTICLE

Yeast glucan particles enable intracellular protein delivery in *Drosophila* without compromising the immune system

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Glucan particles derived from yeast have been recently proposed as potential drug delivery carriers. Here, we demonstrate the potential of glucan particles for protein delivery *in vivo*, using the insect *Drosophila melanogaster* as a model organism. By employing genetic tools, we demonstrate the capacity of yeast glucan particles to spread efficiently through the *Drosophila* body, to enter macrophages and to deliver an active transcription factor protein successfully. Moreover, the glucan particles were nontoxic and induced only minimal immune response. The injection of glucan particles did not impair the ability of *Drosophila* to fight and survive infection by pathogenic bacteria. From this study, *Drosophila* emerges as an excellent model to test and develop drug delivery systems based on glucan particles, specifically aimed to regulate macrophages.

1 Introduction

The design and optimisation of drug delivery systems is an exceptionally parametrically rich problem that requires *in vivo* testing as an integral part of the design loop, since high fidelity prediction of *in vivo* behavior from *in vitro* data is not yet generally possible[1, 2, 3]. The testing of all particular modifications of a drug delivery system in laboratory animals such as mice is not feasible not only for ethical reasons but also due to factors such as poor reproducibility of preclinical studies, time- and financial constraints[4, 5, 6, 7]. While the creation of organ-on-chip models for pre-clinical testing became highly investigated by the scientific community and these tools can prove the pharmacological effect of new drug candidates and confirm the absence of toxicity without harming or sacrificing animals, the cellular response in organ-on-chip models is still far from that in a real living organism[8, 9, 10, 11, 12].

On the other hand, human disease models investigating innate immunity, cancer, cardiovascular diseases, diabetes, infectious diseases, neurodegeneration, Alzheimer's disease, Parkinson's disease, sleep, seizure disorders and cognitive disorders are well known in the fly *Drosophila melanogaster* because many basic biological, physiological, and neurological properties are conserved between mammals and the fruit fly[13, 14, 15, 16, 17, 18, 19, 20]. Nearly 75 % of human disease-causing genes are believed to have a functional homolog in *Drosophila*[21] which makes it suitable for identifying new drug targets and drug candidates[22]. Therefore, we aim to demonstrate that *Drosophila* can serve as a model for the testing of drug delivery systems based on glucan particles (GPs) as a versatile carrier able to deliver bioactive substances *in vivo* and specifically to macrophages.

Macrophages as an essential part of the innate immune response are in the spotlight of the immunology research for their versatile role within living systems[23, 24]. Current research shows that macrophages are not only involved in phagocytosis of bacteria and apoptotic cells but also play essential functions in the formation of developing tissues or in the maintenance of metabolic homeostasis[25, 26]. With an increasing amount of knowledge, it is becoming clear that these immune cells react very plastically to signals from the extracellular space and represent a very heterogeneous population with different organ-specific roles[27]. Under healthy conditions, macrophages can fluently modulate their polarisation program and actively react to changing situation, while chronic dysregulation of their activity frequently causes severe pathologies, such as immune deficiencies, chronic inflammatory diseases, neurodegeneration or metabolic syndrome[28]. The mechanisms behind these diseases are very complex and require the ability to study molecular pathways at the level of cell-specific signaling, as well as of the systemic response of an organism. Owing to their unique ability to engulf foreign objects, macrophages can be exploited for targeted drug delivery more

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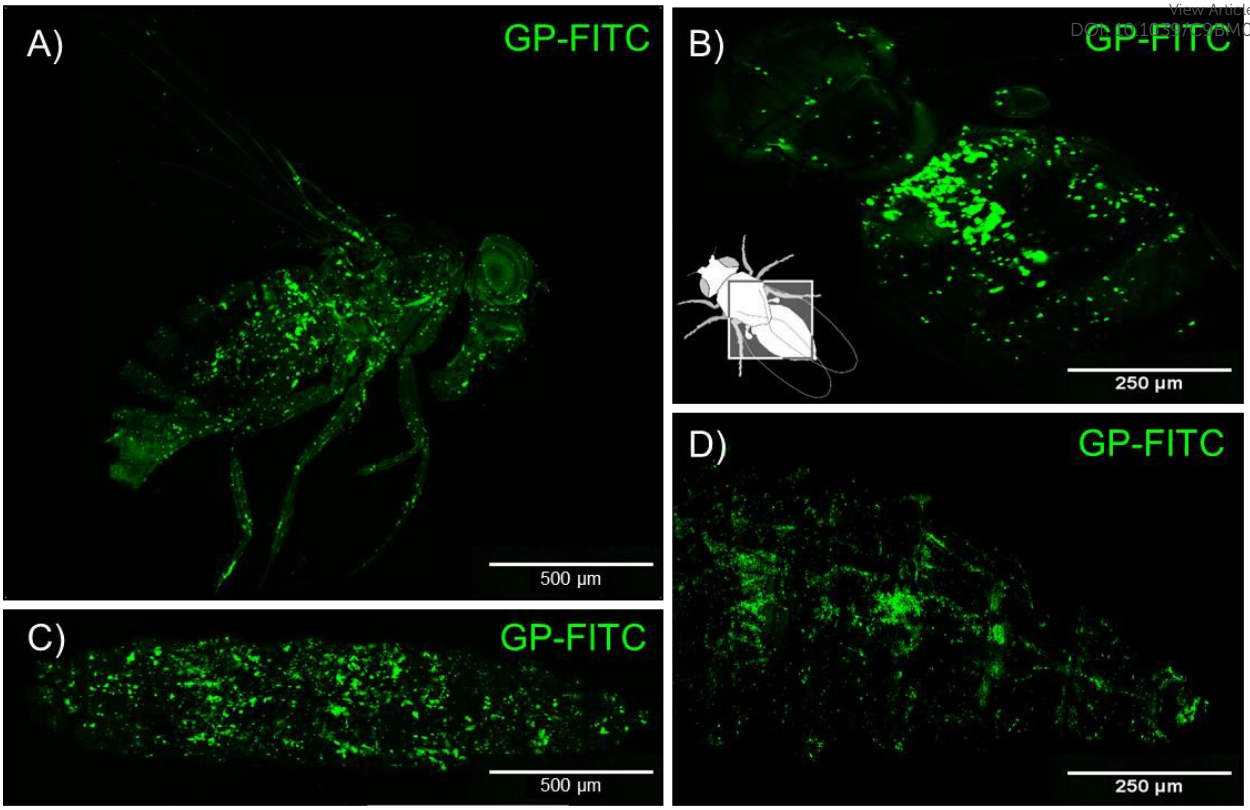

Fig. 1. GP-FITC can spread through the body of both adult flies and larvae after injection, reaching even distal parts of the body. (A) Confocal image of the whole adult fly – lateral view. Green spots represent sites of GP-FITC accumulation 30 minutes after injection. (B) Dorsal view of the adult fly abdomen – GP-FITC particles accumulate in the sites of dorsal hubs – sites around the aorta characteristic by a high occurrence of macrophages. (C) Confocal image of the whole 3rd instar larva – lateral view with head on the right-hand side of the image. (D) Dorsal view of the larval body in detail, GP-FITC particles accumulate in sites of expected sub-epithelial hemocyte patches.

easily than other cell types[29]. This feature, together with their central role in the regulation of many biological processes, makes macrophages a promising target in the development of intelligent drug delivery strategies[30, 31]. Given their motility, macrophages can also serve as Trojan horses for further transport of drug carriers[32, 33, 34].

Key factors that influence the phagocytosis of drug delivery systems by macrophages include particle size, shape, elasticity, and surface chemistry[35]. Macrophage toll-like receptors and other pattern recognition receptors recognize pathogen-associated molecular patterns common to most microbes. Among the molecular motifs recognized by macrophages are beta-glucans, carbohydrate polymers found in the cell walls of fungi, yeast, plants, and bacteria. Several *in vitro* studies have demonstrated the intrinsic adjuvant capacity of *β*-1,3-glucans from yeast and fungi in enhancing the functional activity of macrophages, neutrophils, dendritic cells, and epithelial cells. Particulate β-glucans may serve as a suitable vaccine delivery platform[35] because they are generally recognized as safe since 2007 and established in large animals and humans[36]. Encapsulation of antigens into GPs purified from *Saccharomyces cerevisiae* can serve in the vaccination against diseases such as tularemia and cryptococcosis caused by intracellular pathogens infecting macrophages in the host organism but also in other oral vaccine formulations[37, 38]. A down-regulation of gene expression in macrophages by using the delivery of siRNA in glucan particles was recently tested on mammals[39, 40]. This approach represents a stepping stone for GP delivery systems carrying various compounds as a cargo. Here, we propose *Drosophila* as a model organism combining the features of a simple and inexpensive, yet relevant and well-defined system for the preclinical studies of macrophage-specific drug delivery systems. Specifically, we show that GPs spread through the whole *Drosophila* organism quickly and accumulate in sites of macrophage occurrence. We provide evidence that GPs colocalise with macrophages and are efficiently phagocytosed with only a negligible effect on immune response activation. Crucially, using the Gal4/UAS system, we demonstrate that GPs can deliver a functional molecular cargo into the macrophages *in vivo* to activate gene expression.

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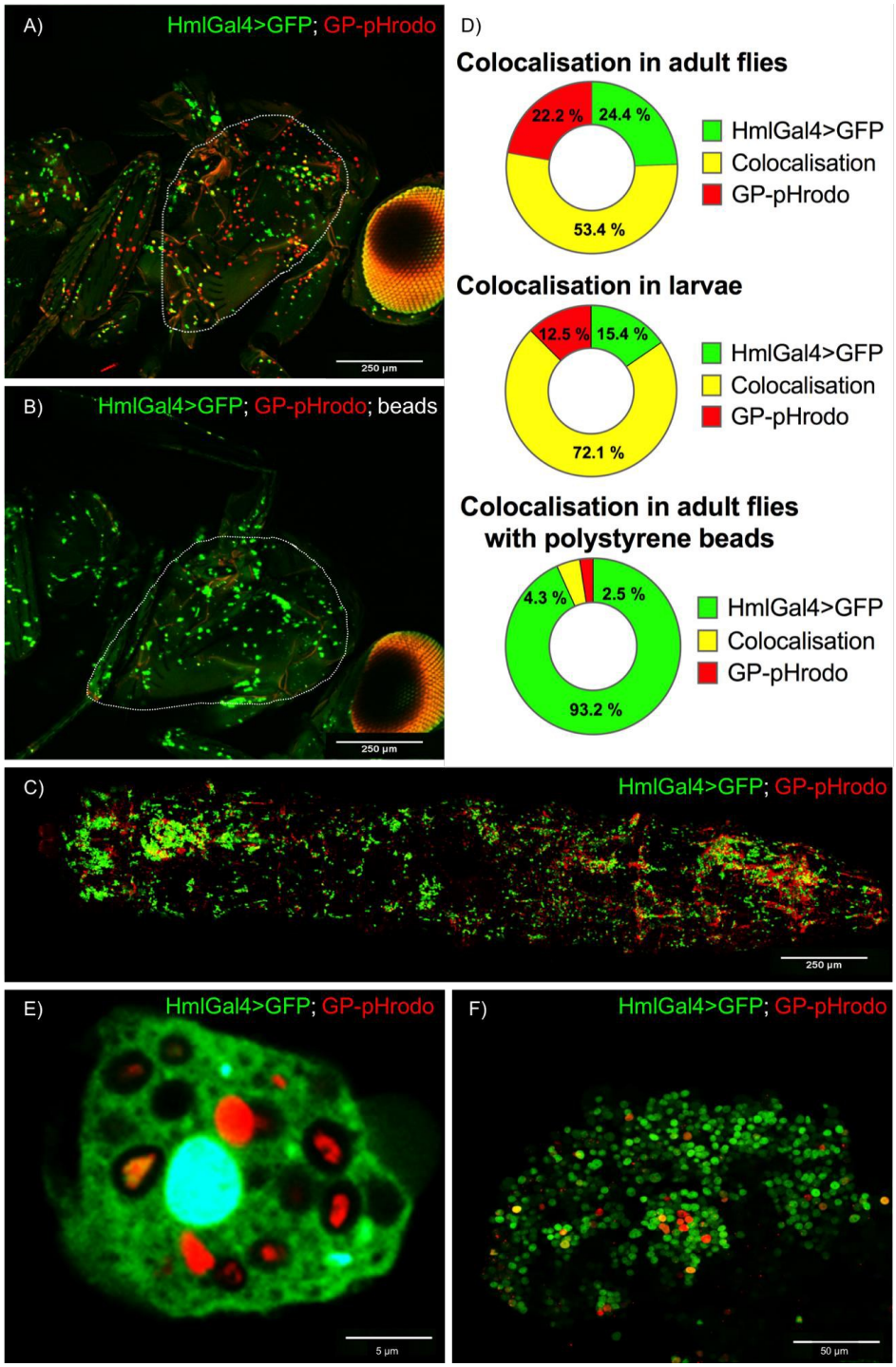

Fig. 2. GP-pHrodo particles (red fluorescent signal) colocalization with Hml al4>GFP macrophages (green fluorescent signal) in adult flies and in 3rd instar larvae. (**A**) Confocal image of an adult fly, **(B)** adult fly pre-treated by the injection of polystyrene beads to saturate the macrophages, **(C)** 3rd instar larva. (**D**) Quantitative analysis of colocalisation, represented by yellow fluorescence (GP internalized by macrophages), compared to regions showing only green fluorescence (empty macrophages) or red fluorescence (GP phagocytosed by other phagocytic cells), evaluated for adult flies, adult flies with abrogated phagocytosis and for larvae. The numbers represent data from 15 counted individuals, counts were made on the standardized confocal images in the defined morphological region of the individuals. (**E**) Detailed confocal image of a single macrophage internalizing GP-pHrodo (blue color for DAPI). (**F**) Dissected lymph gland (hematopoietic tissue) of a larva containing several phagocyting cells.

2 Results

2.1 Glucan particles spread through the whole organism quickly and accumulate in sites of macrophage occurrence

A suspension of GPs modified with fluorescein isothiocyanate (GP-FITC) was introduced into the circulation of living fruit flies and larvae by injection. Feeding, as an alternative delivery method, proved not to be effective (Fig. S1). The amount of GPs injected into the fly can be scaled by dilutions, which helps to define a suitable dose. An appropriate volume injected into the abdomen of an adult fly without damaging its functions and not exceeding the physical limits of the body was found to be 50 nL of a 0.1% (w/w) suspension of GP-FITC particles, which corresponds to approximately 7500 particles. The bright green signal of individual particles observed with confocal fluorescence microscopy gave us information about the GP distribution within the *Drosophila* body at the adult and larval stages (Fig. 1). Immediately after injection, we have observed accumulation of GPs at the site of puncture, but the glucan particles retained their colloidal stability and were able to spread through the hemolymph within 15-20 min after injection spontaneously (Fig. S2). The particles accumulated mostly at the sites densely populated by macrophages. In adult flies, these regions are located in dorsal hubs and along the aorta, but the GPs were able to reach even distal parts of the fly's body such as legs and antennae (Fig. 1A, B). In the larval stadium, GPs accumulates at the sites of high macrophage occurrence – under the cuticle and in locations of hematopoietic pockets and lymph gland (Fig. 1C, D). The fact that GPs are responsible for observed dotted pattern typical for distribution of immune cells was further proven by injection of FITC alone while this treatment resulted in disperse fluorescence in the whole fly body without occurrence of obvious localisation into the particular structures (Fig. S3).

2.2 Glucan particles colocalise with macrophages and are efficiently phagocytosed

To bring evidence that the macrophages truly internalise GPs, we modified GPs with a pH-sensitive fluorescent dye pHrodo (GP-pHrodo). The molecule shows bright red fluorescence in an acidic environment, which occurs explicitly in the phagolysosomes. Thus, the molecule can be used for the detection of phagocytic cells. To show the localisation of phagocytosed GP-pHrodo in macrophages, we have combined GP-pHrodo injection with genetically driven production of green fluorescent protein (GFP) under a hemocyte-specific driver hemolectin (Hml-Gal4>GFP). This combination was used to assess the efficiency of GP internalisation in both 3rd instar larvae and adult flies (Fig. 2A, C). Empty macrophages are visualised in bright green while macrophages containing GPs are yellow. The colocalisation of GPs and macrophages was quantified within defined anatomical regions, the thorax in adult flies and the dorsal side of the A5-abdominal segment in larvae. The high incidence of colocalisation is documented by the fact that 53.4 % of Hml>GFP positive macrophages in adult flies showed the yellow signal of internalised GPs (Fig. 2D). The rate of colocalisation in larvae was even higher (72.1 %, Fig. 2D). Some of the cells only contained the red fluorescent signal, suggesting that cells other than Hml>GFP positive macrophages can also scavenge the GPs. As the HmlGal4 driver is known to mark only approximately 85% of the macrophage population[41], the red signal most likely represents GP phagocytosis by the remaining Hml>GFP negative macrophages. The observation of a single macrophage gave us information about its ability to uptake a high number of GPs in multiple phagolysosomes (Fig. 2E). The dissection of immune tissue in the larval stage of *Drosophila* revealed a somewhat surprising presence of several positive cells also in the lymph gland (Fig. 2F). This observation suggests that some of the lymph gland cells are capable of active phagocytosis.

To further show that the GP-pHrodo positive signal is produced explicitly by phagocytosing cells we carried out a control experiment in which we abrogated the phagocytic activity of macrophages in advance by the injection of polystyrene particles. This pre-treatment resulted in an almost complete dampening of the previously observed signal in adult flies (Fig. 2A, B). To exclude the possibility that the pHrodo molecule plays a role in the phagocytic activation and that a high amount of particles stay in the extracellular space, we confirmed our observations in two additional control experiments. First, we co-injected GP-FITC together with *S. aureus*-pHrodo cell wall fragments commonly used in *Drosophila* to study phagocytosis[42]. Second, we injected GP-FITC into flies carrying a nuclearly localised hemocyte-specific red fluorescent signal (Hml-dsRed). Both of these controls yielded results consistent with the original experiment using GP-pHrodo particles (Fig. S4), *i.e.*, a high degree of colocalisation. Based on these results it can be claimed that macrophages actively internalise GPs and that a pH-sensitive fluorophore modification of the GP delivery system in combination with genetically encoded tools for marking the target tissues can visualise the uptake and localisation of GPs in *Drosophila* macrophages.

2.3 Glucan particles can deliver cargo into the macrophages *in vivo*

We next tested whether GPs can be used for the delivery of bioactive molecules into the macrophages. To exploit the ability of GPs to deliver cargo, we loaded the GPs with a 32-kDa Gal4 protein (GP-Gal4). Gal4 is a transcriptional activator of the upstream activating sequence (UAS), which is frequently used in Drosophila[43] to study targeted gene expression. We injected the GP-Gal4 particles into adult flies carrying a UASmCherry transgenic reporter wherein expression of the red fluorescent protein mCherry is controlled by the UAS. The UAS-dependent production of mCherry can only be triggered by the exogenous Gal4 protein which, moreover, cannot cross cell boundaries and spread into surrounding tissues. Thus, the flies show no

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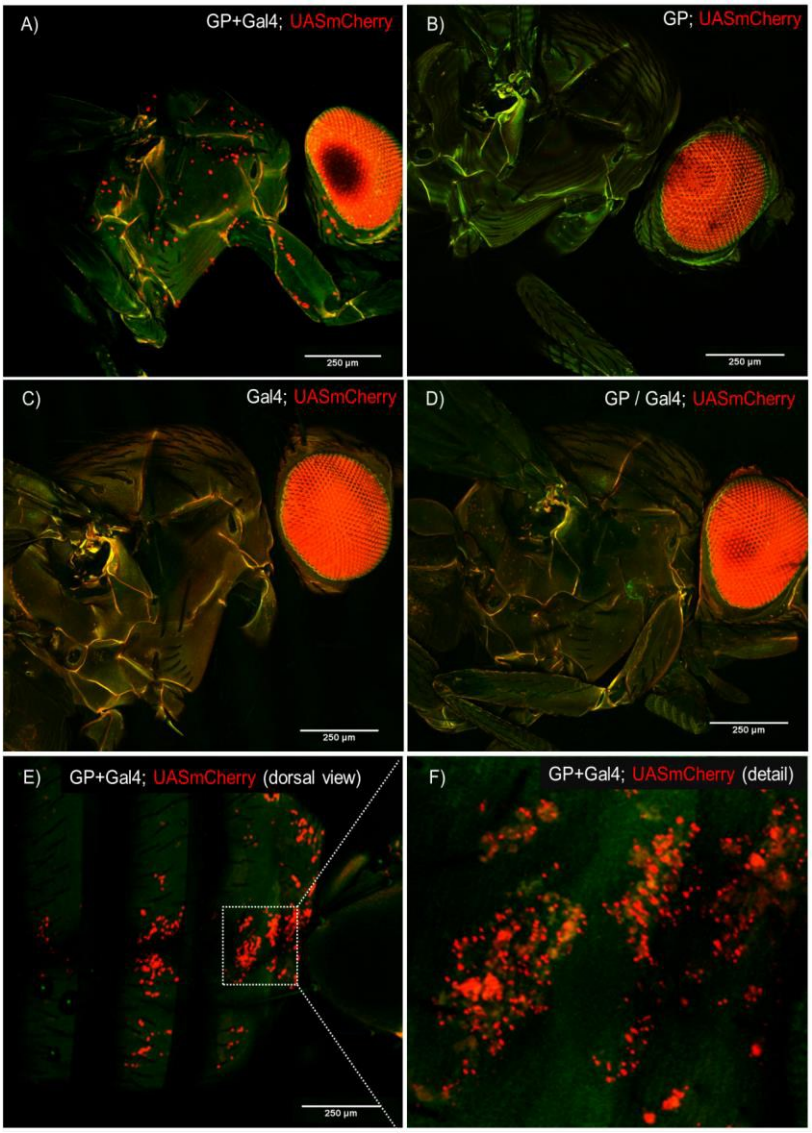

Fig. 3. GP can be used for the delivery of Gal4 protein to trigger the expression of a reporter gene. **(A)** Flies treated by the injection of GP loaded with Gla4 resulted in the activation of UAS mCHerry reporter construct. Coinjection of empty GP together with Gal4 **(B)** as well as the injection of empty GP **(C)** or Gal4 **(D)** individually gave no positive signal. **(E)** Dorsal view of flies injected with Gal4 loaded GP shows a strong fluorescent signal in a pattern typical for macrophages surrounding the aorta **(F)**.

fluorescent signal under normal conditions; however, they produce red fluorescence in the presence of Gal4 in the macrophages. Approximately 60 min following injection of the GP-Gal4 particles into adult flies, the mCherry signal could be detected in the macrophages (Fig. 3A) and also proved by pull down assay (Fig. S5). As expected, no such signal appeared when UASmCherry flies were injected with either empty GPs or the Gal4 protein alone (Fig. 3B, C). Importantly, the coinjection of a mixture of empty GPs with free Gal4 also could not activate mCherry expression (Fig. 3D), demonstrating that the Gal4 protein could only be delivered to the macrophages via the GPs. Further evidence of the unique role of GPs in the delivery of Gal4 to macrophages was provided by employing GP-FITC as the Gal4 carrying particles, and observing their colocalisation with the mCherry signal. The red fluorescence of the mCherry reporter was found only in those macrophages that also contained phagocytosed GP-FITC (Fig. S6). From the drug delivery point of view, this is a very significant result as it offers a new route by which protein-based therapeutic substances can be efficiently delivered to cells. Even though the result of this experiment is clear, it appears that the number of mCherry positive cells in Fig. 3A is lower relative to the number of macrophages targeted with GP-pHrodo particles (Fig. 2A). We hypothesise that in the case of GP-Gal4, the cells have to receive an amount of the Gal4 protein sufficient to trigger a robust detectable expression. In addition to the thorax, we observed many mCherry expressing cells on the dorsal side of the adult fly abdomen (Fig. 3E, F). Consistent with the normal macrophage localisation and function in scavenging foreign objects from the circulation, these cells surrounded the aorta and displayed a robust mCherry signal, perhaps caused by an increased influx of the blood-borne GP-Gal4 complex.

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2.4 Empty glucan particles have a negligible effect on immune response activation

To identify the limits of GPs use in drug delivery and immunology research, we examined the potential impacts of GPs on the activation of the immune response, gene expression, longevity, and resistance to bacterial infection. We first assessed the expression of antimicrobial peptides and the Toll reporter gene Relish in response to the injection of GPs and their modifications. For comparison, we triggered the immune response by injecting 20 000 units of *Streptococcus pneumoniae* (*Sp*), a treatment commonly used to induce sepsis[18]. The data show that contrary to *Sp* injection, the treatment with GPs had only a mild or no effect on the expression of the immune response pathway genes (Fig. 4A). Next, we analysed the survival of individual flies for 30 days following the injections (Fig. 4B). Interestingly, there was a slight but statistically significant decrease in the survival rate of flies injected with empty GPs (Fig. 4B), which correlated with the increased expression of Defensin and Relish mRNAs (Fig. 4A). The effect of empty GPs on the activation of the immune response in comparison with other modified GPs might be explained by the covalently bound fluorescent dyes masking some surface bioactive glucan moieties.

To test whether GPs might interfere with a standard immune response to a pathogen, we deployed GPs 24 h before the injection with *Sp* and monitored individual survival after that. Resistance to *Sp* infection is known to mainly rely on phagocytic activity[44]. Therefore, we compared the survival rate and bacterial growth following GP injection with a situation where phagocytosis was compromised by the injection of polystyrene beads. The results show that relative to the polystyrene beads treatment, the effect of GPs on the survival of *Sp* infected flies was minor, comparable to injection with buffer only (Fig. 4C). Consistently, blocking phagocytosis by polystyrene beads reduced the ability of the flies to suppress bacterial growth, whereas GP injection did not (Fig. 4D). The low immunogenicity and toxicity can be attributed to the complete removal of cellular components from the GPs during their preparation from yeast[45]. Still, the epitopes on the GP surface, particularly *β*-1,3-

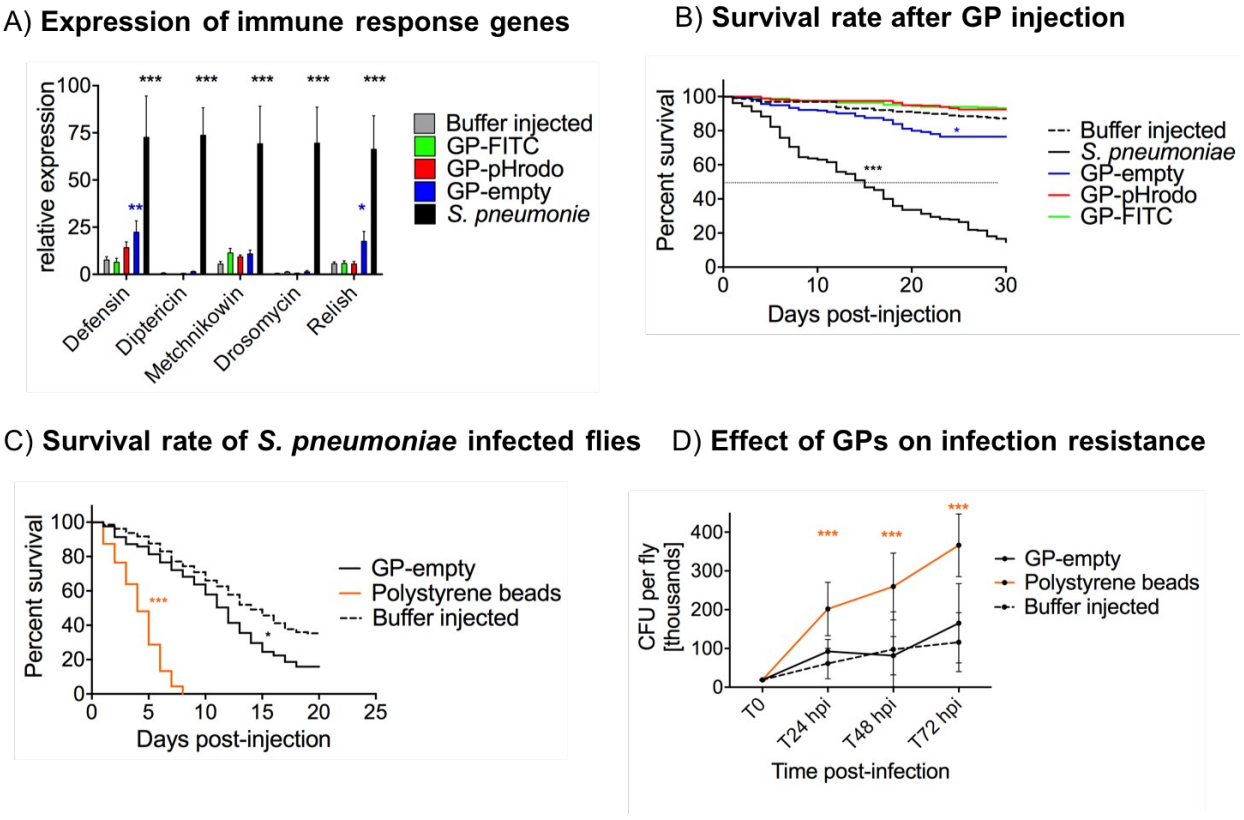

Fig. 4. Injection of GP has a negligible effect on the activation of the immune response, the vitality of individuals and their ability to fight and resist bacterial infection. **(A)** The expression levels of particular immune response readouts (*Defensine*, *Diptericine*, *Metchnikowin*, *Drosomycin,* and *Relish*) were measured by qPCR after the injection of plain glucan particles (blue), pHrodo labeled glucan particles (red) and FITC labeled glucan particles (green) in comparison to the injection of *Streptococcus pneumoniae* (black) as a positive control and buffer (grey) as a negative control. (**B**) The flies that underwent the same treatment were observed for the survival rate for 30 days (each treatment was carried out on more than 300 individuals). (**C**) The survival of bacterial infection was observed after the injection of 20 000 *Sp* units per fly. The flied were pre-treated 24 hours before the infection either by glucan particles, buffer as a negative control, and polystyrene beads abrogating phagocytosis as a positive control. (**D**) In parallel, the number of colony forming units (bacteria) per fly was assayed in this treatment.

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and *β*-1,6-glucans, can be recognised by macrophages and phagocytosed[46]. Similarly, low immunogenicity was also reported after the injection of heat-killed bacterial culture, which does not represent a real danger for the host and is efficiently phagocytosed without massive immune response[47].

3 Discussion

Current progress in macrophage biology identifies macrophage subpopulations residing in different tissues as very heterogeneous but possessing organ-specific functions[48]. Therefore, macrophage-specific targeting can serve as a tool for research into many elusive diseases. Macrophage targeting is based on phagocytosis, using specific particles as a "bait" loaded with an active compound to affect the biology of the cells[49]. Glucan polysaccharides were shown to be specifically recognised and to induce phagocytosis in both mammals and *Drosophila*[46, 50, 51, 52]. Glucan particles prepared from *Saccharomyces cerevisiae*[29] could thus serve as a potent delivery system for direct macrophage-specific targeting. These particles can be modified to influence the release of encapsulated cargo[53], which makes them appealing for use in pharmaceutical applications[29]. For example, glucan particles loaded with RNAi were efficiently applied for the regulation of viral infection in shrimps[54]. In mammalian model organisms, glucan particles were used for the direct targeting of liver macrophages to treat a metabolic syndrome[55].

Due to increasing discovery rate of new drug candidates, there is a rising need to replace mammals by more simple, cheaper and ethically acceptable model organisms for *in vivo* preclinical testing[56, 57]. Thanks to more than one-hundred years long history in experimental research and a high degree of similarity with mammals, *Drosophila* could emerge as such a model organism and enable the evaluation of basic features such as toxicity, immunogenicity, and immune recognition. However, the data considering the testing of drug delivery systems in *the Drosophila* model organism are scarce or completely missing[58, 59]. We demonstrate that glucan particles can be used as a system for targeted drug delivery to macrophages in *Drosophila,* with the potential not only in preclinical testing but also for use in insect immunological research. Thanks to the high versatility of genetic tools, the fruit fly is widely used as a model organism for dissecting molecular mechanisms behind macrophage behavior[60, 61]. The unique set of reporters together with the possibility of tissue-specific drivers provide an opportunity to decipher regulatory pathways in processes such as embryonal development, regulation of hematopoietic niche maintenance, activation of central immune pathways, immune response to cancer, and control of resistance and tolerance to infection[62, 63, 64, 65].

Glucan particles injected into the hemolymph were found to be very effectively distributed throughout the *Drosophila* body and phagocytosed with high coverage of all macrophages. The colocalisation of glucan particles was observed in all organs and tissues where the macrophages reside, but there was always a subpopulation of empty macrophages in the injected flies, suggesting some functional plurality in *Drosophila* immune cells. Further analysis of this phenomenon could form the basis of research into "stealth" particles that avoid detection by the immune system[66]. We observed an unusually high accumulation of glucan particles in the dorsal part of the abdomen. In this region there is a high accumulation of macrophages surrounding the aorta and a high flow of the hemolymph; this is also the proposed site of adult hematopoiesis[67], which makes it an attractive target for the *in vivo* testing of hematopoietic agents[68, 69]. The ability of glucan particles to deliver cargo to macrophages *in vivo* was demonstrated by the delivery of Gal4 protein into the target cells using flies encoding the genomic UASmCherry construct and observing the production of a fluorescent reporter. Only Gal4 delivered via glucan particles produced a positive signal; neither the injection of GPs or Gal4 alone nor the coinjection of Gal4 and GPs resulted in phenocopy production.

These results show that the *Drosophila* immune system can efficiently recognise and remove glucan particles from the circulation. Glucans have been documented to have a stimulatory effect on the immune system and trigger a specific anti-fungal immune response in mammals[70]. We, therefore, measured known markers of immune system activation and observed only a mild activation of antimicrobial peptides with almost no impact on the vitality of individuals. Additionally, the immune capacity was analysed by injecting GPs into the flies 24 hours before the reinjection of pathogenic bacteria. Data from this immunological assay clearly show that neither bacterial resistance nor bacterial killing is influenced by GP treatment, suggesting that the injection of GPs does not limit the ability of *Drosophila* to fight bacteria by phagocytosis. We, therefore, believe that the combination of glucan particles as a tool for drug delivery and *Drosophila* as a model organism makes a unique opportunity for *in vivo* testing in the context of cancer, immunity, and metabolism research.

4 Conclusions

We have demonstrated that *Drosophila* is a suitable model organism for testing glucan particles as a macrophage-specific drug delivery system *in vivo*. We proved that GPs can be delivered by injection directly into the circulatory system and that this treatment results in a fast distribution of GPs into the macrophages over the whole body of both adult flies and larvae. We documented that GPs can be used *in vivo* for the delivery of a transcription protein to macrophages. We analysed the impact of GP treatment on the vitality of individuals and elicitation of their immune response in the normal state and response to bacterial infection. All these experiments represent important steps in establishing the use of GPs as a tool in the field of insect immunology research together with the establishment of *Drosophila* as a suitable model organism for *in vivo* testing of drug delivery systems and their modifications.

5 Experimental section

5.1 Glucan particles preparation and modifications

Glucan particles were prepared from baker's yeast[45]. Wet yeast was added into 1M NaOH, the material was heated for 1 hour at 90 °C and then centrifuged at 14,500 G for 5 minutes. The supernatant was discarded, and this step was repeated three times. The alkali-insoluble solids were then mixed with HCl solution added to the dispersion with the resulting pH = 4.5, heated to 75 °C for 2 hours and then centrifuged at 14,500 G for 5 minutes. Insoluble solids were washed 3 times in deionized water, 4 times in isopropanol and finally 2 times in acetone. Every step was followed by centrifugation at 14,500 G for 10 minutes. The final product was lyophilized to form a dry powder. The lyophilised glucan particles were stored in a refrigerator for further use. The size distribution and morphology of the GPs were characterised by static light scattering and scanning electron microscopy, respectively (Fig. S7).

5.2 Synthesis of GP-FITC

To prepare FITC labeled glucan particles, 100 mg GPs, 5 mL of DMSO (PENTA) and 2 mg of FITC (Sigma-Aldrich) were mixed in a round-bottom flask. Then 1 mg of tin-ethyl hexanoate (Sigma-Aldrich) and subsequently 2 drops of pyridine (Lach-Ner) were added into the suspension containing all reactants. The synthesis was carried out for 1 h at 95 °C and mixed by a magnetic stirrer at 500 RPM. After cooling down, the content of the reaction mixture was centrifuged for 3 min at 12,000 G. The supernatant containing unreacted material was discarded. The pellet was washed by water three times, and then by 99.9 % ethanol (PENTA) three times. Finally, the pellet was redispersed in approx. 50 mL of deionised water and dialysed against deionised water for 5 days under dark with an exchange of water every day. After this step, the suspension was lyophilised to obtain a dry powder and stored in a refrigerator for further use. The successful attachment of FITC was verified by fluorescence spectroscopy and laser scanning confocal microscopy (Fig. S7). It has also been verified that the modification of GPs was not detrimental to their colloidal stability (Fig. S7).

5.3 Synthesis of GP-pHrodo

Glucan particles labeled with pHrodo™ we prepared by first dispersing 10 mg of GPs in 100 µL of sodium bicarbonate buffer (pH=9.3, 0.1 M) in an Eppendorf tube, diluted with 900 µL of deionized water and sonicated for 5 min on the sonication bath. This step was followed by the addition of 2 µL of pHrodo™ succinimidyl ester (M_W ~650 g/mol) 10 mM of dimethylsulfoxide (DMSO) solution (pHrodo® Red Microscale Labeling Kit, Thermo Fisher Scientific) to the suspension and left to react for 15 min at room temperature protected from light. The resulting reaction mixture was centrifuged for 3 min at 12,000 G, and the supernatant was discarded. The pellet was then washed once with deionised water, once with 99.9% ethanol (PENTA), three times with acetone and finally dried on the air in the dark and stored in a freezer. The successful attachment of pHrodo was verified by fluorescence spectroscopy at neutral and acidic pH and laser scanning confocal microscopy (Fig. S7). It has also been verified that the modification of GPs was not detrimental to their colloidal stability (Fig. S7).

5.4 Gal4 protein loading

To prepare glucan particles loaded with Gal4 protein, 100 µL of 49.5 µg/mL aqueous solution of the Gal4 protein (Sigma-Aldrich) was pipetted to 10 mg of dry GP or GP-FITC powder placed in an Eppendorf tube. The protein solution was absorbed by the volume of GPs by capillary forces. Then the whole content of the Eppendorf tube was then lyophilised and stored in a freezer. This loading method has been previously proved to be efficient for the encapsulation of substances including vitamin B12, caffeine and bovine serum albumin (BSA)[45]. The release kinetics of BSA from GPs was shown to occur over a typical time of 24 h at 20 °C, with only approx. 10 % released after 2 hours[45]. Therefore, it can be expected that the spontaneous leakage of Gal4 from the GPs over a time period of 30 min will be negligible and the majority of Gal4 will be injected into the fruit flies in the GP-encapsulated form.

5.5 Fly culture and manipulation

All fly strains used in this work were held in standard conditions of 25 °C, 60% humidity and food supply *ad libitum*. We used two different fly strains for the visualization of macrophages: I. combination of macrophage-specific driver Hml Gal4 and reporter gene (enhanced green fluorescent protein eGFP), under control of artificial UAS promoter (final genotype - Hml Gal4; UAS eGFP); II. Hml recombinant protein fused with a dsRed fluorescent label, containing nuclear localisation signal (final genotype Hml dsRed). Both these lines gave us a bright fluorescent signal enabling us to detect macrophages without the need for dissection, using an inverted fluorescent microscope (Olympus IX71) or a confocal microscope (Olympus FluoView 1000). As a control, we used a white fly strain based on the CantonS genotype, commonly used as a control in our laboratory and representing a genetic background for all the lines that were used. The flies were grown on corn-meal medium (8 % cornmeal, 5 % glucose, 4 % yeast, 1 % agar) at 25 °C. For dietary treatment individuals were held on a high-fat diet consisting of 1 % cornmeal, 5 % glucose, 2.5 % yeasts, 1 % agar, and 10 % lard.

5.6 Injections

In all experiments 0.1% (w/w) suspension of GPs or modified GPs in PBS (pH 7.4, 1X, tablets from Sigma-Aldrich) was used. The suspension was prepared by sonication on an ice bath for 5 min at 25 W, and vortexed just before injection to ensure well-dispersed particles. Flies were anesthetized with carbon dioxide. The Eppendorf Femtojet microinjector and a drawn glass needle were used to inject precisely 50 nL of GP suspension, bacteria or buffer into the fly at the cuticle on the ventrolateral side of the abdomen. For FITC injection experiment 50 nL of 6M FITC in PBS was injected into the flies carrying the genotype UAS mCherry. Flies were analyzed 45

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minutes after injection by confocal microscopy. For injections of larvae, the third instar larvae were collected, rinsed in PBS and mounted on a double side tape. The sharpened needle in combination with Femtojet microinjector was used for injections. Larvae showing high melanisation were excluded from further analyses.

5.7 Glucan particle observation and detection of colocalization with hemocytes

The flies were fixed by 4% paraformaldehyde in PBS and imaged using confocal microscopy with maximal projection from five different layers; the same settings of the Z-stack range and the same intensity of lasers were used for all animals. The cells were observed in whole flies to detect possible significant changes in distal parts. The exact number of macrophages was determined by counting Hml>GFP-positive cells within a selected thorax region as depicted in Fig. 2C, D using the Fiji software and compared by Student t-tests using the GraphPad Prism software. The number of GP phagocytosing cells was determined by the injection of GP-pHrodo 40 min prior to fixing flies. To control the spontaneous fluorescence of pHrodo labeled glucan particles, a few flies were fixed immediately after injection and showed no red fluorescent signal. Another control of phagocytosis specific pHrodo signal was made by the pretreatment of flies with the injection of 50 nL of polystyrene beads one hour before the GP injection. The injection of polystyrene beads (1 µm in diameter, Sigma L5530) is known to dampen phagocytosis and resulted in no red fluorescent signal. We used two other strategies to show phagocytosis of glucan particles by the immune cells: I. we coinjected FITC labeled glucan particles together with phagocytosis marker pHrodo Red *S. aureus* Bioparticles (ThermoFisher Scientific); II. we injected FITC labeled glucan particles into the flies carrying HMLdsRed genotype – in all the cases the flies were assayed as described above. For the detection of glucan particle phagocytosis in the larva, we bled the larvae and quantified the colocalisation on a hemocyte sample in a defined region of Bürker chamber.

5.8 Bacterial strains and culturing conditions

Streptococcus pneumoniae strain EJ1 (D39 streptomycin-resistant derivative) was stored at -80 °C in Tryptic Soy Broth (TSB) media containing 25% glycerol. For experiments, bacteria were streaked onto TSB agar plates containing 100 µg/mL streptomycin and incubated at 37 °C overnight; a fresh plate was prepared for each experiment. Single colonies were used to inoculate 3 mL of TSB with 100 000 units of catalase (Sigma C40) and incubated overnight at 37 °C + 5% CO_2 without shaking. The morning cultures were 2x diluted in TSB with a fresh catalase and grown for an additional 4 hours, reaching an approximate 0.4 OD_{600}. Final cultures were concentrated by centrifugation and resuspended in phosphate buffered saline (PBS) so that the concentration corresponded to OD_{600} 2.4 and stored on ice prior to injection.

5.9 Pathogen load measurement

Single flies were homogenised in PBS using a motorized plastic pestle in 1.5 ml tubes. The lysate containing *S. pneumoniae* was plated in spots onto TSB agar plates containing streptomycin in serial dilutions and incubated overnight at 37 °C before manual counting. The pathogen loads of 16 flies were determined for each treatment in each experiment, and at least three independent infection experiments were conducted. The results were combined into one graph (in all presented cases, individual experiments showed comparable results). Values were compared using unpaired t-tests corrected for multiple comparisons using the Holm-Sidak method in the GraphPad Prism software.

5.10 Survival analysis

Two hundred flies were injected for each treatment in one experiment; at least three independent infection experiments were repeated and combined into one survival curve (in all presented cases, individual experiments showed comparable results). Injected flies were placed into vials with 20 flies per vial, transferred to a fresh vial every other day and checked daily to determine mortality. Flies were kept at 29 °C. The survival curves were generated by GraphPad Prism software and analyzed by Log-rank and Gehan-Breslow-Wilcoxon (more weight to deaths at early time points) tests.

5.11 Gene Expression

Gene expression was analysed by quantitative real-time PCR. Whole flies were, and the total RNA was isolated by Trizol reagent (Ambion) according to the manufacturer's protocol. DNA contamination was removed by using Turbo DNAse free kit (Ambion) according to the protocol (37 °C 30 min) with subsequent inactivation of DNAse by DNAse inactivation reagent (5 min spin at 12,000 G at room temperature). Reverse transcription was done by the Superscript III reverse transcriptase (Invitrogen), and the amounts of mRNA of particular genes were quantified using the IQ Sybr Green Supermix Mastermix (BioRAd) on a CFX 1000 Touch Real-time cycler (BioRad). In all cases, the expression was normalized to the expression of Ribosomal protein 49 (Rp49) and values relative to Rp49 amounts were compared and shown in the graphs. The primer sequences can be found in Table 1. Samples were collected from three independent infection experiments with three technical replicates for each trial and compared by unpaired t-tests using the GraphPad Prism software.

5.12 mCHerry pull down assay

For this experiment we injected 50 nL of 0.25%, 1%, and 4% (w/w) solution of Gal4-loaded GP-FITC in PBS into the flies carrying the genotype UAS mCherry. Five hours after injection, 15 flies were collected and frozen at -80 °C. The flies were homogenised and proteins were extracted according to RFP-Trap protocol (Chromotec). Extracted mCherry proteins were analysed by SDS PAGE (10 %) and the size of gained protein was compared with a protein ladder (Precision Plus Protein™ Dual Color Standards, BioRad), as well as with positive control represented by flies carrying mCherry expression.

Conflicts of interest

There are no conflicts to declare.

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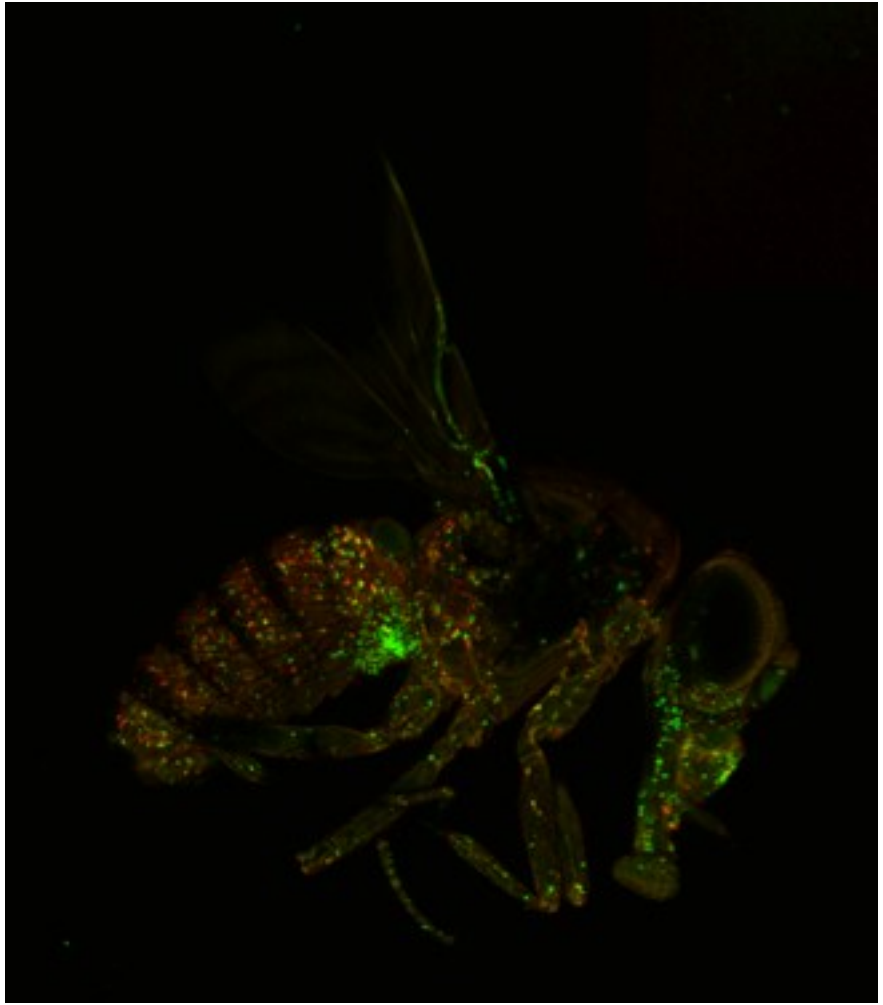
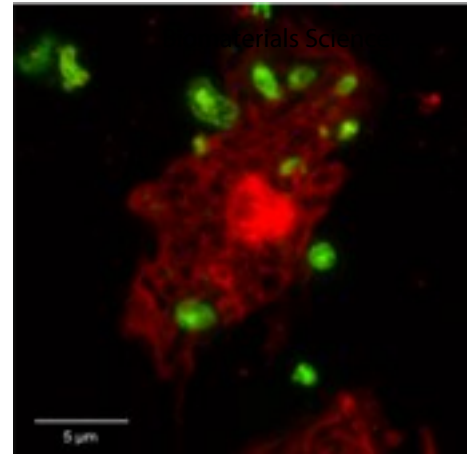
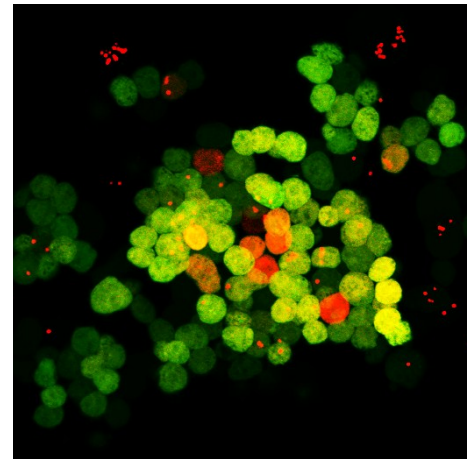


***Drosophila* with injected glucan particles**

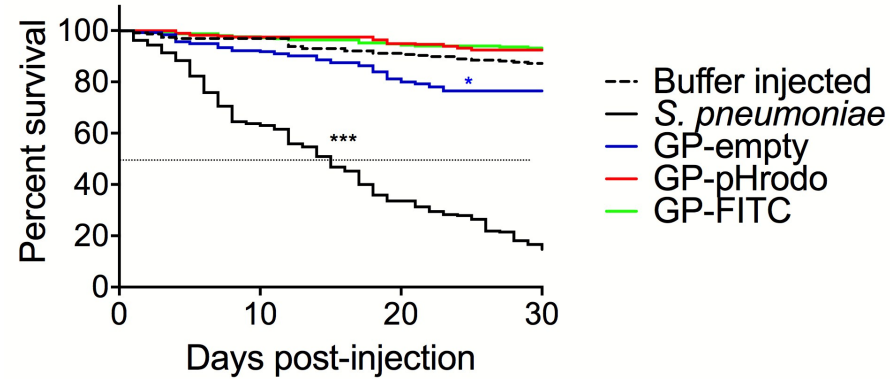


Gal4 macrophage delivery



Lymph glands targeting

Survival rate after injection of glucan particles



Survival rate of *S. pneumoniae* infected flies