Classification and characterization of immune haemocytes in the larvae of the Indian fritillary, *Papilio hyperbius* (Lepidopetra: Nymphalidae)

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Abstract. In this study, haemocytes present in *Papilio hyperbius* Linnaeus were identified and characterized. Six different types of haemocyte were recorded in the haemocoel of this species of insect: prohaemocytes, plasmatocytes, granulocytes, spherulocytes, adipohaemocytes and oenocytoids. Of these the granulocytes were found to be responsible for cell-mediated immune responses such as phagocytosis. Granulocytes that were exposed to immunity inducers (carboxylate-modified polystyrene latex beads [CLBs] and *Escherichia coli*) had fan-like or pod-like structures on their cell membranes. The lysosomes in granulocytes were activated 2 h after injection with *E. coli* and after 12 h, all granulocytes exhibited highly activated lysosomes. After 24 and 48 h, the lysosome activity in granulocytes decreased. Transmission electron microscopy revealed that phagocytosis, which was mediated by granulocytes in the early hours of the *E. coli* infection, led to the formation of one phagosome for one *E. coli* within the cytosol. Moreover, as time passed, endosomes or lysosomes of different size developed. Subsequently, the phagosomes and lysosomes fused and *E. coli* were eliminated. After this series of immune responses, the nuclei of the granulocytes were indistinct and their cellular activity decreased. Hence, as old immune cells were replaced by new ones, active and healthy immune haemocytes were presumed to be maintained in the hemocoel.

INTRODUCTION

The ability of biological organisms to exhibit potent immune response to foreign agents, bacteria, fungi, viruses and parasites is crucial for their survival (Li et al., 2020). Insects have a system of immune responses against foreign agents; their major responses are divided into humoral and cell-mediated immune responses (Carrau et al., 2021). These two distinct responses are initiated concomitantly and complement one another, rather than being independently mediated (without interacting). For example, immune haemocytes of insects perform various cell-mediated immune responses (such as phagocytosis) and may also simultaneously produce and discharge antimicrobial peptides (AMPs) as they react to and fight against pathogens (Ioannis et al., 2021). In other words, while the humoral immune response is mainly mediated by various substances that are produced by the fat body in insects, haemocytes

can also perform related activities. Hence, identifying and characterizing key haemocytes involved in immunity in insects is essential for understanding the overall immunity of a given target species (Cho & Cho, 2019).

The cell-mediated immune response of key haemocytes in insects functions mainly in three ways. Phagocytosis is the most common and easily observed phenomenon; the invading pathogen is rapidly recognized, following which changes occur in the cell membrane, including netting and encapsulation. This allows the cell to efficiently capture and remove the foreign pathogen by phagocytosis (Lee et al., 2016). Furthermore, encapsulation constitutes an immune response against the invasion of a large number of pathogens that are smaller in size than haemocytes (such as bacteria). As one-on-one phagocytosis cannot occur in this scenario, haemocytes secrete sticky substances such as immunolectins, actins and collagens to enclose the patho-

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gens in an equal quantity of haemocytes, thereby securing their removal (Roy et al., 2021). The final method concerns scenarios with foreign agents that are larger than immune cells, such as parasites. As with encapsulation, the haemocytes enclose the invading agent using sticky substances, which they secrete into the surrounding area to ensure the agent's removal (Cerenius & Söderhäll, 2021). As highlighted above, humoral and cell-mediated immune responses occur concomitantly and haemocytes involved in immunity simultaneously secrete large quantities of AMPs (Goodman et al., 2021; Black et al., 2022).

In insects, the cell-mediated immune response is mainly mediated by haemocytes of which there are seven types reported in insects: prohaemocytes, granulocytes, plasmatocytes, oenocytoids, adipohaemocytes, spherulocytes and coagulocytes (Kwon et al., 2014). For example, all seven types are reported for the orders Coleoptera, Blattodea and Lepidoptera, while four to five types are generally reported in the orders Orthoptera, Hemiptera, Trichoptera and Neuroptera (Gupta et al., 1985). However, even within the same order, the specific composition of haemocytes in each species varies (Gupta et al., 1985).

Prohaemocytes, plasmatocytes and granulocytes are found in most species of insects. These three types of haemocytes are thus presumed to have highly significant roles. For example, plasmatocytes and granulocytes, which are the main haemocytes involved in immunity in insects, perform phagocytosis in many insects and are also known to mediate encapsulation by interacting with other haemocytes (Cho & Cho, 2019; Eleftherianos et al., 2021). Thus, when studying cell-mediated immune response in insects, it is first necessary to accurately identify and characterize the target insect's haemocytes. There are many studies on insect haemocytes, but as yet there is no accurate classification of haemocytes (Ribeiro & Brehelin, 2016; Kwon et al., 2014). One such approach could entail the development of common, reliable molecular markers of each specific type of haemocyte for the classification of various insect haemocytes (Feng et al., 2022). In addition, although a variety of staining agents were used during the early era of research on insect haemocytes, they are unsuitable for the accurate analysis of haemocytes in various target insects and are now rarely used.

Therefore, haemocytes in different insects are identified using morphological features, which requires years of research experience. In this regard, we have longstanding experience in the classification of haemocytes in various insects (Kwon et al., 2014; Hwang et al., 2015; Lee et al., 2016; Cho & Cho, 2019). As such, we are able to classify the seven types of insect haemocytes in any target species with considerable accuracy. For example, each specific type of haemocyte involved in immunity in insects can be identified by injection of an immunity inducer and the subsequent real-time monitoring of changes in cellular morphology (Kwon et al., 2014). This approach is the most effective method for identifying specific types of haemocytes involved in immunity in many insects. The most distinct feature of these haemocytes is the ability of their cell

membranes to rapidly form a vast net (netting) and encapsulate pathogens (Cho & Cho, 2019). Such rapid changes in the morphology of the cell membrane are reported for haemocytes in many species (Giglio et al., 2008).

Artificially injecting a foreign substance into the body of an insect can activate haemocytes via three main signalling pathways (Toll, immune deficiency [IMD] and Janus Kinase [Jak]). Notably, contact between a foreign body and haemocytes involved in immunity instantly leads to its phagocytosis in their cytosol; this generates a phagosome (Petrignani et al., 2021; Yakovlev et al., 2022). This phagosome then fuses with lysosomes to initiate the process of removing the foreign body (Petrignani et al., 2021). The fusion of the phagosome with lysosomes activates various enzymes (while the pH is maintained at ~4), which can be stained using reagents such as lysotracker. By means of this process, the foreign substance is removed and the activation of lysosomes is terminated. Despite numerous related reports, many aspects of this process remain unanswered, such as the length of time for which the phagosome can be maintained in the cytosol and how the generation of phagosomes induces the formation of lysosomes. Notably, while the exact timing is unknown, it is thought that the generation of phagosomes and lysosomes in insects coincides with when the haemocyte is activated (with respect to their morphology and activation of lysosomes).

Papilio hyperbius, occurs in East Asia, including Korea, belongs to the family Nymphalidae and appears three to four times a year. The larvae feed mainly on violets and have a longer larval period than other butterflies as they undergo up to six instars. They walk on all fours for courting and mating and lay 300–400 eggs. This species pollinates various flowers in the Republic of Korea and is mass breed for educational use. Studying the immune response in such insects will provide valuable data for the development of solutions to the problem of pathogenic infections during breeding. It could also provide basic data on the cell-mediated immune response in species of the order Lepidoptera.

MATERIAL AND METHODS

Rearing the larvae of the Indian fritillary

For breeding in the laboratory, *Papilio hyperbius* Linnaeus (a ratio of three females to one male) were placed in a box (width 26 cm × length 38 cm × height 26 cm) containing *Viola papilionacea* plants, as food. Honey water and moisture were supplied by spraying them several times a day and after mating the females were moved to another box. The eggs were induced to hatch by placing the host plant in another box (width 70 cm × length 70 cm × height 80 cm). The larvae were reared to adults while controlling the population density by moving them to another box when half of the host plants were consumed. For breeding, the temperature was set to 28°C, the relative humidity to $50 \pm 10\%$ and photoperiod to 16L : 8D.

Preparation and identification of haemocytes

A sterile glass Pasteur pipette was used for collecting haemolymph from the larvae of Indian fritillary. To identify the haemocytes in the haemolymph, 50 µL of plasma was obtained from ten last instar larvae via a dorsal puncture. The haemolymph samples were placed in a sterile Eppendorf tube along with (v/v) of an
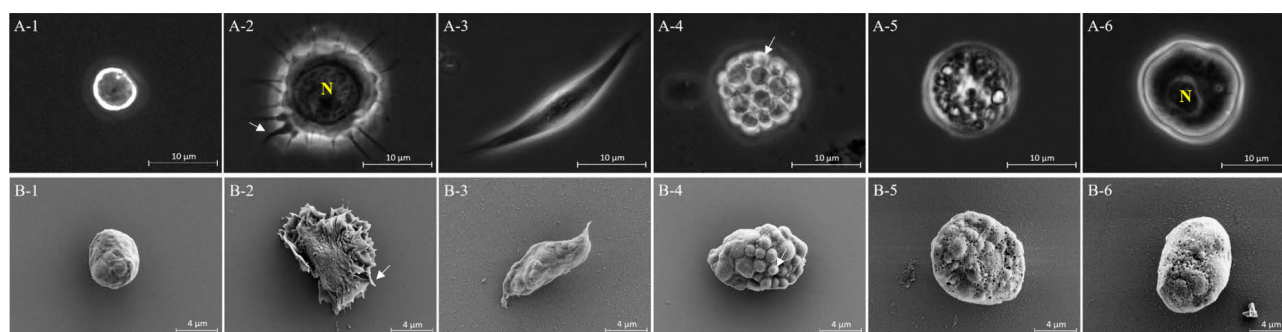
Fig. 1. Differential Interference Contrast (DIC) and scanning electron microscopy (SEM) images of haemocytes from the larvae of the Indian fritillary (*Papilio hyperbius* Linnaeus). There are six types of haemocytes: (A-1 and B-1) prohaemocyte, which is a circular and relatively large cell with long, visible pods on its cell membrane (pods indicated by white arrows) and a relatively large and distinct nucleus (nucleus indicated by N), (A-3 and B-3) plasmatocyte, (A-4 and B-4) adipohaemocyte with glittering granules are indicated by white arrows. (A-5 and B-5) spherulocyte, (A-6 and B-6) oenocytoid with distinct nucleus (indicated by N). DIC images (A-1~A-6) scale bar = 10 µm and SEM images (B-1~B-6) scale bar = 4 µm.

anti-coagulant solution (98 mM NaOH, 186 mM NaCl, 17 mM EDTA, and 41 mM citric acid, pH 4.5) and mixed well. After centrifugation (1,000 g for 10 min at 4°C), the supernatant was removed and the haemocytes (the pellet) were washed with sterile PBS buffer (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate (dibasic, anhydrous), 1.76 mM potassium phosphate (monobasic, anhydrous); pH = 7.4)). The washed samples were then placed in a single tube for use as a sample for optical and electron microscopy. For optical microscopy, over 1,000 haemocytes were photographed in real time to create data base of haemocyte images. This data was analysed with respect to size and shape, based on our past experience of haemocyte classification and reference to various other studies (Kwon et al., 2014; Hwang et al., 2015; Lee et al., 2016; Cho & Cho, 2019). We observed haemocytes under Leica DM2500 upright and Leica DMI 3000B inverted fluorescence microscopes (camera; 2048 × 1536 pixel resolution with the LMD application software version 6.1.).

Injection with carboxylate-modified polystyrene latex beads and *Escherichia coli* K12

Granulocytes and plasmatocytes are the typical haemocytes involved in immunity in insects so their cellular responses were examined. To determine the time-dependent activation for granulocytes, carboxylate-modified polystyrene latex beads (CLBs; 1 µm) and live *E. coli* K12 (10 µl of $6 × 10^7$) were injected into the back of last instar larvae and haemolymph was collected at 2, 4, 12, 24 and 48 h intervals. Translational response was induced through dilutional injections, which resulted immediately in the changes in the morphology of the haemocyte becoming apparent.

To observe lysosomes, we used the acidotropic dye LysoTracker Red (7.5 nM; Molecular Probes). Following the injection of live *E. coli*, haemolymph was collected after 2, 4, 8, 12, and 48 h and the activated lysosomes in the granulocytes were stained. Red lysosomal fluorescence was observed using a Leica DMI 3000B inverted fluorescence microscope and 10,000 haemocytes per sample were determined and quantified using fluorescent microscopy and FACS.

Electron microscopy

For scanning electron microscopy (SEM) a 10 µl sample of haemolymph was sedimented for 3 days on a Thermanox™ 125 coverslip coated with 0.1% poly-l-lysine (Thermo Fisher Scientific, Waltham, USA). Haemocytes attached to the coverslip were fixed in sodium cacodylate buffer (Na(CH^3)^{2}AsO^2, 0.1 M, pH = 6.5; 50 mM-isopropanol 15%, Sigma-Aldrich) containing 2.5% glutaraldehyde. Then, the coverslip was re-fixed with 1% osmi-

um tetroxide (OsO$_4$) and haemocytes dehydrated by progressive bathing in ethanol (70~100%) and critical point dried (Leica® EM CPD030). Finally, cells were sputtered with gold and observed under a SEM Leo 420 scanning electron microscope (LEO Electron Microscopy Ltd., Cambridge, UK). For transmission electron microscopy (TEM), haemocytes were re-fixed with osmium tetroxide 1% (OsO$_4$) and then dehydrated by progressive bathing in ethanol (70~100%) and propylene oxide 100%, embedded in araldite resin (Epon 812) and allowed to polymerase for 48 h at 60°C. Ultrathin sections were obtained using an ultramicrotome (Pabisch Top Ultra 150) and collected on 200-mesh nickel grids. Ultrathin sections were stained with uranyl acetate and precipitated in citrate for 5 min and the sections observed under a JEX-1230 transmission electron microscope (JEOL Ltd, Japan).

Fluorescence-activated cell sorting (FACS) analysis

Red lysosomal fluorescence was quantified via fluorescent microscopy and a BD™ FACSCanto flow cytometer (BD Bioscience, San Jose, CA). Red fluorescence was detected in the channel FL3 (610/20 band-pass) and the percentages of red fluorescences were determined for 10,000 haemocytes in every sample. Details of the experimental procedures and data analyses are described in Kwon et al. (2014) and Cho & Cho (2019).

RESULTS

Microscopic characteristics of haemocytes

The morphology of the haemocytes in last instar larvae of *P. hyperbius* Linnaeus are shown in Fig. 1. Analysis of over 1,000 images of haemocytes indicates that they can be consistently divided and classified into six different types (Fig. 1A).

The haemocyte shown in Fig. 1A-1 is a prohaemocyte, which commonly occur in insects. Of the six types of haemocyte, prohaemocytes are relatively small, circular in shape and there are no visible structures on their smooth cell membranes (Fig. 1A-1). The SEM analysis confirmed they are relatively small and round cells with no pseudopodia on the membrane (Fig. 1B-1). These morphological features are characteristic of insect prohaemocytes.

The haemocyte shown in Fig. 1A-2 is a granulocyte, which is a circular and relatively large with long, visible pods on its cell membrane (Fig. 1A-2; various pods indicated by white arrows) and a relatively large and distinct
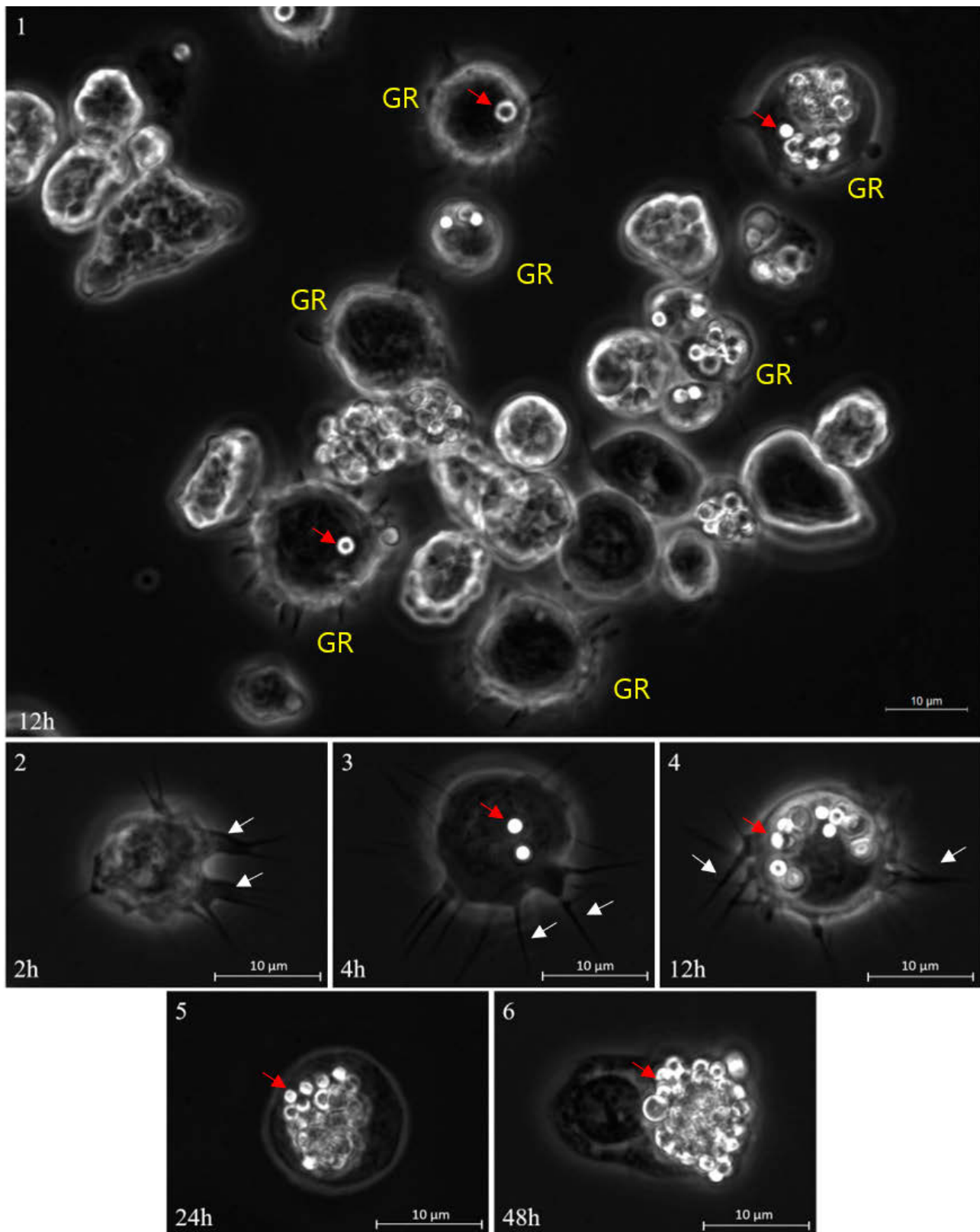
Fig. 2. Differential Interference Contrast (DIC) images of haemocytes stimulated with carboxylate-modified polystyrene latex beads (CLBs). (1) Overall morphology of the haemocytes 12 h after the injection of CLBs (CLBs indicated by red arrows). Among the six types of haemocytes, only granulocytes (indicated by yellow GR) have long and wide pods and nets (hair-like or amoeba-like structures). (2) Granulocytes 2 h after the injection of CLBs (fan-like nets indicated by white arrows). (3) Granulocytes collected 4 h after the injection of CLBs (phagocytosed CLBs indicated by red arrows). (4) Granulocytes viewed 12 h after the injection of CLBs with many CLBs observed together (pod-like structures indicated by white arrows). (5) Twenty-four hours after the injection of CLBs, granulocytes became circular in shape, with negligible movement and activity. (6) 48 h following the injection of CLBs, granulocytes appeared swollen and some even burst.
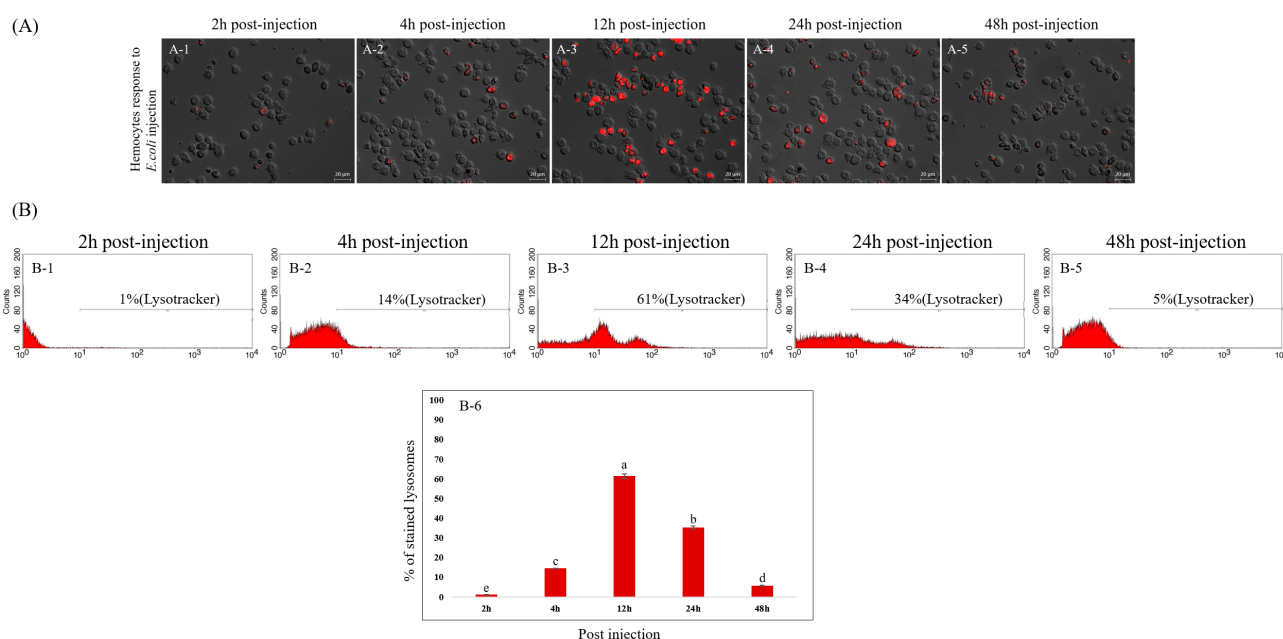
Fig. 3. Microscopic and flow cytometry analysis of LysoTracker Red labelling of granulocyte from the Indian fritillary injected with live *E. coli* K12. (A-1) Activation of lysosomes 2 h after injection with the lysosomes of few granulocytes activated (activated lysosomes [red colour] indicated by white arrows). (A-2) Four hours after injection, the lysosomes of many granulocytes are active. (A-3) 12 h after injection, most lysosomes are highly active (intensely stained). (A-4 and A-5) 24 and 48 h after injection, however, there is a gradual decline in lysosomal activity in granulocytes. (B-1~B-5) Fluorescent microscopy results were quantified using FACS. The percentage of stained lysosomes 2 h after injection was 1%, which increased to 14% at 4 h and 61% at 12 h. It then decreased to 34% at 24 h before declining further to 5% at 48 h. (B-6) The flow cytometry results are expressed as the mean of three independent experiments, each containing three insects (nine insects per condition) using Duncan's multiple range test or one-way ANOVA; *p* value ≤ 0.05. (The X-axis is time post-infection while the Y-axis is the percentage of stained lysosomes in response to *E. coli* infection).

nucleus (Fig. 1A-2; nucleus indicated by N). Opaque granules of varying sizes were often observed in its cytosol. Moreover, SEM analysis also revealed sharp and uneven structures on the cell membrane (Fig. 1B-2; pods indicated by white arrows). The long pods that were observed using optical microscopy could not be seen, presumably because the cells were deformed during the preparation of the samples. The horizontally elliptical haemocyte in Fig. 1A-3 is a typical insect plasmatocyte. SEM analysis also confirmed the elliptical shape of this cell, which has sharp edges on both sides (Fig. 1B-3). Granulocytes and plasmatocytes are both known to be involved in the immune response of insects and were recorded in this study.

The haemocyte in Fig. 1A-4 is presumed to be an adipohaemocyte, which is typical of insects. The cytosol contains an abundance of relatively large and transparent granules (Fig. 1A-4; glittering granules indicated by white arrows). These glittering granules obscured the nucleus under optical microscopy. SEM also revealed relatively large-sized granules (Fig. 1B-4; granules indicated by white arrows). The haemocyte in Fig. 1A-5 is a spherulocyte. In insects, these haemocytes are very similar in size, shape and in their cytoplasmic granules to granulocytes (Fig. 1A-2), but differ in that they do not have pods or nets on their cell membranes. Another feature is their unclear nucleus. SEM analysis further revealed that the cell membrane had a smooth surface, without pods or nets (unlike granulocytes; Fig. 1B-5). The haemocyte in Fig. 1A-6 is an oenocytoid, which characteristically has a highly opaque cytosol. In addition, the nucleus is relatively small compared to the

overall size of the cell and there are no structures visible on the cell membrane (Fig. 1A-6; nucleus indicated by N). In general, oenocytoids are the largest haemocytes in insects, but the target insect in this study, however, has relatively small oenocytoids (Fig. 1B-6).

Immunological activation of granulocytes

Fig. 2(1) shows the overall morphology of this haemocyte 12 h after the injection of CLBs. Among the six types of haemocytes, only granulocytes have long and wide pods and nets (hair-like or amoeba-like structures; Fig. 2(1); granulocytes and CLBs indicated by GR and red arrows, respectively). Granulocytes can also readily phagocytose CLBs in their cytosol. Thus, granulocytes are the main haemocytes involved in the immunity response in *P. hyperbius* Linnaeus. Phagocytosed CLBs were also observed in some plasmatocytes, although not as frequently as in granulocytes.

Fig. 2(2) shows granulocytes 2 h after the injection of CLBs and the initial formation of wide and large nets on the cell membrane (fan-like nets indicated by white arrows). These irregular shaped nets are very sticky. However, phagocytosed CLBs were not observed in the cytosol of most granulocytes. Fig. 2(3) shows granulocytes 4 h after the injection of CLBs in which phagocytosed CLBs are present in the cytosol of many granulocytes at this time (CLBs indicated by red arrows). Subsequently, the nets of granulocytes became thinner and very long (pod-like structures indicated by white arrows). Many CLBs were present in the cytosol of granulocytes viewed 12 h after the injec-
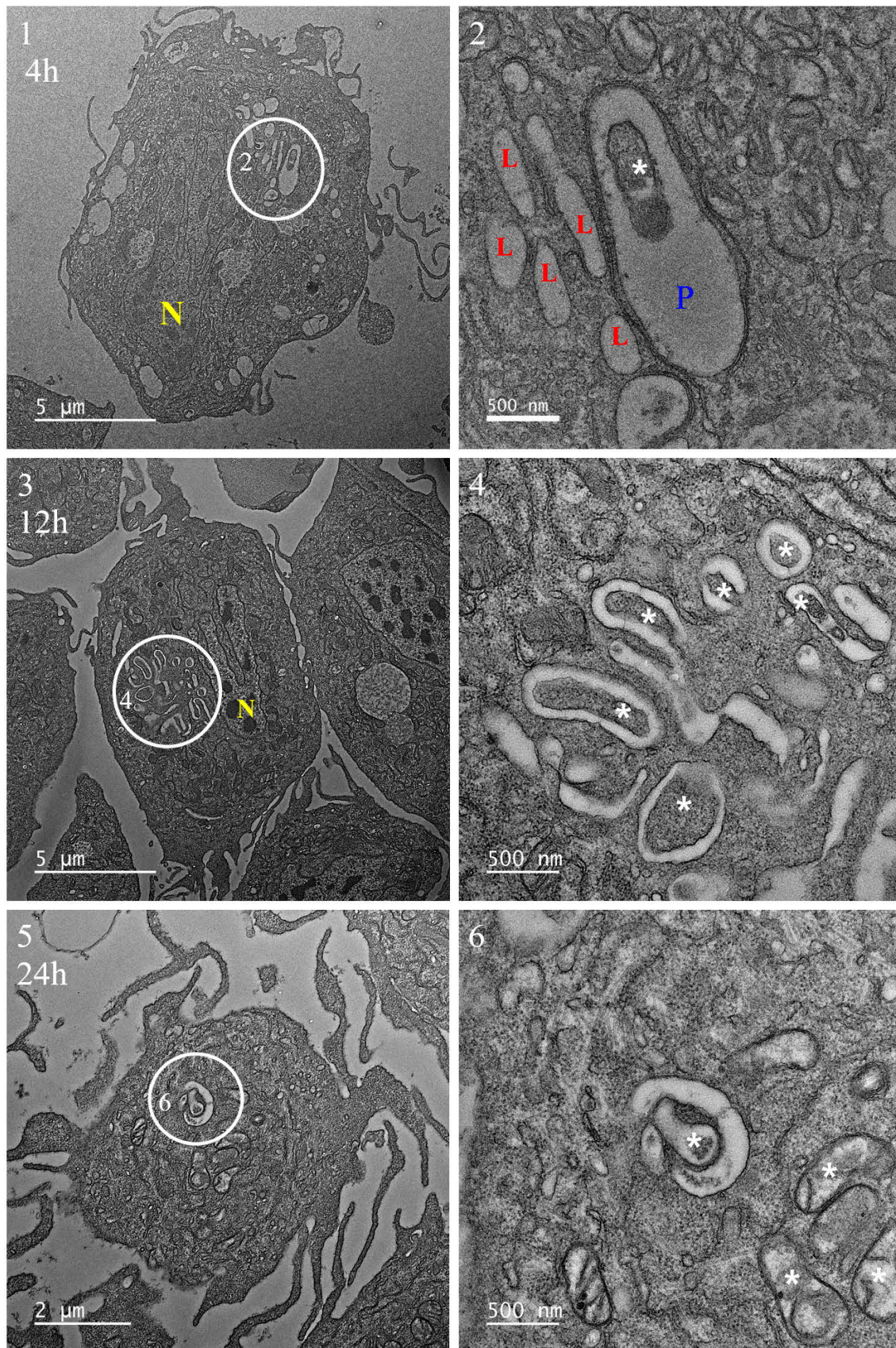
Fig. 4. Transmission electron microscopy (TEM) images of granulocytes in the Indian fritillary injected with live *E. coli* K12. Haemolymph was collected 4, 12 and 24 h after the injection of *E. coli*; TEM images were then mainly taken of granulocytes. (1 and 2) Four hours after injection, phagosomes with *E. coli* occcurred in granulocytes (nucleus indicated by N, *E. coli* indicated by asterisks, phagosome indicated by P). (3 and 4) Twelve hours after infection, granulocytes were only observed with phagosomes or mature lysosomes with *E. coli* in the cytosol (nucleus indicated by N, *E. coli* indicated by asterisks). (5 and 6) Twenty-four hours after infection, newly formed phagosomes, endosomes or lysosomes could no longer be seen in the cytosol of granulocytes (*E. coli* indicated by asterisks). White circles in panels 2, 4, and 6 delimit areas shown at higher magnification in panels 1, 3, and 5, respectively.

tion of CLBs. Moreover, their cell membranes have thin and very long forms of pod-like structures (Fig. 2(4); pod-like structures indicated by white arrows). Twenty-four hours after the injection of CLBs, granulocytes undergo rapid changes in shape. The thin, long pod-like structures were no longer visible on many granulocytes. Moreover, the cells became circular in shape, with negligible movements and activities (Fig. 2(5)). After 48 h the granulocytes contained many CLBs, were swollen and some even burst (Fig. 2(6)).

FACS analysis of haemocytes involved in the immune response

Fig. 2 shows granulocytes that have phagocytosed foreign substances (i.e., formed phagosomes) and it is presumed that a series of steps must have occurred to remove said foreign substances via the formation of endosomes and lysosomes in the cytosol. Fig. 3A-1 shows the activation of lysosomes 2 h after injection with few granulocytes showing lysosomal activity (activated lysosomes [red colour] indicated by white arrows). Four hours after injection, granulocytes with active lysosomes were abundant and after 12 h most had highly activated (intensely stained) lysosomes (Figs 3A-2 and A-3). These granulocytes were also thin with elongated pods (Fig. 2). Twenty-four and 48 h after injection, however, there was a gradual decrease in the lysosomal activity in the granulocytes (Figs 3A-4 and A-5). In line with the results observed after the injection of CLBs, the activity of the granulocytes decreased steadily after 24 h. Hence, the cell-mediated immune response of the granulocytes in *P. hyperbius* Linnaeus reached its maximum 12 h after the pathogenic infection, which was followed by the removal of the pathogen and gradual decrease in immune activity.

Fluorescent microscopy results were quantified using FACS (Fig. 3B). The percentage of stained lysosomes 2 h after injection was 1%, which increased to 14% at 4 h and then 61% at 12 h. It then decreased to 34% at 24 h before falling further to 5% at 48 h (Figs 3B-1 to B-5). This supports the fluorescent microscopy results, indicating that the activation of cell-mediated immune response reached its maximum level after 12 h, before gradually decreasing. Fig. 3B-6 shows time-dependent FACS (based on three measurements) after *E. coli* injection. All measurements were similar and the mean and statistical values are depicted in Fig. 3B-6.

TEM of granulocytes

To verify whether the injected *E. coli* were actually phagocytosed by granulocytes (by phagosome formation) and whether lysosomes fused with phagosomes, the cells were imaged using TEM (Fig. 4). For this, haemolymph was collected 4, 12 and 24 h after the injection of *E. coli* and photographs were taken of the granulocytes (Figs 4(1), (3), and (5); Figs 4(2), (4), and (6) show magnified images). Fig. 4(1) and 4(2) show that phagosomes of *E. coli* formed in granulocytes by cytoplasmic phagocytosis (Figs 4(1) and (2); nucleus indicated by N, *E. coli* indicated by asterisks, phagosome indicated by P). Vacuoles

of varying sizes detected in the vicinity of phagosomes were presumed to be endosomes or lysosomes (Fig. 4(2); endosomes or lysosomes indicated by L). The results thus indicate that the formation of endosomes or lysosomes followed the formation of phagosomes via phagocytosis 4 h after infection.

Twelve hours after infection, granulocytes were only observed as phagosomes or mature lysosomes with *E. coli* in the cytosol. Furthermore, the previously observed vacuoles of varying sizes (endosomes or lysosomes) were no longer present in the vicinity of the phagosomes (Figs 4(3) and (4); nucleus indicated by N, *E. coli* by asterisks). In addition, one *E. coli* was observed in each phagosome or mature lysosome in most cases (Fig. 4(4)). This indicates that one phagosome formed for each *E. coli* in the cytosol following injection-initiated phagocytosis. Then, in time, endosomes or lysosomes of varying sizes formed and fused with phagosomes to complete the formation of mature lysosomes, which contributed to the removal of *E. coli*.

Twenty-four hours after infection, newly formed phagosomes, endosomes, or lysosomes could no longer be seen in the cytosol of granulocytes. Moreover, *E. coli* were no longer present in mature lysosomes (Figs 4(5) and (6); *E. coli* indicated by asterisks). In addition, the nucleus became less visible in the granulocytes that were involved in this immune response (Fig. 4(5)). In *P. hyperbius* Linnaeus, cell-mediated immune response appeared to reach its maximum level at 12 h, and then, from 24 h onwards, it subsided as *E. coli* in lysosomes fused with phagosomes and were removed and the cell then lost its function in terms of immunity.

DISCUSSION

Based on the results presented in this study, the following conclusions can be drawn. First, there are six types of haemocytes in *P. hyperbius* Linnaeus larvae: prohaemocytes, plasmatocytes, granulocytes, spherulocytes, adipohaemocytes and oenocytoids, of which the granulocytes are responsible for the cell-mediated immune response (such as phagocytosis). Second, in the early stage of an infection the granulocytes increase in size and their membranes take the form of large nets. In time, these nets change into long thin pods. Third, in the early stage of an infection, granulocytes have phagosomes in their cytosol containing pathogens undergoing phagocytosis and endosomes or lysosomes are also present in the vicinity. Fourth, each phagosome contains one *E. coli* and after 24 h the phagosomes disappear from the cytosol, which marks the completion of the cell-mediated immune response. Fifth, the granulocytes that were activated in the immune response are likely to disappear entirely.

There are seven types of haemocytes in insects: prohaemocytes, plasmatocytes, granulocytes, spherulocytes, adipohemocytes, coagulocytes and oenocytoids (Gupta at al., 1985). All of these haemocytes occur in Lepidoptera, although it is likely that each species has a distinct set. For example, the silkworm (*Bombyx mori*) has five types of haemocyte: prohaemocytes, plasmatocytes, granulocytes,

spherulocytes and oenocytoids (Lavine & Strand, 2002; Ribeiro & Brehelin, 2006; Strand, 2008) and the Asian corn borer (*Ostrinia furnacalis*), five types, among which granulocytes are the most important (Shen et al., 2016). It is not easy to identify and classify the haemocytes in insects. Various staining agents were used in the past to classify insect haemocytes, but this method is inaccurate and unsuitable for many insects (Kwon et al., 2014). For example, in the case of fruit flies and mosquitoes, there are only three to four types of blood cells, and in the case of Coleoptera and Lepidoptera, most of them have six to seven types of blood cells. In other words, the types of blood cells differ depending on the species and even if the same types of blood cells occur in each species, there are many cases of the dyes not staining them.

Previous studies have classified and characterized haemocytes based only their morphological characteristics (Kwon et al., 2014; Hwang et al., 2015; Lee et al., 2016; Cho & Cho, 2019). Based on such evidence, haemocytes in insects can be consistently identified. For example, pro-haemocytes, granulocytes, plasmatocytes and oenocytoids are easily identified by their morphology. In other words, the morphology of these four types of haemocyte is distinct in insects. By way of illustration, prohaemocytes are the smallest and relative to their size have the largest nucleus, oenocytoids are the largest and have a highly opaque cytosol and the granulocytes and plasmatocytes are the main haemocytes involved in the immune response, the cell membranes of which consist of nets or pods of varying sizes. While these morphological characteristics are generally consistent in insects, other types of haemocyte, such as adipohaemocytes, spherulocytes and coagulocytes, may differ. In order to reduce potential errors in identification, there is a need to develop specific molecular markers for each insect haemocyte (Feng et al., 2022).

Various haemocytes are known to be involved, directly or indirectly, in the immune response in insects (Kwon et al., 2014), with the plasmatocytes and granulocytes mainly involved the cell-mediated immune response against pathogens (phagocytosis, encapsulation, and nodulation). The injection of CLBs with subsequent imaging at set time intervals has previously been used to identify the haemocytes involved in activities such as phagocytosis. Rapid morphological changes occurred in the granulocytes of *P. hyperbius* Linnaeus, indicating phagocytosis along with a much lower level in the plasmatocytes. Therefore, we collected and analysed haemolymph at set time intervals after the injection of CLBs and *E. coli*. However, this method was unsuitable for the accurate monitoring of the cellular immune response by in vivo real-time microscopy, possibly because cells may have undergone morphological changes during the collection of haemolymph or during the preparation of the sample. To minimize such errors, TEM was used to capture various morphological images of haemocytes, notably, that most granulocytes had phagocytosed *E. coli*.

As shown in Figs 4(1) and 4(2), TEM analysis revealed insights into the immune activity of granulocytes after infection with *E. coli*. Interesting phenomena were revealed, including phagosome generation, lysosome formation, the removal of the pathogen in the phagosome and the reduced cellular activity on completion of the immune processes. Indeed, it is reported that on completion of the immune response haemocytes are gradually phagocytosed by newly formed haemocytes and cleared from the haemocoel of cricket larvae (Cho & Cho, 2019). Moreover, although more insects should be investigated, as shown in Figs 2(5), (6), and 4(5), the activity of cells involved in the immune response declines over time. It is likely that this occurs frequently in insects. Hence, it is likely that old cells are replaced by new cells, thus ensuring that a suitable level of active and healthy haemocytes is maintained in the hemocoel. Fig. 4 shows that the generation of phagosomes is induced by one pathogenic entity at a time and several lysosomes form in the cytosol and fuse with phagosomes resulting in the removal of the pathogen. This series of activities of cells involved in the immune response is also reported in mammals (Luzio et al., 2007; Lancaster et al., 2021).

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