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RESEARCH ARTICLE

Activation of cellular immune response in insect model host *Galleria mellonella* by fungal α-1,3-glucan

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One sentence summary: The changes in the hemocytogram accompanied by nodule formation and changes in apolipophorin III localization, indicated the involvement of the insect cellular immune response to immunization with fungal α-1,3-glucan.

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ABSTRACT

Alpha-1,3-glucan, in addition to β-1,3-glucan, is an important polysaccharide component of fungal cell walls. It is reported for many fungal species, including human pathogenic genera: *Aspergillus*, *Blastomyces*, *Coccidioides*, *Cryptococcus*, *Histoplasma* and *Pneumocystis*, plant pathogens, e.g. *Magnaporthe oryzae* and entomopathogens, e.g. *Metarhizium acridum*. In human and plant pathogenic fungi, α-1,3-glucan is considered as a shield for the β-1,3-glucan layer preventing recognition of the pathogen by the host. However, its role in induction of immune response is not clear. In the present study, the cellular immune response of the greater wax moth *Galleria mellonella* to *Aspergillus niger* α-1,3-glucan was investigated for the first time. The changes detected in the total hemocyte count (THC) and differential hemocyte count (DHC), formation of hemocyte aggregates and changes in apolipophorin III localization indicated activation of *G. mellonella* cellular mechanisms in response to immunization with *A. niger* α-1,3-glucan. Our results, which have clearly demonstrated the response of the insect immune system to this fungal cell wall component, will help in understanding the α-1,3-glucan role in immune response against fungal pathogens not only in insects but also in mammals, including humans.

Keywords: α-1,3-glucan; *Aspergillus niger*; *Galleria mellonella*; insect immune response; hemocytes; nodulation; apolipophorin III

INTRODUCTION

Alpha-1,3-glucan, in addition to β-1,3-glucan, is an important polysaccharide component of fungal cell walls. It performs structural and mechanical functions, and serves as reserve material. Alpha-1,3-glucan is present in the cell walls of many species of the classes Ascomycetes and Basidiomycetes. This component constitutes 9–32% of cell wall polysaccharides in filamentous fungi belonging to the genus *Aspergillus*, and as much as 46.5% in *Histoplasma capsulatum* (Kanetsuna *et al.* 1974; Choma *et al.* 2013). The presence of α-1,3-glucan has been demonstrated in the cell walls of entomopathogenic fungi, e.g. *Metarhizium*

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acridum (Zhang *et al*. 2019; Zhao *et al*. 2019), *Fusarium* spp. (Schoffelmeer *et al*. 1999; Navarro-Velasco *et al*. 2011; Santos *et al*. 2020) and *Aspergillus flavus* (Leger, Staples and Roberts 1993; Seo, Akiyoshi and Ohnishi 1999). In comparison to the well-described β-1,3-glucan, the biological activities of α-1,3-glucan remain to be elucidated. As indicated by literature data, in human pathogenic fungi *Blastomyces dermatitidis*, *H. capsulatum* and *Paracoccidioides brasiliensis*, and plant pathogenic fungi, e.g. *Metarhizium oryzae* (Geoghegan, Steinberg and Gurr 2017), α-1,3-glucan can serve as a shield for the β-1,3-glucan layer, preventing recognition of the fungal pathogen by the host and allowing development of infection (Hogan, Klein and Levitz 1996; Pinto, Barreto-Bergter and Taborda 2005; Rappleye, Eissenberg and Goldman 2007).

The greater wax moth *Galleria mellonella* has been widely used as a model organism in studies on insect innate immunity and host-pathogen interactions. Research conducted using *G. mellonella* provided essential information on the virulence factors of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* and strategies developed by these pathogens to overcome the insect immune response (Götz, Matha and Vilcinskas 1997; Ortiz-Urquiza *et al*. 2010; Dubovskiy *et al*. 2013). Due to the similarities in the functioning of insect and mammalian innate immunity and the strong correlations in bacterial and fungal virulence in *G. mellonella* and mice, *G. mellonella* larvae have been widely exploited as an alternative model host for studying the pathogenicity and virulence factors of pathogens, including human pathogenic bacteria and fungi (Jander, Rahme and Ausubel 2000; Salamitou *et al*. 2000; Brennan *et al*. 2002; Loré *et al*. 2012). For example, *G. mellonella* larvae have been used in research on human pathogenic fungi, such as *Candida albicans*, *Cryptococcus neoformans*, *A. fumigatus*, *A. flavus*, *F. oxysporum*, *Pneumocystis murina* and *Microsporum* spp. (Mylonakis *et al*. 2005; Kavanagh and Fallon 2010; Lionakis 2011; Desbois and Coote 2012).

Insect innate immunity relies on humoral and cellular immune response mechanisms. The involvement of hemocytes in the cellular response is an important element of the insect's defense reactions, in which foreign bodies are removed, depending on the size, in the process of phagocytosis, nodulation, or encapsulation (Lapointe, Dunphy and Mandato 2012). Lepidoptera hemocytes can be divided into five types: prohemocytes, plasmatocytes, granulocytes, spherulocytes and oenocytoids (Tan *et al*. 2013; Vogelweith *et al*. 2016; Wu, Liu, Yi 2016; Blanco *et al*. 2017; Wojda 2017; Boguś *et al*. 2018). Granulocytes and plasmatocytes, both involved in cellular immune response, constitute the majority of hemocytes circulating in the hemolymph and are the only hemocytes capable of adhesion (Strand 2008). The circular shape of plasmatocytes changes after adhesion to various surfaces due to the formation of pseudopodia—they can then be 10–15 μm wide and 20–30 μm long. Granulocytes are spherical hemocytes with a small nucleus and numerous granules present in the cytoplasm. Upon recognition of a foreign body, the material contained in the granules undergoes exocytosis, playing the role of opsonins and attractants in relation to other hemocytes (Lapointe, Dunhy, Mandato 2012; Salem *et al*. 2014). Spherulocytes and oenocytoids are non-adherent hemocytes. Spherulocytes contain numerous round and oval spheres bound to the cell membrane. These hemocytes are involved in the transport of cuticle components. Oenocytoids are round or oval cells with a small nucleus. They contain components of the phenoloxidase system; hence, they are involved in the melanization process. Oenocytoids can also release nucleic acids, which are alarm signals informing about an infection (Altincicek *et al*. 2008).

The total number of hemocytes and the number of individual types of hemocytes depends on the insect's developmental stage and environmental conditions, including diet and stress factors (Vogelwieth *et al*. 2016; Blanco *et al*. 2017; Boguś *et al*. 2018). The activation of cellular response mechanisms can be evidenced by a change in the total number of hemocytes (total hemocyte count, THC) and a change in the proportions of individual types of hemocytes (differential hemocyte count, DHC). The participation of hemocytes in the immune response is associated, inter alia, with the neutralization of foreign bodies via separation from the rest of the body in the form of structures called nodules or capsules. It has been shown that despite the difference in the type of the foreign body sequestered in these processes, the spatial structure of nodules and capsules is similar (Ratcliffe and Gagen 1976; 1977). In Lepidoptera, granulocytes and plasmatocytes are involved in formation of nodules and capsules. Depending on the insect species, melanin may be also deposited in these structures (Nappi *et al*. 2000; Hillyer 2016).

Apolipophorin III (apoLp-III) is an 18-kDa multifunctional protein, involved in lipid transport and immune response, which occurs abundantly primarily in hemolymph, but is also detected in *G. mellonella* hemocytes and the fat body (Weers and Ryan 2006; Zdybicka-Barabas and Cytryńska 2013; Wen *et al*. 2016). It has been shown that, in response to infection, the level of apoLp-III in various tissues of *G. mellonella* larvae varies in a pathogen- and time-dependent manner, which indicates its involvement in immune response (Zdybicka-Barabas and Cytryńska 2011; Wen *et al*. 2016; Stączek *et al*. 2018). ApoLp-III can bind bacterial and fungal cell wall components such as lipoteichoic acid (LTA), lipopolysaccharide (LPS) and β-1,3-glucan, acting as a pattern recognition receptor, participates in the detoxification of bacterial endotoxins, and exhibits antimicrobial activity (Halwani and Dunphy 1997; Halwani, Niven, Dunphy 2000; Pratt and Weers 2004; Whitten *et al*. 2004; Zdybicka-Barabas *et al*. 2011; Oztug, Martinon and Weers 2012). ApoLp-III may act as a signal molecule transmitting information of infection to hemocytes by formation of low-density lipophorins (LDLp), which are then taken up by granulocytes (Dettloff *et al*. 2001; Niere *et al*. 2001). In addition, apoLp-III has been shown to be a factor modulating insect cellular response. By affecting the adherent properties of hemocytes, it supports the phagocytosis of yeast cells by *G. mellonella* plasmatocytes and, after binding with lipids, enhances the process of encapsulation and nodulation (Zakarian *et al*. 2002; Whitten *et al*. 2004).

Our recent paper demonstrated that inoculation of *G. mellonella* larvae with *Aspergillus niger* α-1,3-glucan led to inhibition of phenoloxidase activity in hemolymph shortly after injection, indicating this cell wall polysaccharide as a fungal virulence factor involved in impairment of the insect immune response (Stączek *et al*. 2020).

In the present study, cellular immune response of *G. mellonella* larvae to immunization with *A. niger* α-1,3-glucan was investigated. Changes in the total number of hemocytes (THC) and in the proportions of individual types of hemocytes (DHC) were analyzed. In addition, formation of hemocyte aggregates (nodules) was examined. In order to evaluate the involvement of apoLp-III in the *G. mellonella* immune response to *A. niger* α-1,3-glucan, changes in its hemolymph level and localization in larval hemocytes after immunization with this component of the fungal cell wall were investigated.

MATERIALS AND METHODS

Microorganism and isolation of α-1,3-glucan

The filamentous fungus *A. niger* CBS 554.65 (wild type; CBS Fungal Biodiversity Centre, Netherlands) was grown on solid PDA medium (5% potato extract, 0.5% dextrose and 1.6% agar) at 30°C until conidia were obtained and then stored at 4°C (Zdybicka-Barabas and Cytryńska 2010; Stączek *et al.* 2020).

Isolation of α-1,3-glucan from *A. niger* cell walls and its chemical characteristics were described in detail previously (Wiater *et al.* 2015). Before use in the experiments, purified α-1,3-glucan was suspended in 30% dimethylsulfoxide (DMSO) and sonicated (5 × 1 min; 30 s pulse and 30 s off) with a Sonicator Ultrasonic Processor XL 2020 (20 KHz Frequency, MISONIX, New York, United States).

Immunization of insects

The greater wax moth *Galleria mellonella* larvae (Lepidoptera: Pyralidae) were reared in a continuous laboratory culture on honeybee nest debris at 30°C in the dark. Last instar larvae were used for the study. The larvae were immunized with *A. niger* α-1,3-glucan (5 μg dose in 3 μL 30% DMSO) or *A. niger* conidia (1×10^5 dose in 3 μL non-pyrogenic water). For each experiment a fresh batch of conidial suspension with viability of approx. 90% was prepared from a fresh *A. niger* culture. The viability of conidia was determined by a colony forming unit (CFU) assay. The control groups were given 3 μL of 30% DMSO (an average final concentration in larval hemolymph was 1.13%) or non-pyrogenic water, respectively. Non-immunized larvae were also used in the experiments. Hemolymph and hemocytes were collected 0.25, 0.5, 1, 2, 4 and 24 h after the immunization. Additionally, for analysis of nodule formation, the hemolymph samples were collected 48 and 72 h after the treatment.

Hemolymph collection and analysis of the apoLp-III level in cell-free hemolymph

Hemolymph was pooled from five larvae per group (20 μL per larva) into chilled Eppendorf tubes containing a few crystals of phenylthiourea (PTU) to prevent melanization. The hemolymph samples were centrifuged at 200 × *g* for 5 min to pellet hemocytes and then subsequently centrifuged at 20 000 × *g* for 10 min at 4°C to remove cell debris (Mak, Zdybicka-Barabas and Cytryńska 2010; Zdybicka-Barabas *et al.* 2015). The cell-free hemolymph was stored in −20°C until use. The protein concentration in the cell-free hemolymph was assessed using the Bradford method (Bradford 1976).

For analysis of the apoLp-III level, the cell-free hemolymph samples (3 μg of total protein) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 13.8% gels according to Laemmli (1970) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Massachusetts, United States). For apoLp-III detection, the membranes were probed with rabbit polyclonal anti-*G.mellonella* apoLp-III antibodies (1:2500; Agrisera, Vännäs, Sweden, custom ordered) and subsequently with goat anti-rabbit secondary antibodies (1:30 000; Sigma-Aldrich, Saint Louis, United States) conjugated with alkaline phosphatase. The immunoreactive bands were visualized by incubation with *p*-nitro blue tetrazolium chloride (NBT, Sigma-Aldrich) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Thermo Scientific, Waltham, United States; Blake *et al.* 1984). The documentation and densitometric analysis of the protein bands was carried out using the ChemiDoc Imaging System (BioRad, Hercules, United States).

Immunolocalization of apoLp-III in *G. mellonella* hemocytes

The hemocytes were obtained essentially as described before (Stączek *et al.* 2018). In brief, the hemolymph was collected individually from eight larvae per group (in two repetitions) into ice-cold insect physiological saline (IPS; 0.1 M Tris-HCl pH 6.9, 150 mM NaCl and 5 mM KCl) containing 0.02 mg/mL PTU and used immediately for preparation of monolayers in 8-well Multitest Slides (MPBiomedicals, Waltham, United States) with the method described in our previous report (Stączek *et al.* 2018). The hemocyte monolayers were fixed with 3.7% paraformaldehyde in Tris-buffered saline (TBS; 50 mM Tris-HCl pH 7.5 and 150 mM NaCl). The hemocytes were gently permeabilized by 0.2% Triton X-100 in TBS. The application of this detergent caused mild changes in the cell membrane of hemocytes, allowing penetration of antibodies into the cell. The hemocytes in the monolayers were then blocked with 5% BSA in TBS. ApoLp-III in the hemocytes was detected using primary antibodies directed against *G. mellonella* apoLp-III (final concentration 0.1 mg/mL) and then with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit secondary antibodies (1:200; Sigma-Aldrich). The hemocyte nuclei were stained with Hoechst 32 258 (Sigma-Aldrich; Stączek *et al.* 2018). The hemocytes were imaged at 40 × magnification using a laser scanning confocal microscope LSM 5 Pascal (Carl Zeiss, Oberkochen, Germany).

Analysis of changes in the hemocytogram and nodule formation

The involvement of hemocytes in the response against *A. niger* α-1,3-glucan and conidia was assessed by analyzing changes in the hemocytogram. The analyses were performed for 15 individuals in each group (5 larvae per group in each of the three independent experiments). Hemolymph collected individually from each larva was immediately diluted 5-fold in an anticoagulant solution (100 mM glucose, 93 mM NaCl, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA and pH 4.6; Bergin *et al.* 2005). The number of hemocytes of individual larva (THC) was counted in 60 squares of the Bürker chamber grid using a contrast phase microscope (Olympus BH-2; magnification 40×). The number of hemocytes (N_H) found in 1 μL of hemolymph was calculated according to the formula:

$$N_H = \frac{\bar{n} \cdot a}{V},$$

where,

N_H—the number of hemocytes in 1 μL of hemolymph,
$\bar{n}$—average number of hemocytes in one square of the Bürker chamber,
a—dilution of the hemolymph,
V—nominal volume of the hemolymph in one square of the Bürker chamber.

For determination of the DHC, the hemocytes counted as described above were divided into four categories, i.e. plasmatocytes, granulocytes, spherulocytes and oenocytoids, based on their morphological features (Ratcliffe, Mead and Renwrantz 1986; Fig. 2A and B). The results are presented as absolute

numerical values (THC) and the percentage (%) of individual types of hemocytes in the studied population of cells (DHC).

For analysis of hemocyte aggregates and nodule formation, the larvae (five individuals per group) were immunized with α-1,3-glucan or DMSO and the hemolymph was collected 0.25–72 h after the treatment. Nodules formed *in vivo* were observed under a phase-contrast microscope (Olympus BH-2; magnification 20×) in 10 μL of freshly obtained hemolymph. The presence of melanized nodules was also analyzed inside the larval body after dissection.

Statistics

All the experiments were performed in three independent occasions. The statistical significance of the differences in the mean values between the groups was assessed using the method of single- and multi-factorial analysis of variance (ANOVA). The results were presented as mean value ± standard deviation (SD). The ranges of the statistical significance of the results were marked with asterisks: $^{*}P < 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$, or letters of the alphabet, where statistically significant differences between the means in the studied groups were indicated by the lack of a common letter. Calculations were made using the Statistica program (StatSoft, Palo Alto, United States, TIBCO Software).

RESULTS

Changes in *G. mellonella* hemocytogram upon immunization with *A. niger* α-1,3-glucan

The THC and their individual types (DHC) in the hemolymph of larvae immunized with *A. niger* α-1,3-glucan, *A. niger* conidia, DMSO and water was determined. Despite the significant differences in the total number of hemocytes and the number of certain types of hemocytes (Tables 1 and 2), their percentage in the hemocyte population of each individual larva in the given experiment was similar (Fig. 1).

The analysis of THC showed that, 2 and 4 h after the immunization with α-1,3-glucan and DMSO, the average number of all hemocytes (1.6–2.0 × 10^4 in 1 μL of hemolymph) was almost twice as high as the number in the non-immunized larvae (Table 1) and was mainly due to an increase in the number of plasmatocytes (2 h post-treatment) and granulocytes (4 h post-treatment). Interestingly, a statistically significant increase in the THC after the α-1,3-glucan administration, compared to the DMSO immunization, was found 0.5 h post-treatment and could be connected to an increase in the number of granulocytes (Table 1).

After the injection of *A. niger* conidia, a statistically significant increase in the number of hemocytes, compared to the number of hemocytes in the non-immunized larvae, was observed as early as 1 h post-treatment (Table 2). In contrast to the other immunogens, this condition lasted until 24 h after injection. The increase in THC 1–24 h after the immunization was mainly due to an increase in the number of granulocytes as well as spherulocytes. The number of granulocytes increased at all the time points tested. A statistically significant increase in the number of hemocytes after the administration of conidia, compared to the water injection, was found 1, 4 and 24 h after the immunization (Table 2).

A clear change observed 15 min after the injection of each of the immunogens tested was the approx. 2-fold decrease in the number of plasmatocytes compared to their number in the non-immunized insects. The largest decrease by approx. 60% was noted after the administration of α-1,3-glucan (Tables 1 and 2).

An interesting observation was the increase in the number of spherulocytes detected at the different time points after the immunization with each immunogen, e.g. 1–24 h after the treatment with *A. niger* conidia. In turn, 2 and 4 h after the immunization with α-1,3-glucan, the average number of spherulocytes increased more than 3-fold, reaching the highest values in relation to their number determined after the immunization with the other immunogens (Tables 1 and 2).

The number of oenocytoids in the hemolymph of larvae immunized with α-1,3-glucan increased 4- and 3-fold at 4 h and 24 h after the administration, respectively, relative to the number of these hemocytes in the hemolymph of the non-immunized insects. A similar increase was observed in the DMSO-immunized larvae 2 and 24 h post-treatment (Table 1). An approx. 3-fold increase in oenocytoid counts was noted 4 h and 24 h after the administration of water and *A. niger* conidia, respectively (Table 2).

The analysis of the percentage of individual types of hemocytes (DHC) in the hemolymph of the non-immunized larvae showed that granulocytes, plasmatocytes, spherulocytes and oenocytoids accounted for approx. 52, 41, 6.1 and 0.9% of all hemocytes (Fig. 1). It was detected that 15 min after the administration of α-1,3-glucan, DMSO, *A. niger* conidia and water, the percentage of granulocytes in the hemocyte population increased to approx. 70% (68% after the immunization with α-1,3-glucan), which was accompanied by a decrease in the plasmatocytes. However, based on the analysis of the absolute number of hemocytes, it can be concluded that the dominance of granulocytes at that time was due to the decrease in the number of plasmatocytes rather than an increase in the number of granulocytes (Table 1). The exception was the administration of *A. niger* conidia, where 15 min after immunization, the decrease in the number of plasmatocytes was accompanied by an increase in the number of granulocytes (Table 2).

The observed proportions of these two types of hemocytes were maintained up to 1 h after the administration of α-1,3-glucan. Noteworthy is the dominant granulocyte prevalence at almost all the time points after the administration of α-1,3-glucan, DMSO and *A. niger* conidia, except for hemolymph collected 24 h after the immunization with α-1,3-glucan, as a statistically significantly greater number of plasmatocytes relative to the DMSO administration was noticed. Interestingly, 2 h after the immunization of the larvae with α-1,3-glucan and water, an equal level of granulocytes and plasmatocytes was found, whereas an increase in the contribution of plasmatocytes was noticed in the case of DMSO. The proportion of spherulocytes and oenocytoids increased, respectively, 2–4 h and 4 h after the administration of α-1,3-glucan, compared to larvae immunized with DMSO. It was also noted that, 4 h after the immunization with *A. niger* conidia, the oenocytoid content decreased compared to that in the water-injected larvae (Fig. 1).

Nodule formation in *G. mellonella* hemocel after α-1,3-glucan injection

The presence of hemocyte aggregates, constituting the initial stage of nodule formation, was analyzed in the larval hemolymph collected 0.25–72 h after the administration of *A. niger* α-1,3-glucan or DMSO and in the hemolymph of the non-immunized larvae. No hemocyte aggregates were found in the

Table 1. Changes in the total number of hemocytes (THC) and hemocytes of individual types (DHC) after immunization of *G. mellonella* larvae with *A. niger* α-1,3-glucan (A) and DMSO (B). The larvae (five individuals per group) were immunized with α-1,3-glucan (5 μg) or 30% DMSO. Hemocytes were counted in the Bürker chamber in preparations from freshly harvested hemolymph 0.25, 0.5, 1, 2, 4 and 24 h after the immunization and identified based on their morphology. The table shows the number of hemocytes in 1 µL of hemolymph (±SD). The level of significance determined for the differences between the mean values obtained for α-1,3-glucan and DMSO is marked with asterisks: $^{*}P < 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$. Values marked with different letters within one group (individual groups are presented in the table columns) differ statistically, $P < 0.05$. N—hemocytes of non-immunized larvae.

Time [h]	Total hemocyte count	Granulocytes	Plasmatocytes	Spherulocytes	Oenocytoids
(A) α-1,3-glucan					
N	10 698 ± 1616^{a}	5401 ± 1119^{a}	4750 ± 356^{a}	474 ± 249^{a}	73 ± 65^{a}
0.25	8596 ± 1280^{ab}	5896 ± 999^{ab}	1971 ± 499^{b}	558 ± 34^{ab***}	171 ± 104^{ab}
0.5	16 000 ± 1686^{c*}	10 868 ± 1100^{c}	4431 ± 1507^{ac}	493 ± 194^{abc}	208 ± 75^{abc}
1	14 349 ± 7366^{abcd}	9115 ± 4464^{abcd}	4406 ± 3081^{abcd}	646 ± 236^{abcd}	182 ± 69^{abcd}
2	17 008 ± 2693^{cde}	7029 ± 1643^{abde}	7996 ± 3513^{cde}	1779 ± 802^{e}	204 ± 135^{abcde}
4	18 963 ± 5186^{cdef}	12 358 ± 3952^{cdf}	4763 ± 1443^{acdef}	1538 ± 416^{e*}	304 ± 113^{bcdef}
24	14 604 ± 4368^{acdef}	5036 ± 1898^{abde}	8646 ± 2552^{cde}	708 ± 358^{abcd}	214 ± 91^{bcdef}
(B) DMSO					
N	10 698 ± 1616^{a}	5401 ± 1119^{a}	4750 ± 356^{a}	474 ± 249^{a}	73 ± 65^{a}
0.25	9868 ± 1243^{ab}	6778 ± 1034^{ab}	2174 ± 122^{b}	736 ± 43^{ab}	181 ± 11^{ab}
0.5	12 979 ± 567^{ac}	8653 ± 1346^{bc}	3417 ± 1121^{abc}	799 ± 277^{abc}	111 ± 43^{abc}
1	18 021 ± 7463^{abcd}	11 306 ± 4960^{abcd}	5451 ± 2629^{abcd}	1097 ± 280^{bcd}	167 ± 127^{abcd}
2	16 549 ± 2496^{cde}	5785 ± 1564^{abcde}	9778 ± 1107de	660 ± 349^{abcde}	326 ± 175^{bcde}
4	20 552 ± 4640^{cdef}	11 906 ± 2166^{cdf}	7896 ± 2622^{acdef}	615 ± 221^{abcdef}	135 ± 74^{abcdef}
24	19 542 ± 9035^{abcdef}	12 819 ± 5840^{abcdef}	5889 ± 3067^{abcdef}	632 ± 67^{abcdef}	201 ± 154^{bcdef}

Table 2. Changes in the total number of hemocytes (THC) and hemocytes of individual types (DHC) after immunization of *G. mellonella* larvae with *A. niger* spores (A) and water (B). The larvae (five individuals per group) were immunized with *A. niger* spores (1×10^5) or water. Hemocytes were counted in the Bürker chamber in preparations from freshly harvested hemolymph 0.25, 0.5, 1, 2, 4 and 24 h after the immunization and identified based on their morphology. The table shows the number of hemocytes in 1 µL of hemolymph (±SD). The level of significance determined for the differences between the mean values obtained for spores and water is marked with asterisks: $^{*}P < 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$. Values marked with different letters within one group (individual groups are presented in the table columns) differ statistically, $P < 0.05$. N—hemocytes of non-immunized larvae.

Time (h)	Total hemocyte count	Granulocytes	Plasmatocytes	Spherulocytes	Oenocytoids
(A) *A. niger* conidia					
N	10 698 ± 1616^{a}	5401 ± 1119^{a}	4750 ± 356^{a}	474 ± 249^{a}	73 ± 65^{a}
0.25	12 775 ± 1504^{ab}	8804 ± 1724^{b}	2350 ± 298	1429 ± 386^{b*}	192 ± 85^{ab}
0.5	12 071 ± 2074^{abc}	7596 ± 1467^{bc}	3088 ± 576^{c}	1233 ± 129^{bc}	154 ± 67^{abc}
1	16 317 ± 1690^{d*}	10 642 ± 1080^{bcd***}	4471 ± 857^{acd}	1054 ± 540^{abcd}	150 ± 48^{abcd}
2	15 550 ± 2816^{bcde}	9967 ± 1911^{bcde}	4283 ± 1264^{acde}	1121 ± 374^{bcde}	179 ± 75^{abcde}
4	19 208 ± 1568*	12 908 ± 1001^{ef**}	5021 ± 953^{adef}	1204 ± 262^{bcdef}	75 ± 56^{acdef**}
24	17 904 ± 3535^{de*}	12 146 ± 2868^{bdef*}	4300 ± 1442^{acdef}	1258 ± 555^{bcdef}	200 ± 38^{bcde}
(B) water					
N	10 698 ± 1616^{a}	5401 ± 1119^{a}	4750 ± 356^{a}	474 ± 249^{a}	73 ± 65^{a}
0.25	10 013 ± 5416^{ab}	6588 ± 3734^{ab}	2658 ± 1697^{b}	650 ± 234^{ab}	117 ± 28^{ab}
0.5	11 554 ± 3892^{abc}	7333 ± 2378^{abc}	2942 ± 1185^{bc}	1125 ± 575^{abc}	154 ± 92^{abc}
1	12 177 ± 2580^{abcd}	6484 ± 1224^{abcd}	4849 ± 2801^{abcd}	750 ± 316^{abcd}	94 ± 91^{abcd}
2	12 688 ± 3854^{abcde}	7633 ± 2845^{abcde}	4142 ± 781^{abcde}	767 ± 553^{abcde}	146 ± 26^{abcde}
4	15 263 ± 2234^{bcdef}	9883 ± 1477^{ce}	4046 ± 1555^{abcdef}	1125 ± 296^{cdef}	208 ± 66^{cdef}
24	10 719 ± 3060^{abcdef}	6469 ± 1948^{abcde}	2594 ± 1029^{bcdf}	1490 ± 417^{cef}	167 ± 70^{abcdef}

hemolymph of the non-immunized insects, whereas, the aggregates were present in the hemolymph at all the time points after the treatment with α-1,3-glucan and DMSO. The highest number of aggregates was detected 2 h after the immunization, and their number was 3-fold higher after the administration of α-1,3-glucan than after the DMSO injection: 7.5 (±2.5) and 2.5 (±1.5), respectively, in 10 µL of hemolymph. Interestingly, a considerable number of spherulocytes was visible within the aggregates, suggesting involvement of this type of hemocytes in *G. mellonella* cellular immune response (Fig. 2).

The microscopic changes were accompanied by well visible changes in the macroscopic image after dissection of the larval bodies (Fig. 3). A total of 2 h after the immunization with α-1,3-glucan and DMSO, only single dark spots were hardly visible in both cases. However, 24, 48 and 72 h after the injection of α-1,3-glucan, dark structures, corresponding to melanized

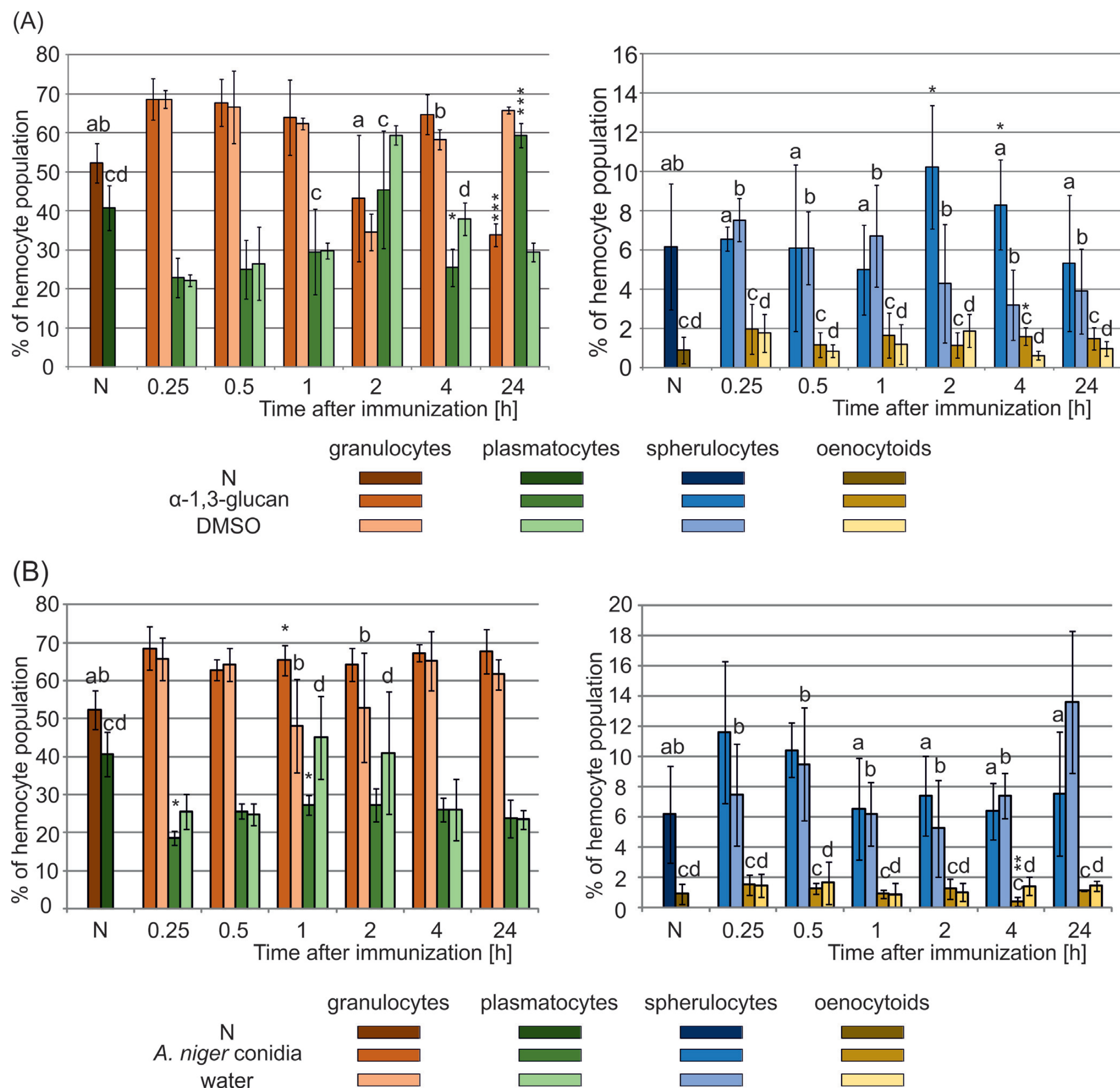

Figure 1. Relative content of particular hemocyte types in the population (DHC) after immunization of *G. mellonella* with *A. niger* α-1,3-glucan, DMSO, *A. niger* conidia and water. The larvae were immunized with **(A)** *A. niger* α-1,3-glucan (5 μg per larva) or 30% DMSO and **(B)** *A. niger* conidia (1×10^5 per larva), or water. Hemocytes were counted in the Bürker chamber in preparations from freshly harvested hemolymph 0.25–24 h after the immunization and identified based on their morphology. The number of all hemocytes found in 1 μL of freshly collected hemolymph was assumed as 100%. The results were presented as a mean value (±SD). The level of significance determined for the differences between the mean values obtained for α-1,3-glucan and DMSO as well as conidia and water are marked with asterisks: $^{*}P < 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$. The statistical significance of differences between the values determining the relative content of a given hemocyte type in the hemocyte population of the non-immunized insects and at the different time points after the immunization is marked by the lack of a common letter ($P < 0.05$). N—hemocytes of non-immunized larvae.

nodules, adhering to the fat body and internal surface of the larval body were detected. Such structures were not found after the DMSO injection (Fig. 3).

Changes in the apoLp-III level in hemolymph after α-1,3-glucan injection

The presence of apoLp-III in the hemolymph of the non-immunized insects and after the administration of the immunogens was confirmed by immunoblotting using specific antibodies. It was shown that the level of apoLp-III increased in hemolymph collected 1 h after the α-1,3-glucan administration in comparison with the DMSO-immunized insects; at the other time points examined, the differences were not statistically significant (Fig. 4A). Following the administration of *A. niger* conidia (1×10^5 per larva), there was no increase in the level of apoLp-III in hemolymph. In this case, the level of apoLp-III was lower than that determined in the hemolymph of the water-injected larvae (Fig. 4B). Statistically significant differences in the apoLp-III levels were noted in hemolymph collected 0.25, 2 and 24 h after the administration of *A. niger* conidia in comparison to the water-injected larvae.

Effect of α-1,3-glucan injection on apoLp-III localization in *G. mellonella* hemocytes

Images of hemocytes incubated with specific primary antibodies and FITC-labeled secondary antibodies obtained by fluorescence microscopy showed intracellular localization of apoLp-III (Fig. 5). Due to the adherent properties of these

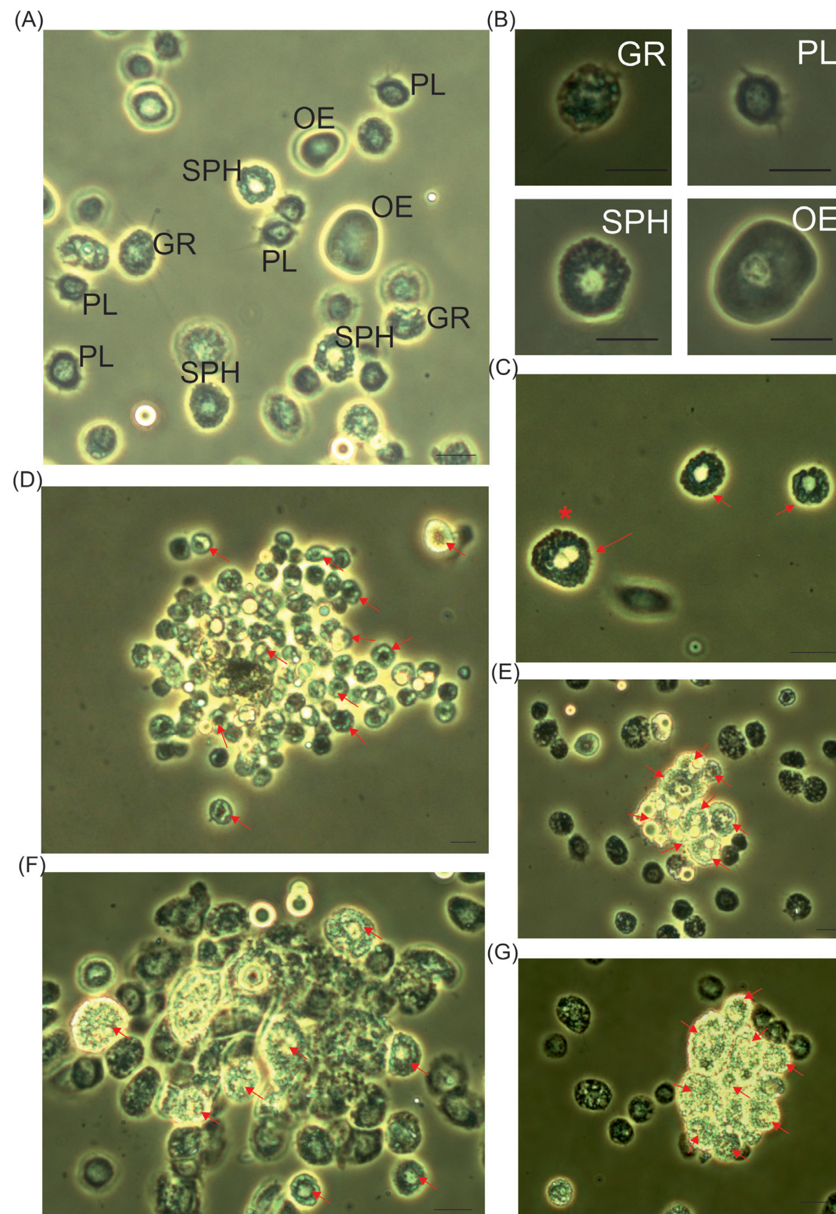

Figure 2. Participation of hemocytes in the *G. mellonella* cellular response to *A. niger* α-1,3-glucan. (**A** and **B**) Images of hemocytes present in the hemolymph of non-immunized *G. mellonella* larvae. PL—plasmatocyte, GR—granulocyte, SPH—spherulocyte, OE—oenocytoid. **(C)** Image of spherulocytes present in the hemolymph of *G. mellonella* larvae. An asterisk marks the spherulocyte most likely during mitotic division. **(D)** Representative image of the aggregate formed *in vivo* in *G. mellonella* hemocel 1 h after the α-1,3-glucan injection. **(D–G)** Images indicating a significant contribution of spherulocytes in the formation of aggregates *in vivo* after the α-1,3-glucan injection. The presence of spherulocytes is marked with red arrows. The images were obtained using a phase contrast microscope. Magnification 40× (A, B, C, F and G), 20× (D and E). Scale—10 μm.

hemocytes, granulocytes and plasmatocytes were present in the monolayers. It was noticed that the localization of apoLp-III depends on the type of hemocytes. Green fluorescence indicating the presence of apoLp-III was only visible in granulocytes (Fig. 5). In contrast, blue stained cell nuclei were visible in plasmatocytes and granulocytes. Particularly marked reduction of green fluorescence was observed in hemocytes 4 and 24 h after the immunization with α-1,3-glucan opposite to the larvae immunized with DMSO and non-immunized insects (Fig. 6A). This experiment additionally revealed changes in the number of hemocytes. A total of 15 min after the immunization with α-1,3-glucan, fewer cells were found in the preparations. The reduction in the number of hemocytes was noted especially in the case of plasmatocytes, which was confirmed by the analysis of the hemocytogram (Table 1and Fig. 1). A large number of strongly flattened plasmatocytes with long protrusions was observed in the monolayers formed from hemocytes collected 4 h after the α-1,3-glucan administration, which could indicate an increase in their adherent properties (Fig. 6A).

In contrast to the changes observed after the α-1,3-glucan immunization, indicating a change in the content of apoLp-III in hemocytes, green fluorescence was well detectable in the hemocytes after the administration of water or *A. niger* conidia at all the time points after the immunization (Fig. 6B). Small green granules that may indicate apoLp-III clusters within hemocytes were observed particularly after the injection of conidia.

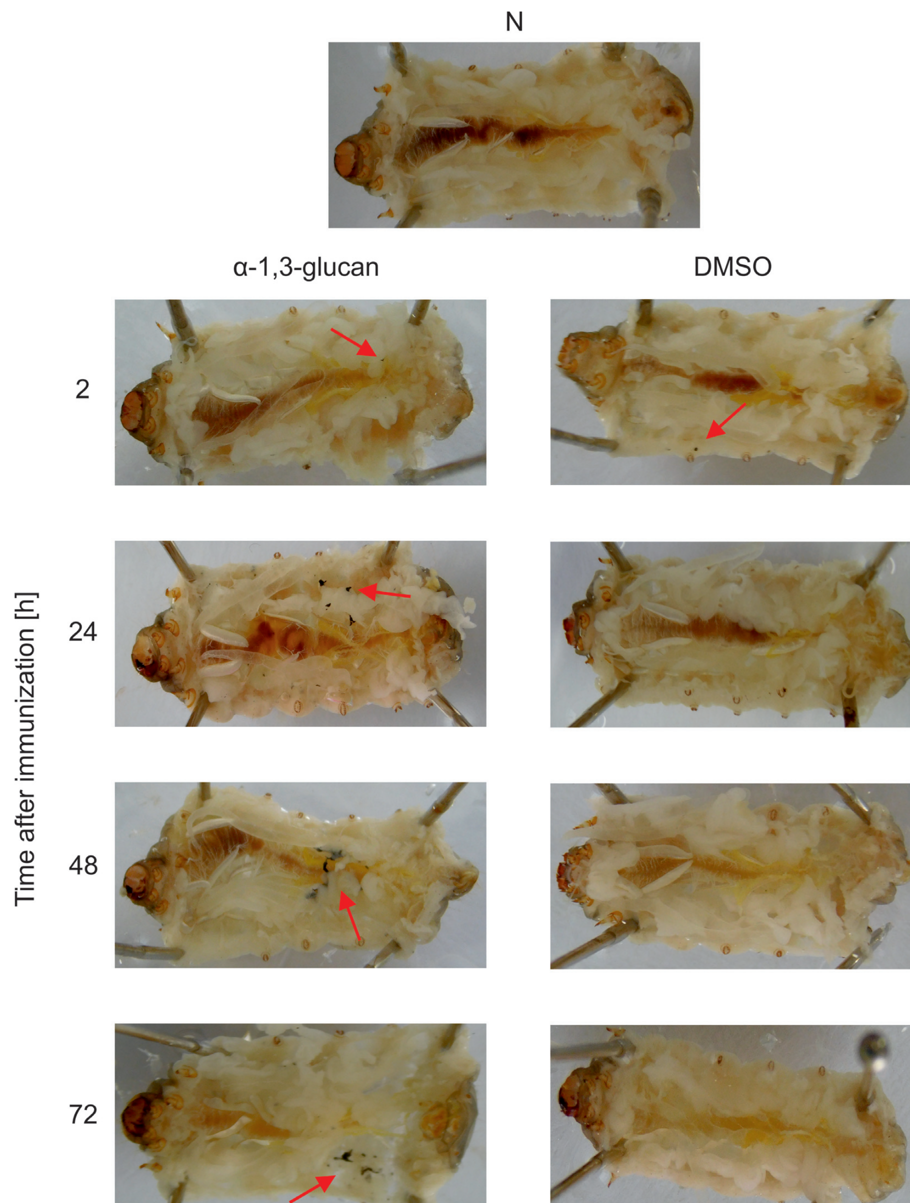

Figure 3. Macroscopic image of melanized nodules formed in *G. mellonella* hemocel after immunization with *A. niger* α-1,3-glucan. Representative images of caterpillars after dissection performed 2 , 24, 48 and 72 h after the immunization with α-1,3-glucan or DMSO are presented ($n = 3$). A non-immunized larva (N) is also shown. The melanized nodules are marked with red arrows.

Similarly to the administration of α-1,3-glucan, a decrease in the number of hemocytes was observed in the monolayers prepared from hemocytes collected shortly (0.25 h) after the immunization with *A. niger* conidia (Fig. 6B).

DISCUSSION

The insect immune response to fungal pathogens or their cell wall components can be assessed by analyzing humoral and cellular mechanisms. The most commonly determined parameters include the change in the number of hemocytes involved in cellular response as well as humoral response mechanisms, such as the induction of antimicrobial peptide synthesis and activation of the phenoloxidase system (Sheehan and Kavanagh 2018; Trevijano-Contador and Zaragoza 2019).

In the present study, the role of fungal α-1,3-glucan as a possible elicitor of cellular response was evaluated in the *G. mellonella* model host. The experiments showed that, like other Lepidoptera species, the dominant types of hemocytes in *G. mellonella* hemolymph are granulocytes and plasmatocytes, constituting 52% and 41% of all hemocytes, respectively (Falleiros, Bombonato and Gregório 2003; Stoepler *et al.* 2013; Vogelweith *et al.* 2016). Spherulocytes and oenocytoids accounted for 6.1% and 0.9% of all *G. mellonella* circulating hemocytes, respectively. The results obtained are consistent with data published by Ratcliffe, Mead and Renwrantz (1986), who estimated the percentage of granulocytes, plasmatocytes, spherulocytes and oenocytoids at 48%, 47%, 3% and 1%, respectively.

Changes in the total number of hemocytes are considered an important factor indicating the reaction of the insect immune system to recognition of a foreign body (Cytryńska *et al.* 2007;

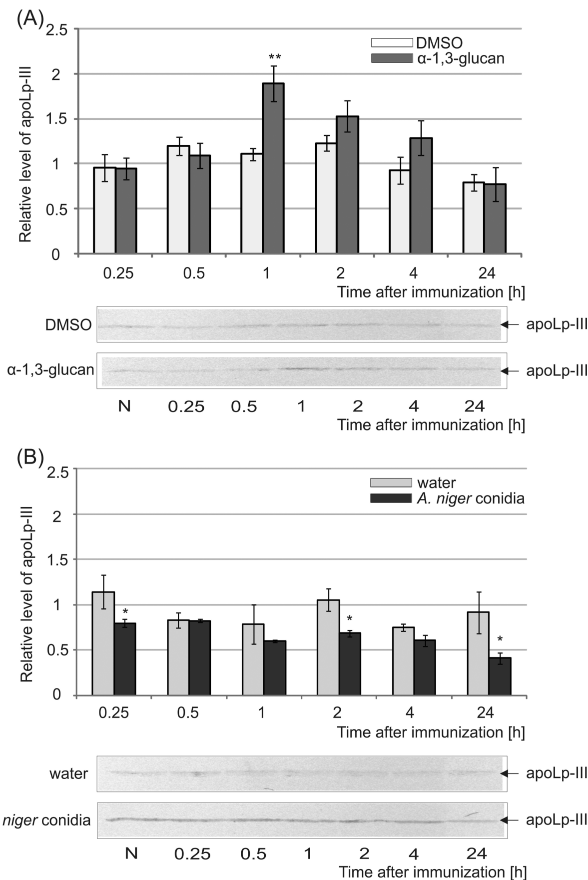

Figure 4. Apolipophorin III level in the hemolymph of *G. mellonella* larvae immunized with *A. niger* α-1,3-glucan **(A)** or *A. niger* conidia **(B)**. Hemolymph samples (3 μg) of non-immunized larvae (N) and samples collected 0.25–24 h after the immunization with α-1,3-glucan or DMSO and *A. niger* conidia (1×10^5) or water were separated by SDS-PAGE and transferred onto a PVDF membrane. After incubation with primary anti-apoLp-III and secondary antibodies conjugated with alkaline phosphatase, protein bands of approximately 18 kDa corresponding to the position of apoLp-III were detected (representative membranes are presented below diagrams). A densitometric analysis of the obtained bands was carried out, assuming the relative value of N = 1. The level of significance determined for the differences between the mean values from three independent experiments obtained for α-1,3-glucan and DMSO and for *A. niger* conidia and water is marked with asterisks: $^*P < 0.05$ and $^{**}P \leq 0.01$.

Carton, Poire and Nappi 2008; Dubovskiy *et al.* 2016). The involvement of hemocytes in *G. mellonella* response to *A. niger* α-1,3-glucan and conidia was reflected, among others, in the decrease in the number of plasmatocytes 0.25 h post-treatment. The decrease in the number of circulating plasmatocytes may indicate their involvement in the nodulation process, because plasmatocytes are the main component of nodules (Browne, Heelan and Kavanagh 2013; Mesa-Arango *et al.* 2013; Gago *et al.* 2014; Grizanova *et al.* 2018). As reported, a few minutes after bacterial immunization of *G. mellonella* and *Pieris brassicae* larvae as well as *in vitro* incubation of *G. mellonella* hemocytes with the entomopathogenic fungus *Conidiobolus coronatus*, aggregates were formed, which then led to formation of capsules (Gagen and Ratcliffe 1976; Boguś *et al.* 2007). Depending on the insect species and type of the foreign body, the process of nodule/capsule formation continues from 2–24 h after immunization (Carton, Poirie and Nappi 2008). In most cases, the nodule/capsule is clearly visible after 24 h; however, as suggested by Ratcliffe and Gagen (1977), 72 h are needed for full formation of this structure. The formation of hemocyte aggregates in *G. mellonella* hemolymph *in vivo* was noted at all the time points after the administration of *A. niger* α-1,3-glucan. In addition, the dissection of the larvae showed the presence of melanized nodules, clearly indicating activation of cellular immune response to this fungal cell wall component. Similarly, the presence of melanized capsules was found *in vitro* after incubation of *G. mellonella* hemocytes with *C. coronatus* (Boguś *et al.* 2007). A total of 6 h after immunization of *G. mellonella* larvae with *A. fumigatus* conidia, small-sized melanized structures were also visualized; they were clearly visible after 24 h (Sheehan, Clarke and Kavanagh 2018). A total of 4 h after the immunization with *A. niger* α-1,3-glucan, the *G. mellonella* plasmatocytes exhibited greater ability to adhere (hyper-spreading hemocytes). This may further indicate stronger activation of the cellular response after the administration of this component of the fungal cell wall. Similar observations were reported in the literature, e.g. 24 h after *G. mellonella* infection with *C. coronatus* and after *M. sexta* infection with *B. bassiana* (Dean *et al.* 2004; Boguś *et al.* 2018).

A characteristic phenomenon observed in animals is the increase in the number of immune cells in response to infection (Fuchs *et al.* 2010). At 2 h and 4 h after the immunization with α-1,3-glucan and DMSO, an almost 2-fold increase in the total number of hemocytes was noted, which resulted from an increase in the number of plasmatocytes and granulocytes, respectively. After the administration of *A. niger* conidia, an increase was noted after 1 h. It was mainly associated with the increase in the number of granulocytes. This may reflect their key role in insect immunity, since they are involved in hemolymph clotting in addition to being involved in cellular responses. It is worth noting that the increase in the number of hemocytes in *G. mellonella* hemolymph to a level of $1.6 \times 10^4/1\ \mu$L of hemolymph occurred most quickly after the administration of α-1,3-glucan. An increase in the number of hemocytes indicates a successful fight against the invading foreign body. Sheehan and Kavanagh (2018) showed a 25% increase in the number of hemocytes 6 h after immunization of *G. mellonella* with *Saccharomyces cerevisiae* β-1,3-glucan. In other studies, the total number of hemocytes almost doubled 24 h after immunization of *G. mellonella* larvae with 5 μg of β-glucan (Mowlds *et al.* 2010). Similarly, immunization of *G. mellonella* with *A. fumigatus* conidia led to an increase in the number of hemocytes (Sheehan, Clarke and Kavanagh 2018).

An increase in the number of spherulocytes was noted after the immunization of the *G. mellonella* larvae with *A. niger* α-1,3-glucan and administration of *A. niger* conidia. Although Ribeiro and Brehelin (2006) found that these cells do not take active part in the cellular response, our study revealed that these hemocytes participated in the formation of aggregates, which are the first stage in nodule formation. Interestingly, Ochiai, Niki and Ashida (1992) showed that β-glucan recognition proteins (βGRP) involved in the non-self recognition could be found in both *B. mori* granulocytes and spherulocytes. Studies on aphids *Acyrthosiphon pisum* demonstrated that spherulocytes could participate in hemolymph coagulation, energy accumulation and lipid transport (Schmitz *et al.* 2012).

Our results demonstrated an over 3-fold increase in the number of oenocytoids in the hemolymph of immunized *G. mellonella*. The increase may be explained by the fact that oenocytoids are responsible for the synthesis and release of phenoloxidase (PO), whose activity determines the proper course of the melanization process (Lavine and Strand 2002; Kanost, Jiang and Yu 2004; Eleftherianos and Revenis 2011). Interestingly, our previous study provided evidence on temporary inhibition of PO

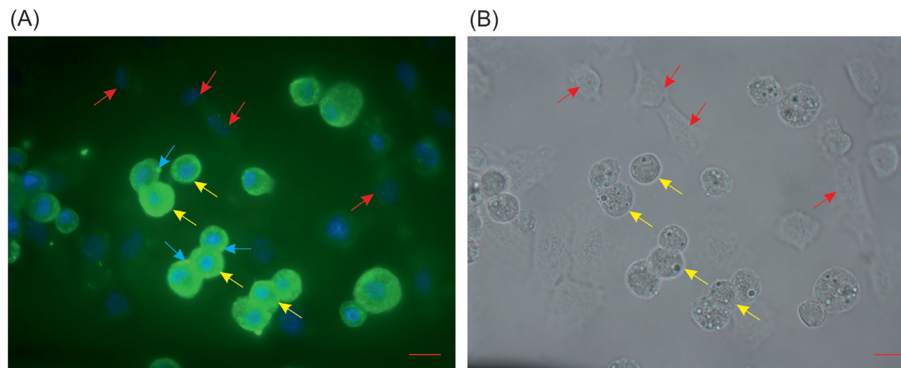

Figure 5. Detection of apolipophorin III in *G. mellonella* hemocytes. Hemocytes collected from non-immunized larvae were fixed on a slide and incubated with primary anti-apoLp-III and secondary FITC-labeled antibodies. Green fluorescence corresponds to the apoLp-III localization in the cells. Hemocyte nuclei were stained with Hoechst 32 258 (blue fluorescence). Images were taken using a laser scanning confocal microscope at a wavelength $\lambda = 495/517$ nm **(A)** and in transmitted light **(B)**. Red arrows—plasmatocytes, yellow arrows—granulocytes, blue arrows—apoLp-III localization. Magnification 100×. Scale–10 μm.

activity in the hemolymph of *G. mellonella* larvae after immunization with *A. niger* α-1,3-glucan and *A. niger* conidia (Stączek *et al.* 2020). Considering this, it is possible that the *G. mellonella* immune system increases the number of oenocytoids in order to ensure an adequate level of PO activity for further melanization processes.

Literature data indicate that the level of apoLp-III in *G. mellonella* hemolymph changes depending on the pathogen and on the time after immunization, and these changes are accompanied by changes in the level of this protein in hemocytes and the fat body (Zdybicka-Barabas and Cytryńska 2011; Zdybicka-Barabas *et al.* 2015; Stączek *et al.* 2018). In this study, a transient increase in the apoLp-III level in the *G. mellonella* cell-free hemolymph was noted 1 h after the immunization with *A. niger* α-1,3-glucan, which may have been connected with fast release from the fat body and/or hemocytes where it is synthesized and stored (Kim *et al.* 2004; Weers and Ryan 2006). In contrast, when *A. niger* conidia were administered, a decrease in the apoLp-III level in the hemolymph was noted which may have been caused by apoLp-III binding to the conidia surfaces. *G. mellonella* apoLp-III was demonstrated to bind to *Aspergillus oryzae* and *F. oxysporum* conidia (Zdybicka-Barabas *et al.* 2012). Similarly, a decrease in the apoLp-III level was found after immunization of *G. mellonella* with other filamentous fungi containing α-1,3-glucan in the cell wall, i.e. *F. oxysporum* and *A. fumigatus* (Schoffelmeer *et al.* 1999; Sheehan, Clarke and Kavanagh 2018).

Changes in the level of apoLp-III in *G. mellonella* hemolymph after immunization with various immunogens may be related to its role as a molecule transmitting the signal of infection to hemocytes and the fat body, which is associated with its interaction with lipids and lipophorin particles (Dettloff *et al.* 2001; Dettloff, Kaiser and Wiesner 2001; Niere *et al.* 2001). The present study analyzed the localization of apoLp-III in the *G. mellonella* hemocytes after the administration of *A. niger* α-1,3-glucan and conidia. The presence of apoLp-III was mainly found in *G. mellonella* granulocytes, which is consistent with the results presented by Dettloff, Kaiser and Wiesner (2001). A decrease in the apoLp-III level in hemocytes was noted especially 4 and 24 h after immunization with α-1,3-glucan. Kim *et al.* (2004) showed that, 0.5 h after immunization with *E. coli*, granulocytes containing apoLp-III inside the granules were degranulated. This phenomenon could explain the decrease in the apoLp-III level in the *G. mellonella* hemocytes in our study. On the other hand, presence of the apoLp-III clusters in *G. mellonella* hemocytes, especially 24 h after the immunization with *A. niger* conidia, may reflect the role of apoLp-III as a signal molecule, transmitting information about infection to hemocytes involved in the cellular response. The apoLp-III translocation to hemocytes may also explain the decrease in the apoLp-III level in *G. mellonella* cell-free hemolymph after the administration of *A. niger* conidia.

As mentioned earlier, the function of apoLp-III as a signaling molecule is performed in LDLp, i.e. after binding to apolipophorin I (apoLp-I), apolipophorin II (apoLp-II) and lipids (Dettloff and Wiesner 1998). For example, 0.25 h and 1 h after challenging *G. mellonella* larvae with *F. oxysporum* conidia, the content of apoLp-III in complexes with apoLp-I/apoLp-II in hemolymph increased 2- and 4-fold, respectively, compared to its levels in non-immunized insects (Stączek *et al.* 2018). Interestingly, apoLp-I and apoLp-II bound to *A. niger* α-1,3-glucan (unpublished results) and therefore their participation in the formation of LDLp with apoLp-III in hemolymph may be limited, which results in signal transmission disorders to granulocytes and, consequently, a lower level of apoLp-III in hemocytes. This may be another mechanism used by pathogens to delay or limit infection signal transduction and thereby block the host's immune response.

In summary, our results demonstrated the reaction of the cellular arm of *G. mellonella* immune system to *A. niger* α-1,3-glucan. Although differences in hemocytogram between α-1,3-glucan- and DMSO-immunized insects were statistically significant only for THC 0.5 h post-treatment and in selected time points for spherulocyte DHC, administration of α-1,3-glucan, in contrast to DMSO, induced considerably formation of hemocyte aggregates. Moreover, melanized nodules were found only in α-1,3-glucan-immunized larvae. These findings indicated: (i) recognition of α-1,3-glucan by *G. mellonella* immune system, (ii) activation of cellular immune response by this polysaccharide that manifested with nodule formation. An increased level of apoLp-III in the hemolymph 1 h post-treatment with α-1,3-glucan may additionally trigger the cellular response associated with formation of nodules. Thus, one can suggest that presence of α-1,3-glucan in fungal cell wall stimulates the host defense reactions and helps in fighting against fungal infection. On the other hand, apoLp-III was hardly detected in granulocytes of α-1,3-glucan-immunized larvae. Given apoLp-III role as a signaling molecule in immune response, one can suggest that this cell wall component delays other host defense reactions. These results, together with those reported in our previous study (Stączek *et al.* 2020), indicate that fungal α-1,3-glucan can exert two types of effects on the insect immune system. On the one

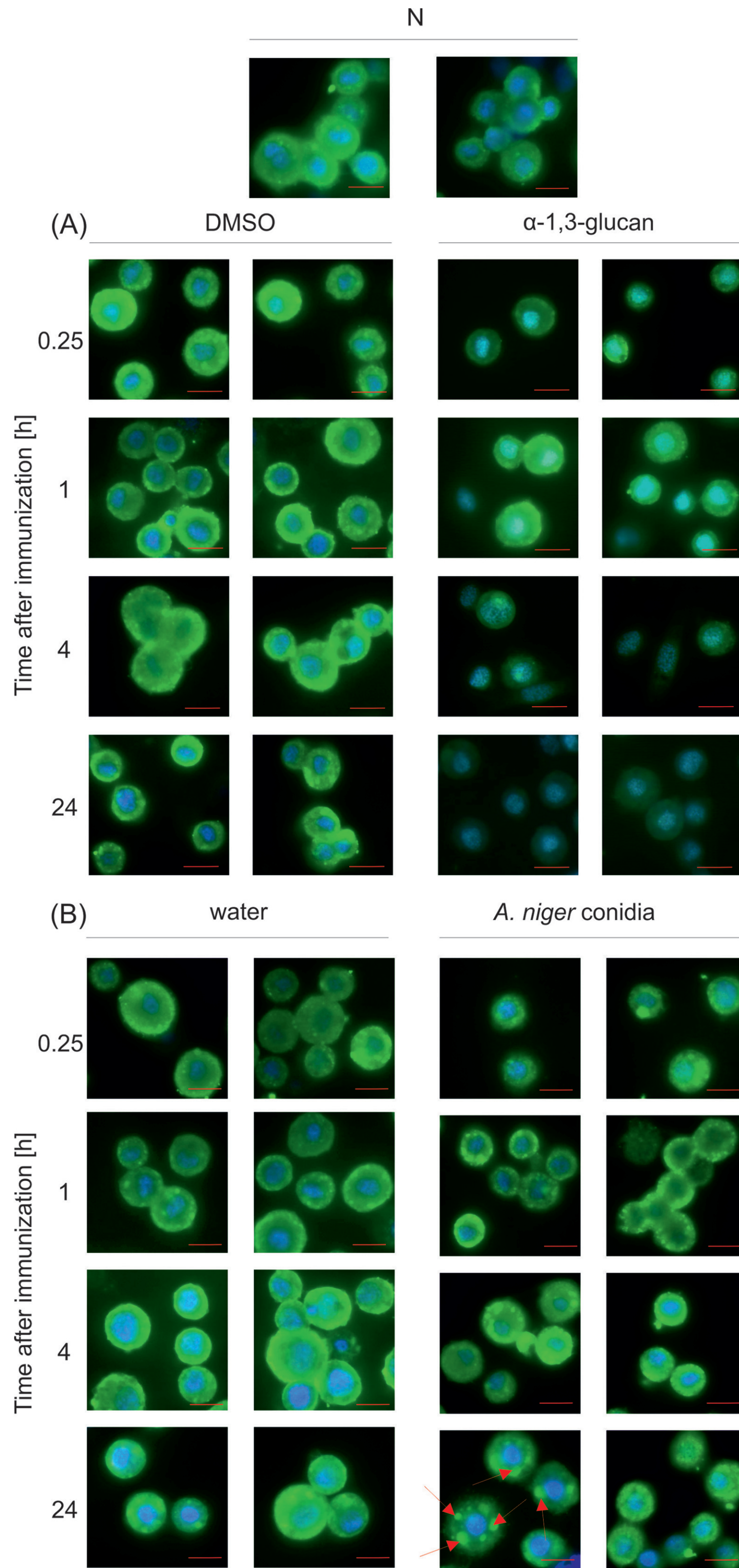

Figure 6. Immunodetection of apolipophorin III in hemocytes of *G. mellonella* larvae immunized with *A. niger* α-1,3-glucan **(A)** or *A. niger* conidia **(B)**. Hemocytes were obtained from non-immunized (N) larvae and 0.25, 1, 4 and 24 h after the immunization with α-1,3-glucan or DMSO and *A. niger* conidia or water. Hemocytes were then incubated with primary anti-apoLp-III and secondary FITC-labeled antibodies. Green fluorescence corresponds to the localization of apoLp-III in cells. Hemocyte nuclei were stained with Hoechst 32258 (blue fluorescence). Representative images taken with a laser confocal scanning microscope are presented. Arrows indicate apoLp-III clusters inside hemocytes. Magnification 100×. Scale—10 μm.

hand, it can induce defense processes. On the other hand, it can act as a virulence factor through limiting apoLp-III action and inhibition of PO activity, i.e. an enzyme engaged in early steps of immune response against pathogens. Therefore, the balance or imbalance between the two effects may determine the outcome of a fungal infection. Taking into consideration that exposure of α-1,3-glucan during germination of filamentous fungi is essential for development of germ tubes (Barreto-Bergter and Figueiredo 2014), a proper immune reaction to this component may be important for control of fungal infection. As *G. mellonella* is widely used as an alternative model in studies on host-fungal pathogen interactions, our results will help in understanding the role of α-1,3-glucan in immune response against fungal pathogens not only in insects but also in mammals, including humans.

AUTHOR CONTRIBUTIONS

S.S. and A.Z.-B. performed the experiments and analyzed data, M.P and A.W. purified and characterized α-1,3-glucan, S.S. and M.C. wrote the manuscript, S.S. A.Z.-B. and M.C. contributed to the conception and design of the research and revised and finalized the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of interest. None declared.

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