Full length article

Investigating the internal system of defense of Gastropoda *Aplysia depilans* (Gmelin, 1791): Focus on hemocytes

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A R T I C L E I N F O

A B S T R A C T

The internal defense system of mollusks represents an efficient protection against pathogens and parasites, involving several biological immune processes, such as phagocytosis, encapsulation, cytotoxicity, and antigenic recognition of self/non-self. Mollusks possess professional, migratory, and circulating cells that play a key role in the defense of the organism, the hemocytes. Several studies have been performed on hemocytes from different mollusks, but, to date, these cells are still scarcely explored. Different hemocyte populations have been found, according to the presence or absence of granules, size, and the species of mollusks studied. Our study aims to deepen the knowledge of the hemocytes of the gastropod *Aplysia depilans* using morphological techniques and light and confocal microscopy, testing Toll-like receptor 2, inducible nitric oxide synthetase, and nicotinic acetylcholine receptor alpha 7 subunit. Our results show two hemocyte populations distinguishable by size, and presence/absence of granules in the cytoplasm, strongly positive for the antibodies tested, suggesting for the first time the presence of these receptors on the surface of sea hare hemocytes by immunohistochemistry. These data help in the understanding of the immune system of this gastropod, providing additional data for comprehending the evolution of the defense response in metazoan phylogenesis.

1. Introduction

The phylum Mollusca belongs to the protostomes Lophotrocozoa, one of the three metazoan lineages, along with Ecdisozoa protostomes and deuterostomes [1]. Over the centuries, gastropods have colonized marine, brackish, and terrestrial environments, adopting herbivorous, carnivorous, endoparasitic, and symbiont-mediated chemoautotrophic lifestyles. Gastropods began developing more than 542 million years ago in the Precambrian and were already widespread in the Upper Cambrian (488–501 million years ago) [2]. There are currently between 40.000 and 150.000 species alive, with sizes ranging from less than 1 mm to about a meter [3]. Most gastropod species are shell-bearing and benthic, but some have abandoned the shell and others have adopted fully pelagic lifestyles. The soft and moist surface of the body of gastropods is protected by a mucus-producing ciliated epithelium, which provides an initial physical trap and barrier to pathogens [4].

Mollusks possess sophisticated and specific immune responses [5]. The study of molluscan immunology has revealed several defense mechanisms similar to those of vertebrates. The process to produce toxic reactive oxygen species toward pathogens by molluscan hemocytes may be homologous to that responsible for the cytotoxic respiratory burst of vertebrate phagocytes [6]. Gastropod mollusks have an internal defense system with a well-developed ability to discriminate between self and non-self. Recognition, as in all animals characterized by coelomic cavities, is carried out mainly by the cellular and humoral components of the hemolymph.

The open circulatory system of gastropods is populated by professional circulating and migratory cells, the hemocytes, which play a defense role through phagocytosis and encapsulation [7,8]. Hemocytes are also involved in wound healing, nerve repair, shell formation and restoration, and tissue remodeling [9]. They are responsible for the synthesis and release of several defense-related factors, such as reactive oxygen species, lectins and antimicrobial peptides, acetylcholine (ACh), and nitric oxide (NO). The vast majority of gastropods have two circulating hemocytes: spread hemocytes (SHs) and round hemocytes (RHs), with two peculiar morphologies [10]. In *Lymnaea stagnalis* (Linnaeus, 1758), a single-cell type with both morphologies has been described, where the round form is the young stage and the spread form the mature stage [11]. SHs produce agglutinins, bind Concavalin A, have phagocytic characteristics, stick to glass, and contain muramic acid. RHs show several traits, including non-phagocytic characteristics, a lack of glass adhesion, cytotoxic activity, and possess many markers typical of vertebrate T lymphocytes. Furthermore, SHs and RHs play a role in foreign tissue recognition: SHs can encapsulate and phagocytize foreign material, while RHs, with their natural killer (NK) activity, can act like cytotoxic T lymphocytes or vertebrate NK cells. Therefore, it can be concluded that RHs have characteristics similar to vertebrate T lymphocytes, while SHs are related to the macrophage family [12]. Moreover, phagocytosis maintains uniform characteristics from protozoa to mammals and humans [10].

Toll-like receptors (TLRs) have been recognized as fundamental players in the vertebrate innate immune response, implicated in the recognition of a wide range of bacterial, fungal, and viral molecular patterns. In many invertebrates, TLRs have been linked to pathogen detection and subsequent immune effector production through the activation of nuclear factor kappa of activated B cells (NF-κB) signal [13]. Genomic studies have also revealed that some invertebrates possess a very large repertoire of TLRs, as in the sea urchin *Strongylocentrotus purpuratus* (Stimpson, 1857) [14]. However, a similar number of membrane-bound receptors have been found in other deuterostomes and protostomes, including the pacific oyster *Magallana gigas* (Thunberg, 1793) [15].

Franchini et al. (1995) [16], identified an immunoreactive nitric oxide synthetase (NOS)-like protein in molluscan hemocytes. NO is involved in several cell signaling events and, depending on the cellular environment, can promote cell survival or cell death [17]. NO has been implicated in many other biological functions, such as development and neurotransmission [18], immune response [19–21], feeding behavior and chemosensory activation [22], olfaction [23], and stress response [24].

Nicotinic acetylcholine receptors (nAChRs) belong to a superfamily of ligand-bound pentameric ion channel proteins [25]. Mammals have a large number of nAChRs throughout their neural systems, where they control neurotransmitter release, cell excitability, and neuronal integration, all of which are vital for physiological homeostasis in relation to pain processing, immunological responses, and stress [26]. nAChRs are also found in non-nervous systems, such as muscles, macrophages, lymphoid tissues, and skin [26,27]. An important role for nAChR subunit alpha7 is the modulation of the inflammatory response, where perturbation of its expression *in vivo* significantly increases tumor necrosis factor release [28].

Our study aims to provide additional information on the internal defense system of *Aplysia depilans* (Gmelin, 1791), using immunohistochemistry to characterize hemocytes with TLR2, inducible NOS (iNOS), and nAChR-alpha7 antibodies for the first time, thus increasing the basic knowledge of gastropod immunity, which has not yet been extensively explored.

2. Materials and methods

2.1. Samples and tissue preparation

Samples of *A. depilans* were treated according to standard techniques for light microscopy. Sections 4 μm thick, obtained by rotary microtome (LEICA 2065 Supercut, Nussloch, Germany, Europe), were positioned on each slide. Then, slides underwent deparaffinization in xylene and rehydration using graduated alcohol solutions ranging from 100% to 30% alcohol to distilled water.

2.1.1. Histology

May–Grunwald Giemsa (MGG) (04–081802, BioOptica Milano S.p. A., Milan, Europe) were used to stain the sections [29,30]. Following the recommended procedure of the manufacturer, we deparaffinized the slides and rehydrated them in ethanol 70°. Then, we made a buffer solution with 20 ml of distilled water and 10 drops of concentrate buffer solution, applied it to the sections, and let it sit for 2 min. The slides were then drained, and 10 drops of May-Grunwald staining were mixed with 5 drops of buffer solution. Then, we used 10 ml of buffer solution to wash the slides. Next, we added 5 drops of Giemsa staining and 10 drops of buffer solution, and we let the solution to sit for 12 min. In the end, we differentiated it in ethanol 95° for 10 s, ethanol 100° for 30 s, and again ethanol 100° for 30 s. Finally, the slides were clarified in xylene and then mounted with Eukitt™.

2.1.2. Immunofluorescence

Slices already deparaffinized and rehydrated received a treatment 2.5% solution of bovine serum albumin (BSA). The sections were then incubated to anti-TLR2, anti-nAChR-alpha7, and anti-iNOS primary antibodies [31]. After one night, the incubation of secondary antibodies was performed. The slices were mounted using Vectashield (Vector Labs, Burlingame, CA, USA) to avoid photobleaching. Experiments were performed without the primary antibodies as a negative control. To ensure the immunopositivity of the primary antibodies, rat skin tissues were employed as a positive control [32,33].

A confocal laser scanning microscope with a META module (Zeiss LSM DUO, Carl Zeiss MicroImaging GmbH, Jena, Germany, Europe) was used to examine the slices. Two different laser types — helium-neon (543 nm) and argon (458 nm) — were used to create optical slices of fluorescence samples. With Zen 2011 (LSM 700 Zeiss software, Oberkochen, Germany, Europe), the visuals were enhanced. Each photo was taken as quickly as possible to avoid photodegradation. Using Adobe Photoshop CC version 2019 (Adobe Systems, San Jose, CA, USA), the digital images were inserted in a figure composite. The "Display profile" function of Zen 2011 was then used to assess the fluorescence intensity curves. Table 1 contains information regarding antibodies. Furthermore, "2.5D" function of Zen 2011 was used to create a graphic elaboration of the immunoreactivity of cells.

Table 1
Antibodies data.

Antibody	Supplier	Dilution	Animal source
TLR2	Active Motif, La Hulpe, Belgium, Europe.	1:100	Rabbit
nAchR-alpha7	Alomone Labs, Ltd., Jerusalem, Israel.	1:200	Rabbit
iNOS	Santa Cruz Biotechnology, Inc., Dallas, TX, U.S.A.	1:200	Mouse
Alexa Fluor 488 Donkey anti-Mouse IgG FITC conjugated	Molecular Probes, Invitrogen	1:300	Donkey
Alexa Fluor 594 Donkey anti-Rabbit IgG TRITC conjugated	Molecular Probes, Invitrogen	1:300	Donkey

2.1.3. Quantitative analysis

Ten sections and twenty fields per sample were examined to collect data for the quantitative analysis. The ImageJ software version 1.53e was used to evaluate the cell positivity. The image was converted to 8 bits, a "Threshold" filter was used to remove the background, and the plugin "Analyze particles" was then used to determine the number of cells. SigmaPlot version 14.0 was used to count the number of hemocytes that were positive for TLR2, nAChr-alpha7, and iNOS in each field (Systat Software, San Jose, CA, USA). The normally distributed data were analyzed with One-way ANOVA and a Student's t-test. The mean values and standard deviations (SD) of the data are displayed: **p ≤ 0.01, *p ≤ 0.05.

3. Results

The epidermis of *A. depilans* appears as a simple monostratified columnar epithelium. Among the epithelial cells, mucous unicellular glands are notable. The epidermis rests on a broad layer of loose connective tissue (the subcutis) which rests on a muscular layer. The epidermal epithelial cells are well-organized and compact, with a nucleus in a basal position (Fig. 1). Numerous hemocytes scattered in the subcutis and near the epidermis are evident. By MGG staining we highlighted two hemocyte populations, distinguishable by size and stain affinity. Hemocytes in pink, some granular and others with abundant cytoplasmic portion, lacking granularity can be seen. We identified granular and generally smaller in size hemocytes in blue (Fig. 1).

Immunohistochemically, by confocal microscopy, our results show hemocytes of different sizes, both strongly positive for TLR2. They are diffuse in the subcutis and especially immediately below the epidermis,

as already noted with histological staining (Fig. 2). TLR2 positivity is evident for the whole cell or only in some portions, suggesting a probable presence of granules in hemocytes with full positivity. However, this finding would need further investigation to be confirmed.

We also highlight the presence of hemocytes immunoreactive for iNOS and nAChR-alpha7. Hemocytes of both sizes are also positive for these two antibodies, mostly the granular ones. Our data show an occurrence of colocalization between these antibodies, as demonstrated by the "display profile" function of the confocal microscope, suggesting that these hemocytes express both receptors on their surface (Fig. 3).

Using the "2.5D" function of the confocal microscope, we developed a graphic elaboration that highlights the immunopositivity of *A. depilans* hemocytes to the antibodies tested (Fig. 4).

Furthermore, the number of hemocytes positive for TLR2 was similar to those positive for iNOS and nAChR-alpha7, as evidenced by quantitative analysis (Table 2).

4. Discussion

Mollusks have an efficient internal cellular response against invasion by pathogens or foreign substances [7]. The innate immunity of mollusks provides the first line of defense, including physical and chemical barriers, and humoral and cellular components with a non-specific response. The cellular immune response in gastropods relies on hemocytes [34], involved in cellular defense by phagocytosis and encapsulation of invading foreign particles and in the production of cytotoxic molecules [35]. They are also implicated in various physiological responses, such as tissue wound healing, shell formation, nutrient transport, metabolite excretion, and gas exchange [36]. *Aplysia* species have
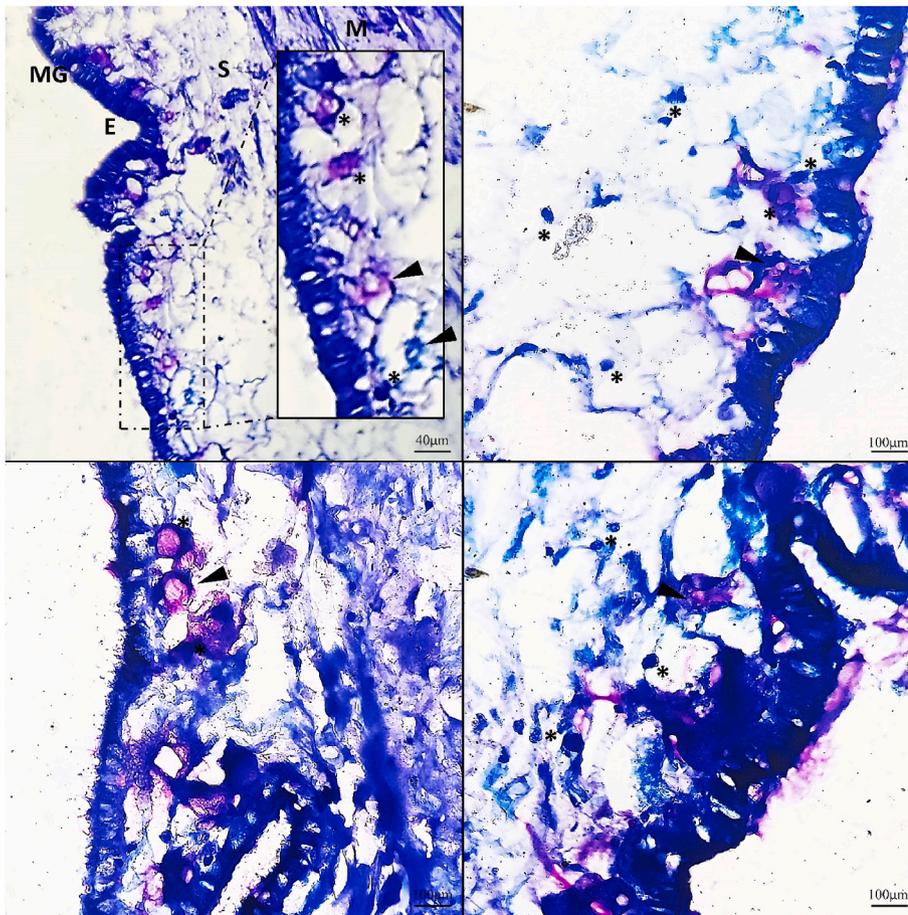

Fig. 1. Transversal section of *A. depilans* skin stained by MGG, 40x and 100x. A simple monostratified epithelium with columnar cells and mucous glands is evident. A subcutis of loose connective tissue under the epidermis and a muscular layer can be distinguished. Hemocytes in pink and blue are distinguishable, some granular (*), others with a large cytoplasmic space lacking granules (arrowheads). The inset shows an enlargement of one field. E = epidermis; MG = mucus glands; S = subcutis; M = muscular layer.

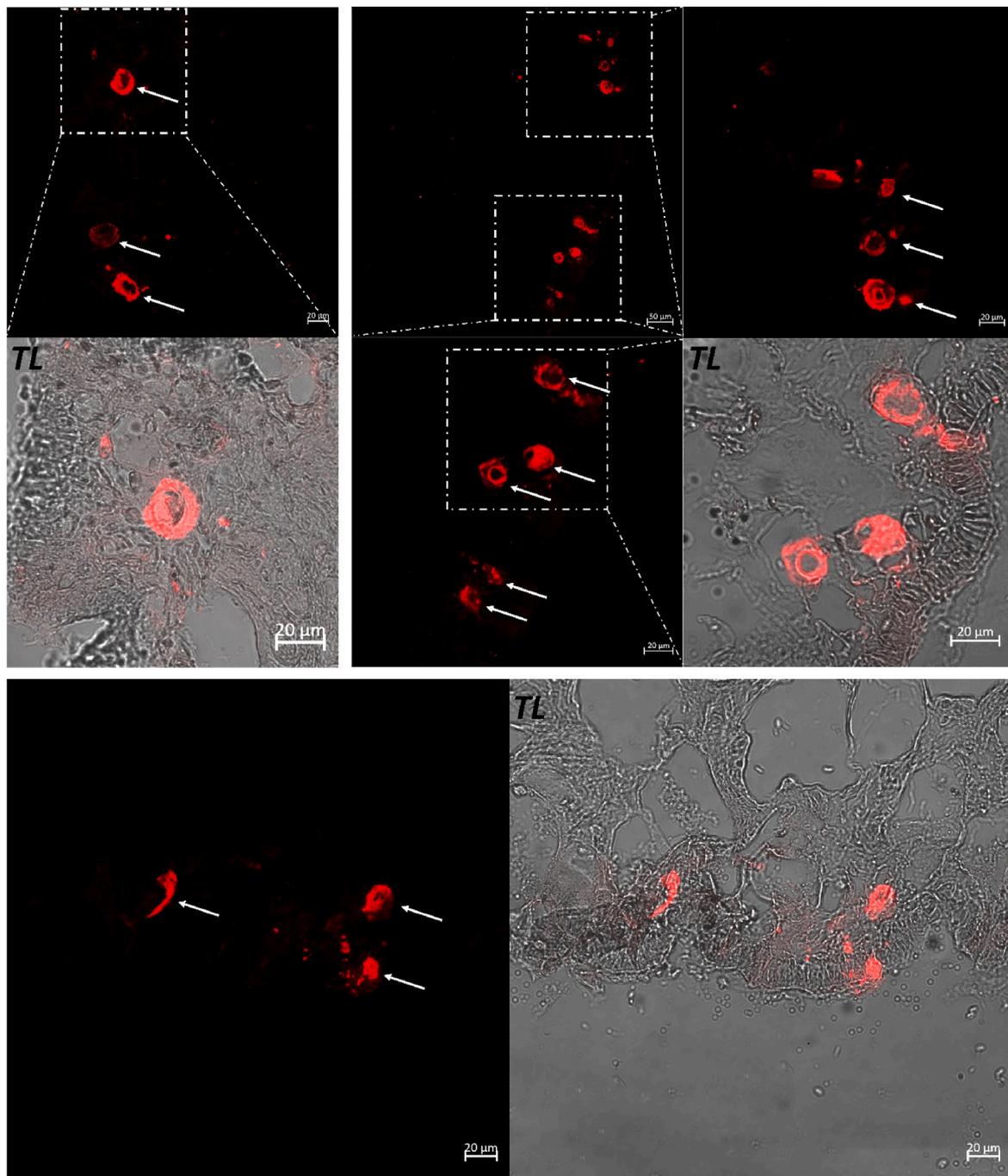iNOS and nAChR-alpha7, as evidenced by quantitative analysis (Table 2).

Fig. 2. Transversal section of *A. depilans* skin, confocal microscopy, TLR2 immunoreaction. Hemocytes of different sizes, spread in the subcutis and near the epidermis strongly positive for TLR2 are evident (arrows). Insets provide a magnification of the field. Transmitted light (TL) images help to understand the localization of hemocytes in the tissue.

been widely used in neurobiology, toxicology, and ecology for their chemical defense and bioactive molecules. Despite their large presence in ecosystems and their relevance as model animals, their internal defense system has been studied to a limited extent.

Hemocytes in mollusks can be classified into two main types at least: granulocytes and agranular hyalinocytes [37]. However, there is variability in the number of hemocyte lineages described in different mollusks [38]. Granulocytes of *Biomphalaria glabrata* (Say, 1818) are characterized by extensive pseudopod production in all directions. These cells are adhesive and measure about 24 μm in their longest and 16 μm in their widest dimensions. They constitute about 87% of the hemocyte population [39]. Hyalinocytes are smaller in size than

granulocytes and are generally spherical or slightly oval. Granules are sparse, and the only pseudopods formed are lobular and not extensive. Hyalinocytes measure about 6.9 and 6.6 μm and constitute 13% of the hemocyte population [39]. Only one type of hemocyte, the equivalent of the granulocyte, has been described in *Viviparus ater* (De Cristofori and Jan 1832). This cell is characterized by irregular shape, pseudopod formation, round or oval nucleus, and abundant cytoplasm with inclusions and numerous vacuoles [40]. Three distinct hemocyte types from the sea hares *Aplysia kurodai* (Baba, 1937), *Aplysia juliana* (Quoy & Gaimard, 1832), and *Aplysia oculifera* (Adams & Reeve, 1850) have been described and classified as granulocytes, hyalinocytes, and blastiform cells using flow cytometry and light microscopy. While hyalinocytes
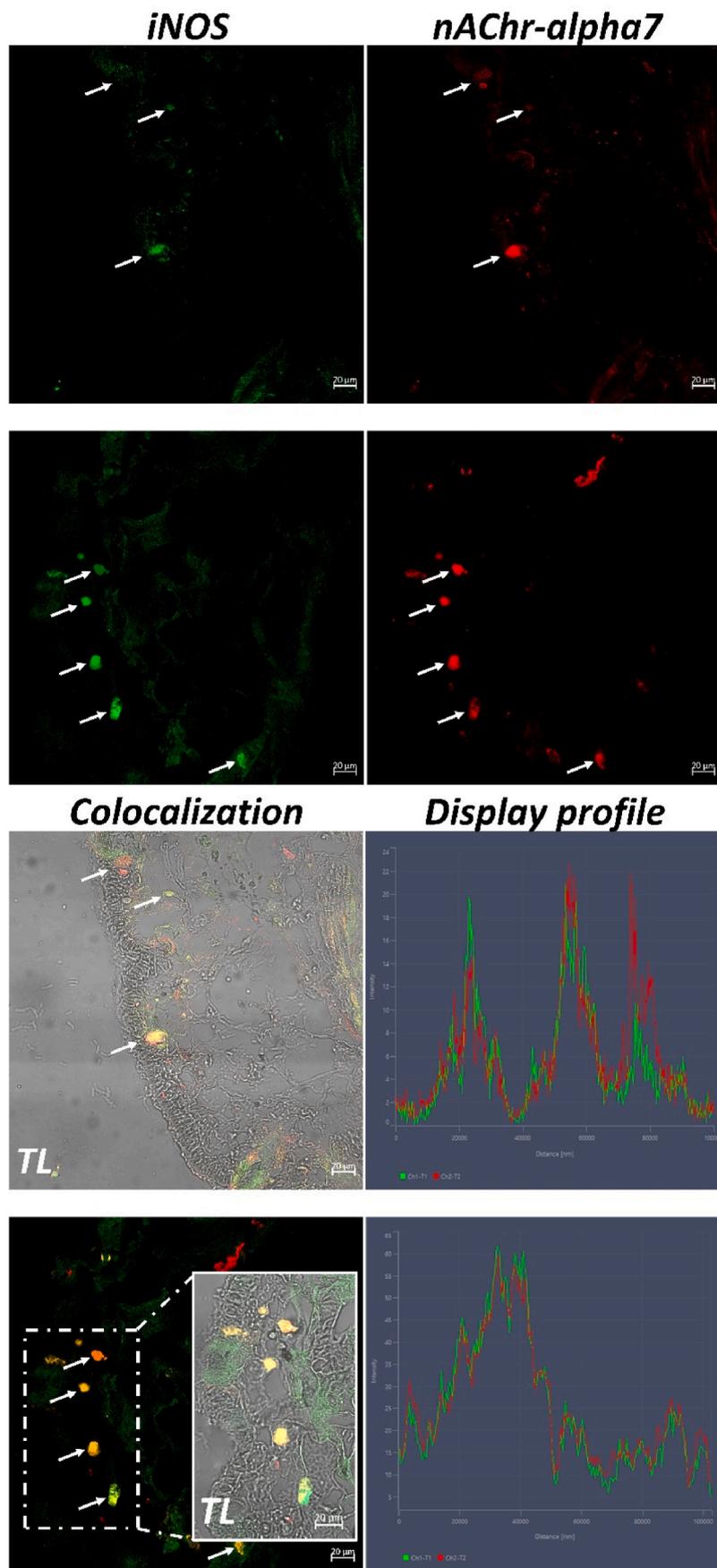
Fig. 3. Transversal section of *A. depilans* skin, confocal microscopy, iNOS, and nAchR-alpha7 immunoreaction. Hemocytes of different sizes near the epidermis and in the loose connective tissue are evident and immunopositive to iNOS (green) (arrows) and nAchR-alpha7 (red) (arrows). Antibody colocalization is evident (arrows), as also demonstrated by the "display profile" function. Insets provide field magnification, and transmitted light (TL) helps to localize immunoreactive hemocytes in the tissue.
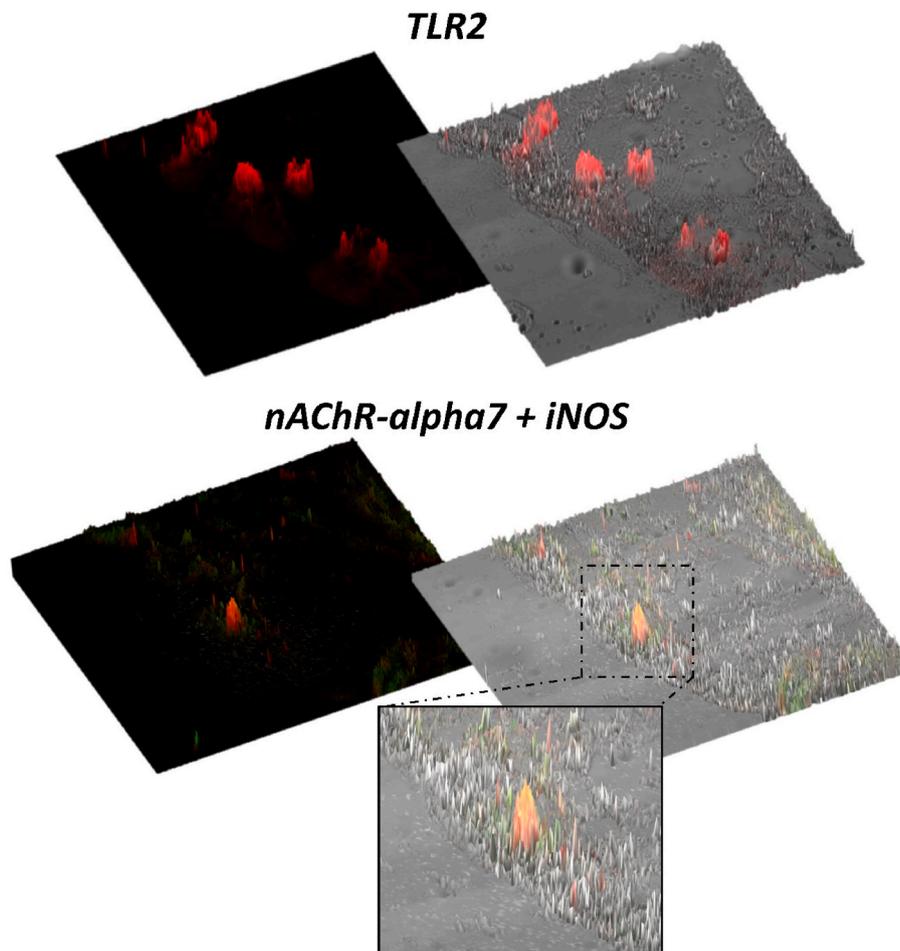
TLR2

nAChR-alpha7 + iNOS

Fig. 4. Graphical representation by "2.5D″ confocal microscope function of the positivity of hemocytes to the antibodies tested. Inset provides a field magnification.

Table 2
Quantitative analysis data. (mean values $\pm$ standard deviation) ($n=3$).

	No. of hemocytes[a]
TLR2	381.56 ± 65.32**
iNOS	337.29 ± 54.10*
nAChR-alpha7	349.21 ± 42.53*
iNOS + nAChR-alpha7	324.84 ± 39.16**

**p $\leq$ 0.01, *p $\leq$ 0.05 –
 [a] One-way ANOVA and Student's *t*-test were used to compare the means.

lacked granules in the cytoplasm, granulocytes were distinguished by their abundance. Small, round, blastiform cells with extremely thin cytoplasm were seen. Granulocytes and hyalinocytes are involved in phagocytosis and the generation of reactive oxygen species, according to flow cytometry. Despite the species, flow cytometry and microscopy revealed that the hemocyte types and their functions were the same [36]. Moreover, three cell types were described based on size, ultrastructure, and internal complexity. In *B. glabrata*, three subpopulations of hemocytes have been described: large hemocytes, medium hemocytes, and small hemocytes [41]. Asymmetrical shape, low nucleus/cytoplasm ratio, cytoplasm with numerous mitochondria, thick glycogen particles, and conspicuous extensions are features of large hemocytes. Medium hemocytes have a higher nucleus/cytoplasm ratio and are more symmetrical. Small hemocytes are lacking secretory granules, have a high nucleus-to-cytoplasm ratio, and are filled with organelles. Using flow cytometry, light microscopy, and transmission

electron microscopy (TEM), three different forms of circulating hemocytes—hyalinocytes, agranulocytes, and granulocytes—were identified in *Pomacea canaliculate* (Lamarck, 1822) [42]. A study by Martin et al. (2007) analyzed the hemocytes of the opisthobranch *Aplysia californica* (Cooper, 1863) and the vetigastropoda *Megathura crenulata* (Sowerby I, 1825). They exhibited a single type of hemocyte. The most striking difference between the hemocytes of the two mollusks is related to the morphology of the cells: the hemocytes of *A. californica* are spherical cells with 2–3 wing lamellipodia, while those of *M. crenulata* are simply ovoid, they have a central nucleus and spaces in the cytoplasm that absorb the neutral red dye. None of the hemocytes from *A. californica* or *M. crenulata* had the appearance of granulocytes described for other molluscan species [43].

According to these studies, our data in *A. depilans* show histologically large, nearly spherical, asymmetrically shaped hemocytes stained pink-red with MGG staining. Under light microscopy, we distinguished two hemocyte populations. The second population shows smaller, roundish or ovoid hemocytes, stained blue-violet with MGG, following the stain properties, that highlights eosinophilic granulocytes in pink-red, and basophils, neutrophils, and monocytes in blue-violet [44]. Consistent with previous studies [36,43] some hemocytes appear with abundant cytoplasm and agranulated, while others are infilled with granules. Further investigation is needed on this finding to substantiate our morpho-functional hypothesis.

TLRs, evolutionarily preserved receptors [45], play a key role in the innate immune response by directly recognizing a plethora of pathogens (typically bacteria, viruses, and fungi) or factors evidencing their presence and transducing signals to the immune cells [46–48]. In previous studies, we have immunohistochemically identified TLR2 in several

metazoans: protochordates tunicates *Styela plicata* (Lesuer, 1823) [49], chordates cyclostomes *Eptatretus cirrhatus* (Forster, 1801) [50], chordates chondrichthyes *Scyliorhinus canicula* (Linnaeus, 1758) [51], chordates osteichthyes *Carassius auratus auratus* (Linnaeus, 1758) [52, 53], *Polypterus senegalus* (Cuvier, 1829), *Lepisosteus oculatus* (Winchell, 1864), *Clarias batrachus* (Linnaeus, 1758) [30], chordates mammal *Stenella coeruleoalba* (Meyen, 1833) [54]. TLR is expressed in hemocytes more than in other tissues and its transcription is upregulated significantly on bacterial and viral challenges. Mollusks appear to maintain a vertebrate-like TLR signaling cascade. Single cysteine cluster (scc) and multiple cysteine cluster (mcc) TLRs, two distinct groups of coexisting structurally dissimilar receptors, are present in both bivalves and gastropods [55]. TLRs, however, are recognized to play a critical role in mollusks immune response mediation [56]. mccTLRs have been linked to antibacterial and antiviral responses in abalone *Haliotis* (Linnaeus, 1758) [57] and a TLR from the snail *B. glabrata* was found to be closely associated with infection-resistant specimens of the trematode *Schistosoma mansoni* (Sambon, 1907) [58]. A study by Kron et al. (2022) shows that the *A. californica* genome encodes for essentially all major components of the canonical TLR signaling cascade. Interestingly it encodes for many more TLRs than in vertebrates [59]. Pila et al. characterized TLR in *B. glabrata*, demonstrating its relevant role during the immune response. This study provided significant evidence that BgTLR is involved in the regulation of parasite infection in this snail [58]. TLRs have been characterized in several other mollusks such as disc abalone *Haliotis discus* (Reeve, 1846), Hawaiian bobtail squid *Euprymna scolopes* (Berry, 1913), zhikong scallop *Chlamys farreri* (Jones & Preston, 1904), soft clam *Mya arenaria* (Linnaeus, 1758), mussels, and oysters [60–63]. All molluscan TLRs that have been functionally evaluated may be involved in antimicrobial immune responses. Phagocytosis along with cytotoxicity and encapsulation are the main hemocytic responses induced by recognition receptors in the snail [64]. Our results, consistent with the aforementioned studies, show for the first time hemocytes of *A. depilans* immunopositive to TLR2, indicating the presence of this receptor on the hemocyte surface and confirming the role of these cells in *Aplysia* immune responses.

Zahoor et al. showed that NO levels in *B. glabrata* hemocytes affected by the parasite *S. mansoni* [65]. Hemocytes from resistant snails had a significantly greater increase in NO production 5 h after contact with the parasite. Similar activities were also observed in *L. stagnalis* hemocytes [66]. In addition, Recombinant human interleukin-2 (IL-2), a known NO stimulant for mammalian macrophages, has been found to considerably increase NO production in *Mytilus galloprovincialis* (Lamarck, 1819) hemocytes [67]. Walker et al. showed that schistosome-sensitive *B. glabrata* snail hemocytes produce more intracellular NO under basal conditions than resistant snail hemocytes [68]. Furthermore, the expression of NOS in hemocytes of *Crassostrea gigas* (Thunberg, 1793) has been demonstrated [69].

Our data show hemocytes of *A. depilans* reactive for iNOS by confocal microscopy, confirming the role of this neuro-immunomodulator in the internal defense system of this gastropod. Moreover, the hemocytes were also positive for nAchR-alpha7. The two antibodies tested are perfectly colocalized, as also demonstrated by the "display profile" function of the confocal microscope. This confirms that *A. depilans* hemocytes are involved not only in the recognition mechanisms but also in macrophagic activities. Additionally, confocal microscopy revealed differences in hemocyte morphology. The presence of nAChR-alpha7 on hemocytes of *A. depilans*, as our data suggest, finds good support in previous studies. The presence of the nicotinic receptor has been demonstrated in *A. californica* neuroendocrine cells [70]. In addition, nAChRs were identified in all vertebrates and invertebrates studied. In mollusks, 12 nAChR subunit genes were detected in the snail *L. stagnalis* [71], and two genes were identified in the bivalve *C. farreri* [72]. In the bivalve mollusk *C. farreri*, all organs, including the adductor muscle, mantle, gills, hepatopancreas, kidney, and gonad, expressed two nAChR genes, and their expression increased in response to Lipopolysaccharides

(LPS) and Tumor Necrosis Factor (TNF)-α stimulation, suggesting an immunomodulatory function [72,73]. A genomic study by Jiao et al. (2019), revealed a massive expansion and diversification of nAChR genes in mollusks. Gene family analysis was conducted with amino acid sequences deduced from all protein-coding genes of nine species: *C. gigas*, *Pinctada fucata* (Gould, 1850), *Lottia gigantea* (Sowerby I, 1834), *A. californica*, *Octopus bimaculoides* (Pickford & McConnaughey, 1949), *Helobdella robusta* (Shankland, Bissen & Weisblat, 1992) *Capitella teleta* (Blake, Grassle & Eckelbarger, 2009), *Danio rerio* (Hamilton, 1882) and *Homo sapiens* (Linnaeus, 1758). Through comparative genomic analysis, huge expansions of nAChR genes were found in mollusks. Some molluscan nAChR genes are expressed before the development of the nervous system, whereas others are connected to immune and stress responses [74]. Additionally, Liu et al. showed that hemocytes can inhibit the immune response in oysters by releasing acetylcholine and norepinephrine through the nAChR. This study shows that *C. gigas* hemocytes can release acetylcholine and that this operates a negative control on the hemocytes themselves in phagocytotic activity, via nAchR-alpha7 on the surface of the hemocytes [75].

In conclusion, our study provides a deeper insight into the internal defense system of *A. depilans*, probably highlighting two different hemocyte populations, distinguishable for size and presence/absence of granules. Furthermore, by confocal microscopy, these hemocytes were characterized for the first time with TLR2, iNOS, and nAChR-alpha7, confirming the involvement of these cells in the immune responses of recognition, phagocytosis, and cytotoxicity of this gastropod. Although the antibodies tested have already been validated in *Aplysia* by previous investigations [59,70,76], further studies, that can corroborate our data, are needed to advance the understanding of molluscan defense mechanisms. However, the results obtained from our research may expand the knowledge of gastropod immunity, to date still little known. Moreover, the identification of different hemocytic populations can help provide new data for taxonomy, classification, and recognition both among sea hare species and, more generally, in gastropods.

Institutional review board statement

Ethical review and approval were waived for this study because no experimental procedures were applied to samples.

Informed consent statement

Not applicable.

CRediT authorship contribution statement

Alessio Alesci: Conceptualization, Formal analysis, Investigation, Data curation, Writing – original draft, preparation, Writing – review & editing. **Angelo Fumia:** Formal analysis, Investigation, Data curation, Visualization. **Marco Albano:** Investigation, Data curation. **Emmanuele Messina:** Investigation, Data curation. **Roberta D'Angelo:** Investigation, Data curation. **Angelica Mangano:** Investigation, Data curation. **Anthea Miller:** Investigation, Data curation. **Nunziacarla Spanò:** Investigation, Data curation, Writing – review & editing. **Serena Savoca:** Investigation, Data curation, Writing – review & editing. **Gioele Capillo:** Investigation, Data curation, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Not applicable.

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