



Integration of molecular and cellular immunity in sea cucumbers: a comprehensive review

Noé Wambreuse^{*} , Guillaume Caulier¹ , Jérôme Delroisse¹ , Igor Eeckhaut^{*}

Biology of Marine Organisms and Biomimetics Unit, Institute for Biosciences, University of Mons, Mons, 7000, Belgium

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ABSTRACT

As non-chordate deuterostomes, echinoderms constitute a key phylum for comparative immunology studies related to vertebrates. Among them, sea cucumbers (Holothuroidea) have attracted growing scientific interest, both for aquaculture applications and for their potential as sources of bioactive compounds. Over the past decades, research on the sea cucumber immune system—especially on immune-related genes—has expanded considerably. This progress has been largely driven by efforts to improve the health and disease resistance of the aquacultured species *Apostichopus japonicus*. In contrast, cellular immunology has received comparatively less attention, and modern molecular approaches have rarely been applied to investigate the functions of sea cucumber immune cells, known as coelomocytes. Although numerous coelomocyte types have been described over the last century, their classification still solely relies on morphological criteria, which likely masks an important functional diversity within these cell populations. This review compiles the current knowledge on both the molecular and cellular components of the sea cucumber immune system, thereby highlighting the existing gaps between these two levels of investigation. The principal immune mechanisms are presented together with their associated molecular and cellular actors. In addition, recent advances linking molecular and cellular immunity are discussed, and future directions are also proposed for the molecular characterisation of coelomocytes at single-cell-type resolution. Overall, this review provides a framework for bridging molecular and cellular immunology in sea cucumbers—two fields that remain largely disconnected—and offers an updated overview of immune components in these economically and ecologically important marine invertebrates.

Introduction

Sea cucumbers (holothuroids) are a group of marine invertebrates that belong to the echinoderms, a non-chordate deuterostome phylum [1]. Since the pioneering work of the Nobel Prize winner Elie Metchnikoff, whose discovery of phagocytosis was inspired by cellular aggregation in sea star larvae [2,3], this phylum has become an important model in comparative immunology [4–6]. Nonetheless, the five classes that compose the echinoderms were not equally studied: while sea urchins were positioned as a central model organism, sea stars and sea cucumbers received less interest, with ophiuroids and crinoids remaining largely understudied [6]. Among echinoderm classes, holothuroids have many unique characteristics, the most conspicuous of which is their secondary bilateral symmetry after metamorphosis [1]. They also display the greatest diversity of immune cell morphotypes, offering a good model to explore immune cell diversification in echinoderms [4,6].

In addition, some species have a high commercial value due to their

exploitation and trading as trepang on the Asian market [7,8]. To respond to the increasing demand for sea cucumber products, aquaculture projects have been developed around the world, with two main species being exploited on a large scale: the Asian temperate species *Apostichopus japonicus* and the Indo-Pacific tropical species *Holothuria scabra* [7,9]. However, sea cucumber aquaculture faces numerous diseases that can drastically reduce production and lead to significant economic losses [9–11]. The most commonly reported is the Skin Ulceration Syndrome (SUS), characterised by a gradual degradation of the integument as well as progressive loss of vital functions [10–13]. Several factors have been identified in the development of this syndrome, including abiotic factors [14,15] and biotic factors such as pathogenic bacteria or viruses [16,17]. Nevertheless, disease outbreaks are generally thought to result from the combined action of several environmental and biological factors rather than from a single causative agent [10,12,18]. Beyond their aquaculture importance, sea cucumbers also represent a valuable source of bioactive compounds with promising

^{*} Corresponding authors.

E-mail addresses: noe.wambreuse@umons.ac.be (N. Wambreuse), igor.eeckhaut@umons.ac.be (I. Eeckhaut).

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pharmaceutical applications. They contain a wide range of biologically active molecules, including anticancer, antioxidant, and antibacterial compounds [19–21]. In particular, immune-related molecules such as antimicrobial peptides are considered promising candidates for the development of new therapeutic agents [22].

In most metazoans, the immune system operates through both molecular and cellular components [23–25]. Molecular defences include the production of several molecules that have a broad function in immune response, including, among others, recognition of pathogens and damage-associated molecular patterns (PAMPs and DAMPs, respectively), pathogen lysis, inflammation and promotion of cellular aggregation [26]. In contrast, cellular defence relies on the direct activity of immune cells through mechanisms such as phagocytosis or encapsulation [23]. In practice, these two levels of immune defence are deeply interconnected: immune molecules are produced by immune cells while immune cells themselves depend on these molecules to mediate and coordinate their functions, notably through signalling pathways such as cytokine-mediated communication. Nevertheless, immunological studies traditionally distinguish humoral response (*i.e.*, encompassing mechanisms relying on simple molecules) and cellular response (*i.e.*, mechanisms achieved by immune cells *per se*) [26].

In holothuroids, cellular immunity is ensured by specialised immune cells called coelomocytes [4,27]. These cells are widely distributed throughout the organisms but are particularly abundant in the coelomic fluids, in which they freely circulate. Owing to their remarkable morphological diversity, different types of coelomocytes have been described during the last century [4,27,28]. However, unlike in sea urchins, research on holothuroid immune cells has continued to rely predominantly on morphological observations, while molecular and functional approaches have been used far less frequently to characterise coelomocytes [6,29]. As a result, specific roles and functions associated with many coelomocyte types remain poorly understood. In recent years, several key studies have sought to clarify these specific functions and update their classification so that it better reflects their role in immunity (*e.g.*, [30–33]). Although these studies offer a perspective on establishing a functional classification of coelomocytes, the functions of most cell types remain elusive and molecular evidence is still lacking to validate most functional hypotheses.

In contrast to cellular immunity, the number of molecular actors of the immunity being characterised has increased substantially over the past two decades ([34]; present study), largely driven by research on the aquacultured species *A. japonicus*. Nevertheless, these molecular components are most often investigated individually, without being integrated into a broader cellular context. Moreover, when the expression of immune-related genes or molecules is reported in coelomocytes, these cells are generally treated as a single homogeneous functional entity. Such an approach overlooks the likely diversity of gene expression patterns and functional specialisations among the different coelomocyte populations, thereby limiting our understanding of the cellular complexity underlying sea cucumber cellular immunity. In parallel, the rapid and continuous increase in the number of identified immune genes has made it difficult to have a clear overview of the molecular immunology in sea cucumbers. The last major attempt to provide such a comprehensive overview was achieved by Xue et al. in 2015 [34]. Since then, the number of studies has dramatically increased, reflecting the need for an updated integrative review.

As a reflection of the fragmentation between molecular and cellular levels, to date, only two studies have paired immune gene expression profiling with corresponding coelomocyte functional assays in the same experimental framework [33,35]. The current literature therefore highlights a disconnect between cellular and molecular immunology, with few studies integrating molecular actors to characterise the functions of specific cell types, and gene functions being rarely interpreted within their cellular context.

This review provides a comprehensive and updated overview of the current state of knowledge on the holothuroid immune system. It

primarily aims to characterise the actors of immunity in holothuroids in the light of recent advances in the field. It mainly focuses on molecular and cellular actors, although other levels of immune organisation, including the role of the organism as a whole and its associated microbiota, are also briefly addressed. In addition, the major mechanisms of the holothuroid immune response are presented. Finally, new advances and future directions in linking molecular and cellular immunology are discussed. In this way, it ultimately aims to link the different levels of organisation that are complementary in the immune response in holothuroids but currently disconnected in the literature, and provide a comprehensive overview of the actors of immunity in these economically valuable marine organisms.

Review strategy and methodology

This review aims to summarise key findings on the immune system of sea cucumbers, with a focus on recent literature. Searches were performed between January 2025 and March 2026 on three databases: Web of Science, ResearchGate, and Google Scholar. While most of the review can be considered a narrative review, for the section on molecular actors of immunity, a systematic search was conducted for articles published between 2006 and 2026. Different “key words” searches were conducted for screening (i) studies that characterised functionally immune genes and (ii) studies that employed omics tools on coelomocytes.

Search terms for immune genes included combinations of “sea cucumber” with: “immune gene”, “immune protein”, “pathogen recognition receptor”, “humoral factor”, “inflammation”, and “apoptosis”. Molecular actors identified in Xue et al. [34] were also individually checked. Genes reported only in a single study and whose immune function was inferred solely from stress-induced differential expression were excluded to retain only functionally validated immune genes. However, some genes sharing homology with key immune genes in vertebrates were retained but marked by an asterisk in the immune gene table. From the studies that were selected, information on the species studied, techniques used, and the spatial expression and expression patterns after “immune stress” (*i.e.*, exposure to PAMPs or pathogenic bacteria) were noted in order to provide descriptive general metrics.

For omics studies on coelomocytes, terms such as “coelomocytes”, “omics”, “proteomics”, “transcriptomics”, “metabolomics”, “mass spectrometry”, “RNA-sequencing” and “single-cell RNA sequencing” were used in combination with “sea cucumber.” Results were screened to ensure high-throughput analyses on coelomocytes. Species, type of omics and “biological question” were noted and summarised in a table.

For cellular actors of immunity, the present review focuses on the functional diversity of coelomocytes and aims to reveal the status of the different cell types with regard to the recent literature. For these reasons, only key studies discussing putative functions of coelomocytes were considered. All images of coelomocytes presented in the manuscript were acquired by the authors. These were acquired using light and electron microscopy following the protocols of Wambreuse et al. [36]. Fluorescent microscopy images of the actin cytoskeleton were acquired using the same process of cell settlement, and the staining was done following the protocols of [37] using Alexa Fluor™ 488 conjugated Phalloidin.

Multi-level immunity of sea cucumbers

Studies on sea cucumber immunity often focus on the immune system *per se*, including immune cells and immune molecules. However, it is important to note that immune defences do not rely exclusively on these components [23]. A substantial proportion of potential infections is prevented by other forms of defence that remain relatively overlooked or insufficiently studied in holothuroids. These additional layers of defence include physical barriers, chemical defences, behavioural responses, and the protective role of the microbiota. Together, they contribute to a complex and integrated immune landscape that extends

beyond the classical cellular and molecular immune system. While cellular and molecular immunology will be extensively detailed in the next sections, it is important to briefly present these different components to provide a more holistic understanding of holothuroid immunity. This first section, therefore, presents the different levels of immunity in holothuroids before addressing the cellular and molecular aspects of their immune system in greater depth.

Behavioural immune response

Behavioural immune responses encompass the processes by which organisms reduce the risk of infection through behavioural adaptations [23] (Fig. 1A). These types of behaviours remain largely under-investigated in echinoderms, although some behaviours reported in the literature may be attributed to immune mechanisms [38–41]. It should be noted that such behaviour often arises in evolution among organisms showing high proximity to conspecifics, which facilitates the transmission of the pathogenic agent [42]. In sea cucumbers, it was recently

demonstrated that the species *H. scabra* displays gregarious behaviour, which appears to be driven by chemical communication based on saponins [43]. Additionally, some species, such as the suspension-feeding holothuroid *Cucumaria frondosa*, can reach very high local densities in the wild [44]. In these two species, conspecific avoidance behaviour has been observed when some individuals were starved or injured, respectively. Such responses may represent adaptive strategies allowing these gregarious organisms to avoid unfavourable or potentially dangerous conditions. In *C. frondosa*, it was even shown that danger-associated signals can trigger an anticipatory immune response, notably through the potential release of stress-related hormones and an increase in the concentration of circulating immune cells within body fluids [39]. More recently, exposure to “alarm cues”, derived from recently dead or injured conspecifics, was tested in the species *A. japonicus* and resulted in a significant shift in some behaviours, including podia attachment time [40,41]. Although direct links between these behavioural changes and specific pathogen infections or diseases remain to be demonstrated, and the specific molecular pathways are still not clearly identified, these

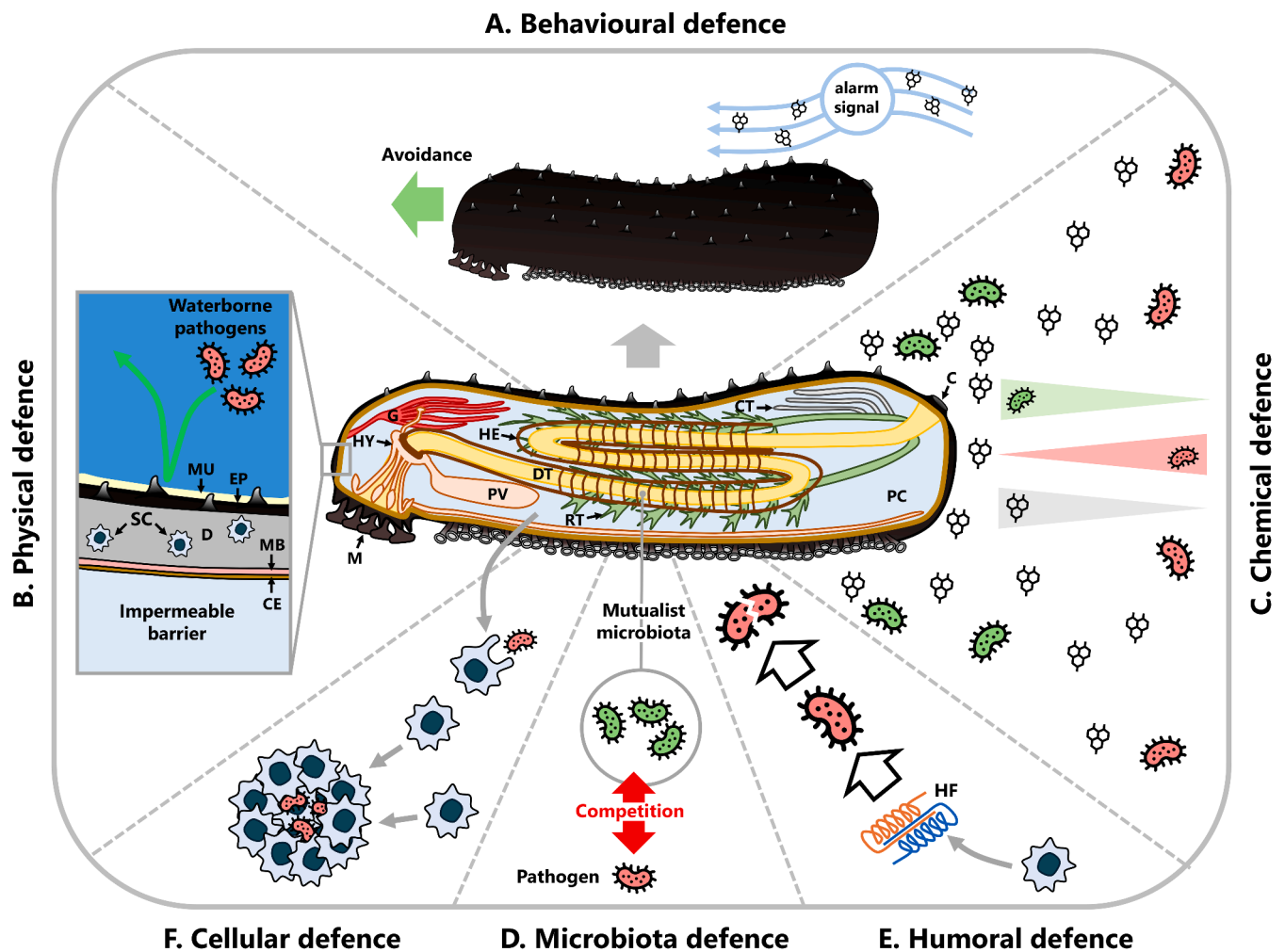


Fig. 1. Overview of immune defence at different levels of organisation in holothuroids (note that some mechanisms remain to be confirmed in holothuroids). **A.** Behavioural immunity: the holothuroid avoids the alarm signal coming from injured conspecifics. **B.** Physical immunity ensured by different tissue layers of the body wall (CE – Coelomic Epithelium; D – Dermis; EP – Epidermis; MB – Muscular Band; MU – Mucus; SC – Sentinel Coelomocytes). **C.** Chemical immunity ensured by the released molecules in the seawater, forming a chemical gradient (in grey) that has an antimicrobial property. Some mutualistic bacteria (in green) might deal with these antibacterial molecules. **D.** Microbiota immunity: mutualistic bacteria may be beneficial by competing with pathogenic bacteria (in red) and interacting with immune defence. **E.** Humoral immunity ensured by molecules (mainly enzymes) called humoral factors, which have broad activity in the immune response. Here, the enzyme is a lysin capable of degrading the bacterial cell wall, leading to cell lysis. **F.** Cellular immunity mediated by coelomocytes from the coelomic cavities. An individual coelomocyte phagocytises an isolated pathogenic bacterium, and several coelomocytes form a coelomocyte aggregate around a group of bacteria. Legend: C – Cloaca; CT – Cuvierian tubules; DT – Digestive tract; G – Gonad; HE – Hemal system; HF – Humoral factor; HY – Hydrovascular system; M – Mouth; PC – Perivisceral cavity; RT – Respiratory tree.

few examples suggest the presence of a chemical communication-driven behaviour aiming at ensuring the organism's survival.

Physical barriers and chemical defences

The physical barriers primarily rely on epithelial tissues that form an impermeable barrier against pathogens through tight junctions between epithelial cells [23] (Fig. 1B). In holothuroids, the body wall constitutes the main barrier against pathogens. It is composed of an epidermis, a thick dermis made mainly of collagen fibres, muscle bands and finally a coelomic epithelium [10,45,46]. In addition, echinoderms have a mutable collagenous tissue that provides them with the ability to quickly stiffen and soften their body walls [47,48]. Although this property serves multiple functions depending on the context, it likely contributes to immune defence by facilitating rapid wound closure, thereby limiting microbial entry and promoting tissue healing. Beyond the body wall, several other potential routes of infection exist for pathogens, especially the different mucosal surfaces [23]. These include the digestive tract, from the mouth to the cloaca, as well as respiratory trees, *i.e.*, large organs filled with seawater pumped from the cloaca and serving as gas exchange organs [49]. Additional entry points for pathogens may include the gonopore, through which gametes are released directly into seawater during reproduction [50]. Furthermore, although the hydro-vascular system connection to the external environment (via the madreporite) is internalised in most holothuroid species [51], it may also constitute a potential route of infection, particularly through the podia or tentacles, which can easily be torn off. It is important to note that while these different tissues act as physical barriers, they are generally associated with sentinel coelomocytes. For instance, in *Holothuria glaberrima*, coelomocytes were localised within the dermis and proliferated in case of injury [46]. In addition to the different tissue layers, the epidermis and mucosa also produce a mucus, *i.e.*, a secreted viscous biological fluid, which also constitutes a key physical barrier [52]. This mucus is generally constituted of mucin (glycoprotein) and different lipidic and salt compounds [52], but can also contain some protective molecules, such as saponin in sea cucumbers [53].

Chemical defences consist of molecules that are secreted by the organism either in the extracellular environment or in the surrounding environment (*i.e.*, out of the organism), and that exhibit antimicrobial activity ([26]; Fig. 1C). These defences encompass a broad diversity of humoral factors secreted into the extracellular environment. In holothuroids, particular attention has been given to saponins, a family of triterpene glycosides widely produced in plants but, among metazoans, found only in asteroids, holothuroids, and sponges [54–57]. Numerous studies have demonstrated that these compounds possess important antimicrobial properties. As an example, saponins from three sea cucumber species have an antifouling effect against the fouling diatom *Cylindrotheca closterium* [57]. In addition, it was recently demonstrated that saponin extracts from *Holothuria leucospilota* have significant antibacterial and antibiofilm formation effects on *Aeromonas hydrophila*, a pathogenic bacterium typically occurring in pisciculture [58]. These molecules are present in the body wall of holothuroids as well as in Cuvierian tubules and mucus and are secreted into the seawater [43,53,59], constituting a chemical defence against opportunistic epibionts, predators and pathogens (Fig. 1C). In addition, these bioactive compounds are thought to play a key role in shaping and regulating the sea cucumber-associated microbiota.

Protective role of the microbiota

It has recently been shown that mutualistic microbiota and the immune system interact in the organism's defence [60]. On the one hand, beneficial microorganisms can compete with pathogenic bacteria for space and resources, thereby limiting pathogen establishment (Fig. 1D). These microbial communities are often actively promoted or at least tolerated by the host. On the other hand, mutualistic bacteria may also

stimulate or modulate the host immune response when required [61]. Although these interactions remain largely underexplored in holothuroids and echinoderms in general [62], preliminary investigations have demonstrated that holothuroids are capable of selecting specific mutualistic microbial communities that provide essential enzymes involved in food digestion [63]. The gut microbiota of different species was also characterised [64,65], notably in stress conditions [66,67]. Among these, it was suggested that some bacteria could confer an immune advantage [65].

More recently, studies revealed the presence of bacterial communities coexisting with coelomocytes within the coelomic fluid [68–70]. Notably, Laguerre et al. [68] demonstrated that the composition of the microbiota in the perivisceral fluid of three echinoderm species, namely *Sphaerechinus granularis* (an echinoid), *Marthasterias glacialis* (an asteroid) and *Holothuria forskali* (a holothuroid), was distinct from that of the surrounding seawater, suggesting a selection process in echinoderm internal bacterial communities. In addition, among the three species, coelomic bacterial diversity was the highest in the holothuroid, consisting mainly of Bacteroidota, Firmicutes and Actinobacteriota. Some could be cultured and displayed antibacterial activities against different bacteria classically reported to be pathogens in aquaculture, suggesting they may have the same activity *in vivo* [68,69].

Furthermore, the coexistence of these bacteria with coelomocytes raises intriguing questions regarding the potential capacity of coelomocytes to selectively recognise and tolerate beneficial microorganisms while targeting harmful ones. The role of the microbiota in holothuroid immunity, therefore, represents a promising and still largely unexplored field that warrants further investigation.

Cellular versus humoral immunity

Cellular and humoral immune responses constitute the principal effector mechanisms of the immune system. Humoral immunity relies on molecules released into the extracellular environment that perform a wide range of immune functions (Fig. 1E). In vertebrates, the humoral response generally refers to the release of immunoglobulin by B-cells [71]. More broadly, however, humoral immunity encompasses a diverse array of molecules involved in processes such as inflammation, pathogen recognition, pathogen lysis, opsonisation and agglutination [26]. In contrast, cellular immunity comprises all defence mechanisms that involve the direct action of immune cells, including processes such as phagocytosis or encapsulation (Fig. 1F).

Molecular actors of immunity

Molecular actors of immunity in holothuroids encompass a large number of molecules that have a broad spectrum of functions, ranging from pathogen recognition to degradation and regulation of the immune response. While several reviews have already contributed to listing these molecules in sea cucumbers and echinoderms [26,34], the number of studies describing new molecular actors during the last decades has substantially increased. This increase seems to be supported by the advent of molecular biology tools and the increasing availability of genomics data, allowing easier screening of homologous immune genes in vertebrates. To have a clear view of molecular studies on the holothuroid immune system, studies using omics tools on coelomocytes and characterising immune genes were screened and are reported below.

Omics studies on coelomocytes

In total, 27 studies employed omics on sea cucumber coelomocytes belonging to five species (Table 1; Fig. 2A). A wide range of omics technologies has been applied, with a primary focus on transcriptomics and proteomics. The most commonly used method is RNA-sequencing, focusing either on microRNA (miRNA; [72–75]) or coding messenger RNA (mRNA; [33,35,36,76–88]). As for the proteomics analyses,

Table 1
Summary of published omics studies employed on holothuroid coelomocytes. Only high-throughput studies were considered. For those, the omics types, the technique used, the model species, and the experimental conditions are indicated.

Omics type	Technique	Species	Experimental condition/ comparison	Reference
Transcriptomics (miRNA)	miRNA-sequencing	<i>A. japonicus</i>	Skin Ulceration Syndrome	[72]
Transcriptomics (miRNA)	miRNA-sequencing	<i>A. japonicus</i>	Skin Ulceration Syndrome	[73]
Transcriptomics (mRNA)	RNA-sequencing	<i>A. japonicus</i>	Lipopolysaccharide challenge	[76]
Proteomics	2-DE + MS/MS	<i>A. japonicus</i>	<i>Vibrio splendidus</i> challenge	[89]
Proteomics	iTRAQ-Based LC-MS/MS	<i>A. japonicus</i>	<i>Vibrio splendidus</i> challenge	[90]
Transcriptomics (mRNA)	RNA-sequencing	<i>A. japonicus</i>	Lipopolysaccharide challenge	[77]
Transcriptomics (mRNA)	RNA-sequencing	<i>A. japonicus</i>	<i>Vibrio splendidus</i> challenge	[78]
Transcriptomics (miRNA)	miRNA-sequencing	<i>A. japonicus</i>	Lipopolysaccharide challenge	[74]
Transcriptomics (miRNA)	miRNA-sequencing	<i>A. japonicus</i>	Skin Ulceration Syndrome	[75]
Transcriptomics (mRNA)	RNA-sequencing	<i>A. japonicus</i>	Skin Ulceration Syndrome	[79]
Proteomics	Label-free LC-MS/MS	<i>A. japonicus</i>	Gender and spawning	[91]
Transcriptomics (mRNA)	RNA-sequencing	<i>H. leucospilota</i>	PAMP challenge	[80]
Transcriptomics (mRNA)	RNA-sequencing	<i>H. leucospilota</i>	Lectin identification	[81]
Transcriptomics (mRNA)	RNA-sequencing	<i>A. japonicus</i>	Air exposure	[82]
Transcriptomics (mRNA)	RNA-sequencing	<i>A. japonicus</i>	Knockdown of the pacifastin gene	[83]
Transcriptomics (mRNA)	RNA-sequencing	<i>A. japonicus</i>	Different coelomocyte subsets	[35]
Proteomics	Label-free LC-MS/MS	<i>H. tubulosa</i>	Extracellular versus intracellular	[92]
Transcriptomics (mRNA)	RNA-sequencing	<i>H. leucospilota</i>	Tissue expression comparison	[63]
Transcriptomics (mRNA)	RNA-sequencing	<i>H. scabra</i>	Lipopolysaccharide challenge	[36]
Transcriptomics (mRNA)	RNA-sequencing	<i>H. forskali</i>	Lipopolysaccharide challenge + different body fluids	[33]
Proteomics	Label-free LC-MS/MS	<i>H. forskali</i>	Different body fluids	[33]
Transcriptomics (mRNA)	RNA-sequencing	<i>A. japonicus</i>	Chitosan oligosaccharide (COS) exposure	[84]
DNA methylomics	DNA-BSP-Sequencing	<i>A. japonicus</i>	Evisceration	[85]
Transcriptomics (mRNA)	RNA-sequencing	<i>A. japonicus</i>	Evisceration	[85]
Transcriptomics (mRNA)	RNA-sequencing	<i>A. japonicus</i>	Evisceration	[86]
Transcriptomics (mRNA)	RNA-sequencing	<i>A. japonicus</i>	Peptidoglycan challenge	[87]
Transcriptomics (mRNA)	RNA-sequencing	<i>A. japonicus</i>	Repeated infection with <i>Vibrio splendidus</i> (Priming)	[88]
Single-cell transcriptomics (mRNA)	Single-cell RNA-sequencing	<i>H. forskali</i>	Cell cluster	[93]
Single-cell transcriptomics (mRNA)	Single-cell RNA-sequencing	<i>A. japonicus</i>	Cell cluster, body fluids and evisceration	[94]

different approaches were used, including 2D gel + MS/MS, iTRAQ-based LC-MS/MS and label-free LC-MS/MS [33,89–92]. Recently, single-cell RNA-sequencing was also employed on circulating coelomocytes in *H. forskali* and *A. japonicus* [93,94]. In addition to proteomics and transcriptomics, DNA methylomics was used to better understand the response to evisceration in *A. japonicus* [85]. Overall, it can be seen that while the first studies aimed to employ these tools mainly to understand the coelomocyte response to pathogen challenges [76,78,89] or to SUS [72,75], recent studies have addressed additional questions, including, for example, gender differences [91], tissue specificity [33], and response to evisceration [85]. Among the 27 studies that employed omics tools on sea cucumber coelomocytes, 74.1% were done in *A. japonicus*. It can also be noticed that the number of omics studies on coelomocytes increased substantially around 2014, corresponding to the emergence of the omics approaches field, and then between 2024 and 2026, reflecting the more common use of such tools in the study of the sea cucumber immune system lately (Fig. 2A).

Immune gene characterisation in sea cucumbers

In total, 108 immune genes were characterised in sea cucumbers, corresponding to a total of 85 studies. Table 2 lists all the immune molecules that were reported, and a whole view of these molecular actors is represented in Fig. 3. For better clarity, immune molecules are classified into three large families according to their function in immunity, including pathogen recognition receptors, humoral factors, and immune response regulators. However, it can be noted that some molecular actors can play several functions and that this classification is not restrictive. Although this list tries to be as complete as possible in the molecular actor diversity, we have chosen to keep genes for which strong functional evidence was provided to consider them as “key immune genes” (i.e., not only differential expression after an immunological stress according to only one study). However, some genes having an annotation that corresponds to important immune genes in vertebrates are still shown in the table, but in this case are flagged by an asterisk. For these last, additional targeted functional studies will be necessary to

confirm their key function in sea cucumber immunity. Based on this list, several metrics could be extracted.

First of all, it can be seen that the number of studies characterising immune genes in sea cucumbers has started to increase substantially from 2014, which seemed to coincide with the emergence of the first omics studies (Fig. 2A). Species distribution analysis reveals that 83.5% of the studies were carried out in *A. japonicus*, followed by *H. leucospilota* (10.6%), *Stichopus monotuberculatus* and *H. scabra* (2.4% both; Fig. 2B). The large domination of *A. japonicus* in the field reflects the aquaculture interest in this species and its high economic value [8,9]. Although all gene functions are expected to be relatively conserved within the class Holothuroidea, the lack of diversity among the species investigated may hinder the detection of interspecific differences. This is especially a concern in this case since the six species investigated belong to only two orders out of seven [95], namely Synallactida and Holothuriida. In comparison, the review by Queiroz and Custódio [29] on coelomocyte diversity in holothuroid species revealed 80 species investigated for four orders. Although the low number of species investigated for immune gene characterisation is consistent with the limited number of species for which genomic and transcriptomic data are available, it would be important to acquire some molecular data for a few representatives of each order to obtain a broad overview of immune gene diversity at the scale of the class.

Overall, it can be noticed that the characterisation of immune genes follows a methodological framework which is quite consistent between studies. First, the gene is cloned and sequenced to obtain functional information from its sequence, such as the presence of specific domains and their associated functions. Next, its expression pattern is investigated by quantitative PCR (qPCR), both following an immunological challenge—generally involving exposure to PAMPs or pathogenic bacteria—and across different tissues. Finally, additional analyses are performed to provide further functional information about the activity of the gene or its encoded protein. Among the immune genes reported in this review, 82.4% were cloned, and the expression patterns of 80.6% were investigated following an immunological challenge (Fig. 2C). However, these analyses alone do not constitute functional

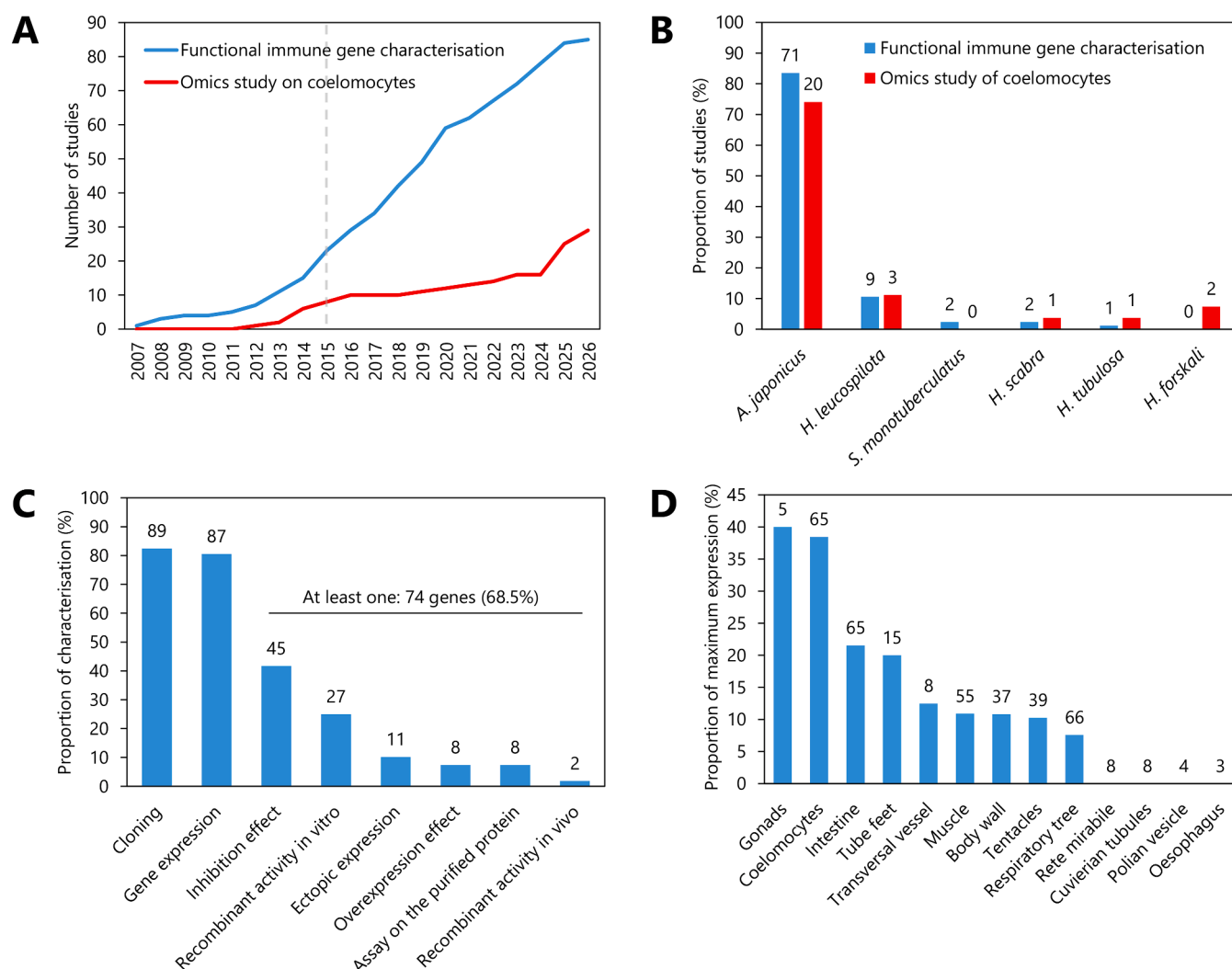


Fig. 2. Evolution of molecular studies on the immune system in sea cucumbers over the last 20 years. **A.** Cumulative number of studies that employed omics approaches on coelomocytes or functionally characterised immune genes in sea cucumbers over the years. The dotted line indicates the most recent review on molecular immunity in sea cucumbers by Xue et al. [34]. Note that 2026 data only include the first three months. **B.** Species distribution of studies that employed omics approaches on coelomocytes or functionally characterised immune genes. The number above each bar indicates the total number of studies conducted on each species. **C.** Proportion of different techniques applied for the characterisation of the immune genes (see Table 2); note that most genes were characterised using different techniques. The exact number of studies is indicated above the bars. **D.** For immune genes whose spatial expression was investigated (see Table 2), the proportion of immune genes showing maximal expression in each tissue. Because not all tissues were investigated for every gene, the proportion was calculated based on the subset of genes for which a given tissue was investigated. This latter number is indicated above the bars for each tissue.

characterisation per se. Overall, 74 immune genes (68.5%) were functionally characterised using at least one functional approach. These include functional fluorescent or colourimetric assays on purified protein, which accounted for 7.4% of the approaches and were more commonly used in older studies. Here, the term “purified” is used to distinguish these proteins from those produced recombinantly. Another approach involves modifying gene expression, either by silencing the gene or, conversely, by increasing its expression, in order to assess its effects on downstream signalling pathways or immune mechanisms. These approaches accounted for 41.7% and 7.4% of the functional characterisation methods, respectively. Functional information can also be obtained through ectopic expression of the gene, *i.e.*, by expressing it in a different cell lineage with a well-characterised physiology, such as HEK293T cells. This approach was used for 10.2% of the immune genes. Finally, recombinant production of the protein enables its activity to be assessed *in vitro* or *in vivo*. These approaches were used for 25.0% and 1.9% of the immune genes, respectively.

Finally, tissue-specific expression patterns were investigated in most

studies. Taken together, these analyses allowed us to identify the tissues in which immune genes were most highly expressed. The three tissues showing the highest expression of immune genes are the gonads (40.0%), the coelomocytes (38.5%), and the intestine (21.5%) (Fig. 2D). High expression of immune genes in gonads, and especially in ovaries, may reflect a maternal transmission of some immune factor at the early stages [96]. High expression in coelomocytes is consistent with their function as immune cells. Regarding the intestine, it is directly in contact with the incoming sediment, including all the microorganisms contained in it, and it thus constitutes a privileged route of infection for pathogens. It can also be exposed to diverse xenobiotics or harmful structures, such as sharp mineral particles or microplastics. Hence, the interest in maintaining a high expression level of immune genes in this tissue lies in its ability to detect threats and promptly initiate an adequate response. This principle also applies to other body parts, such as the respiratory trees, which are directly exposed to seawater entering through the cloaca, as well as the tentacles and podia, which can be easily torn off in sea cucumbers. However, it should be highlighted that all studies did not

Table 2

List of immune genes in sea cucumbers, along with several characteristics, including the species in which they were studied, whether their expression changes following immune stress (stress expression; this includes both PAMPs exposure or bacterial infection), their spatial expression pattern (tissues are ordered by decreasing expression), the level of experimental characterisation, a summary of their function and the study that characterised them. Note that some genes displayed in this table remain to be functionally characterised in sea cucumbers and were included based on a strong assumption that these have a function in the immune system. These last are denoted by an asterisk *. Legend of species: Aj – *Apostichopus japonicus*; Hl – *Holothuria leucospilota*; Hs – *Holothuria scabra*; Ht – *Holothuria tubulosa*; Sm – *Stichopus monotuberculatus*. Legend of tissues: BW – Body Wall; COE – Coelomocytes; CT – Cuvierian Tubules; GO – Gonads; INT – Intestine; MU – Muscles; Ov – Ovaries; PV – Polian Vesicle; RM – Rete Mirabile; RT – Respiratory Tree; TE – Testis; TF – Tube Feet; TT – Tentacles; TV – Transversal Vessel. Legend of characterisation levels: 1 – cloning the full-length sequence; 2 – studying gene expression pattern after an immunological stress; 3 – performing activity assay on the purified protein; 4 – silencing the gene expression to study subsequent effect; 5 – increasing the gene expression to study subsequent effect; 6 – expressing the gene in a model cell lineage to study their effect; 7 – recombinantly produced the protein to study its activity *in vitro*; 8 – recombinantly produced the protein to study its activity *in vivo*.

Full name	Short name	Species	Stress expression	Tissue expression	Function	Characterisation level	Study
Pathogen recognition receptors							
Lectins							
C-type lectin	AjCTL	Aj	Up	MU, INT, BW, RT	Putative PRR; binds monosaccharides	1	[100]
C-type lectin	AjCTL-1	Aj	Up	GO, BW, COE, TT, INT, MU, RT	Binds peptidoglycans and aggregates Gram-positive and Gram-negative bacteria	1, 2, 7	[102]
C-type lectin	AjCTL-2	Aj	Up	COE, INT, TF, RT, GO, BW	Binds galactose motifs and diverse bacteria	1, 2, 7	[101]
Mannan-binding c-type lectin (MBL)	MBL-Aj	Aj	Not tested	Not tested	Putative PRR; binds mannan motifs	2	[103]
T-antigen-specific lectin	HSL	Hs	Up	Not tested (coelomic fluid)	Binds and aggregates diverse bacteria; antibacterial activity	3	[104]
T-antigen-specific lectin	HSL	Hs	Not tested	Not tested (coelomic fluid)	Binds antigens and mediates hemagglutination	3	[105]
Toll-like receptors							
Toll-like receptor (TLR)	AjTLR	Aj	Up	RT, BW, TF, COE, INT	Putative PRR	1, 2	[107]
Toll-like receptor 3 (TLR3)	AjTLR3	Aj	Up	RT, INT, TF, COE, BW	Putative PRR	1, 2	[107]
NOD-like receptors (NLRs)							
NLR family CARD domain-containing protein (NLRC4)	AjNLRC4	Aj	Up	INT, TT, RT, MU, COE	Promotes coelomocyte apoptosis via CASP1 expression	1, 2, 4, 7	[110]
NLR family CARD domain-containing protein (NLRC4)	AjNLRC4	Aj	Not tested	Not tested	Binds pathogenic bacteria and promotes actin-dependent phagocytosis	2, 4, 5	[109]
NOD-like receptor family pyrin domain containing 10 (NLRP10)	AjNLRP10	Aj	Timing dependent	COE, INT, RT, BW, TF, MU	Inhibits CASP1 and MMP37 expression; putatively inhibits apoptosis	1, 2, 4	[111]
NOD-like receptor family pyrin domain-containing 3 (NLRP3)	AjNLRP3	Aj	Up	COE, INT, TT, MU, RT	Putative role in inflammation	1, 2, 4	[112]
Scavenger receptors							
Scavenger receptor B (SR-B)	AjSR-B	Aj	Up	INT, MU, TT, RT, COE	Binds diverse PAMPs, particularly LPS, and aggregates bacteria	1, 2, 7	[115]
Deleted in malignant brain tumours 1 protein (DMBT1)*	AjDMBT1	Aj	Up	Not tested (COE)	Contains an SRCR domain	1	[36]
Other pathogen recognition receptors							
ApeXtrin C-terminal (ApeC)-containing proteins (ACPs)	AjACP1	Aj	Up	Not tested (COE)	Binds pathogenic bacteria and promotes actin-dependent phagocytosis	1, 2, 4, 5, 7	[124]
C-type mannose receptor 2*	Hs-MRC2	Hs	Up	Not tested (COE)	Putative role in pathogen recognition	1	[36]
Fibrinogen-like protein (FREP)	AjFREP	Aj	Up	COE, TT, RT, MU, INT	Putatively binds pathogens and promotes phagocytosis and bacterial clearance	1, 2, 4	[120]
Globular complement component 1q (gC1qR)	AjgC1qR	Aj	Up	TT, COE, INT, RT, MU	Binds PAMPs and putatively activates inflammation	1, 2, 7	[123]
Lipopolysaccharide-binding protein/bactericidal permeability-increasing protein 1 (LPB/BPI1)	AjLPB/BPI1	Aj	Up	TT, COE, INT, MU, TT, RT	Putatively binds LPS and promotes ROS production via Toll signalling	1, 2, 4	[116]
Lipopolysaccharide-binding protein/bactericidal permeability-increasing protein (LBP/BPI2)	AjLBP/BPI2	Aj	Up	INT, COE, MU, TT, RT	Putatively binds LPS	1, 2, 4	[116]
Macrophage mannose receptor 1-like*	Hs-MMR1	Hs	Up	Not tested (COE)	Putative role in pathogen recognition	1	[36]
Peptidoglycan recognition protein (PGRP-S)	AjPGRP-S1	Aj	Up	COE, TF, RT, INT, MU, BW	Binds diverse PAMPs and interacts with innate immune factors	1, 2, 7	[118]

(continued on next page)

Table 2 (continued)

Full name	Short name	Species	Stress expression	Tissue expression	Function	Characterisation level	Study
Peptidoglycan recognition protein S2 (PGRP-S2)	AjPGRP-S2	Aj	Up	TF, COE, RT, INT, MU, BW	Binds diverse PAMPs and promotes complement and PRR expression	1, 2, 4, 7	[119]
Humoral factors							
Agglutinins							
Amassin*	AjAmassin	Aj	UP	Not tested (COE)	Putatively promotes coelomocyte aggregation	1	[76]
Galactose-Binding C-Type Lectin (GBCL)	AjGBCL	Aj	Not tested	Not tested (INT)	Promotes coelomocyte aggregation via galactose binding	1, 2, 3	[81]
Lysins							
Antimicrobial peptides SCAK33 (AMP SCAK33)	SCAK33	Aj	Not tested	Not tested	Antibacterial activity	2, 3, 7	[128]
Holothuroidin 1 (H1), Holothuroidin 2 (H2)	H1, H2	Ht	Not tested	Not tested (COE)	Antibacterial and anti-biofilm activity	3	[22]
c-type lysozyme (c-Lys)	AjCLYZ	Aj	Not tested (up following salinity stress)	TF, MU, BW, COE, INT, TT, RT	Antibacterial activity; putative role in salinity stress response	1, 2, 7	[134]
c-type lysozyme (c-Lys)	AjCLYZ	Aj	Not tested	TF, MU, COE	Putative role in salinity stress response via NKA/NKCC1 downregulation	1, 4	[133]
I-type lysozyme (i-Lys)	AjLys	Aj	Up	Not tested	Inhibits bacterial growth; glycosidase and isopeptidase activity	2, 7	[131]
I-type lysozyme (i-Lys)	Aj-iLys	Aj	Up	TT, RT, COE, BW, INT	Inhibits PAMP-induced apoptosis; isopeptidase activity	1, 2, 3, 4, 7	[132]
Lysin motif-containing protein (LysM-1)	AjLysM-1	Aj	Up	COE, RT, INT, BW, MU	Antibacterial activity; putative role in focal adhesion	1, 2, 7	[135]
WAP domain-containing protein (WAP)	AjSWD-WAP	Aj	Up	BW, RT, COE, MU, INT, TT	Antibacterial activity	1, 2, 7	[136]
Cytokines							
Double Ig interleukin-1 receptor-related (DIGIRR)	AjDIGIRR	Aj	Up	MU, COE, INT, TT, RT	Regulates inflammatory chemotaxis and vacuolisation; inhibits IL-17 expression	1, 2, 4, 5	[165]
Erythroid differentiation-related factor 1-like (EPRF1)*	HI-EPRF1	HI	Unchanged	Not tested (COE)	Putative cytokine	1	[80]
Interleukin 17 ligand and receptor (IL-17 and IL-17R)	AjIL-17, AjIL17R	Aj	Up	Not tested	Activates inflammation via TRAF6 ubiquitination and P65 nuclear translocation	1, 2, 4, 6, 7	[137]
Thrombospondin (TSP) and Thrombopoietin (TPO)*	HI-TSP and HI-TPO	HI	Unchanged	Not tested (COE)	Putative cytokine	1	[80]
Transforming growth factor beta (TGF- β)	AjTGF- β	Aj	Down	BW, TT, MU, COE, INT, RT	Inhibits inflammation; downregulation following bacterial exposure promotes inflammation	1, 2, 4, 8	[138]
Complement and opsonisation							
Complement component 3 (C3)	AjC3, AjC3-2	Aj	Up	COE, BW, RT, INT	Putative central role in complement system	1, 2	[140]
Complement B factor (BF)	AjBF-2	Aj	Up	COE, BW, TT, INT, RT, MU	Serine protease; putative role in complement activation	1, 2	[142]
Complement B factor (BF)	AjBF	Aj	Up	BW, COE, INT, TF, RT	Serine protease; putative role in complement activation	1, 2	[141]
Complement factor H (HF)	AjHF	Aj		Not tested (COE)	Putative role in complement system	1	[76]
Phenoloxidase and melanisation							
Phenoloxidase (PO)	AjPO	Aj	Up	COE, TF, BW, RT, INT, MUT	Catalyses melanin production; putative immune role	1, 2	[149]
Phenoloxidase 1, 2 and 3 (PO1, 2 and 3)	AjPO1, AjPO2, AjPO3	Aj	Not tested	Not tested (COE)	Antibacterial activity	3	[150]
Phenoloxidase 1, 2 and 3 (PO1, 2 and 3)	AjPO1, AjPO2, AjPO3	Aj	Up	Not tested (COE)	Catalyses melanin production	3	[148]
Phenoloxidase II (POII)	AjPOII	Aj	Up	BW, TF, MU, INT, COE, RT	Catalyses melanin production; putative immune role	1, 2	[147]
Phenoloxidase II (POII)	AjPOIII	Aj	Up	BW, TF, MU, RT, COE, INT	Catalyses melanin production; putative immune role	1, 2	[147]
Immune response regulation							
Inflammation signalling							
Gamma-interferon-inducible lysosomal thiol reductase (GILT)	StmGILT	Sm	Up	COE, INT, BW, RT	Interacts with NF- κ B; putative role in inflammation	1, 2, 7	[163]
Heat shock cognate protein 70 (HSC70)	AjHSC70	Aj	Up	Not tested (COE)	Activates NF- κ B and promotes pro-inflammatory factor expression	1, 2, 4	[160]

(continued on next page)

Table 2 (continued)

Full name	Short name	Species	Stress expression	Tissue expression	Function	Characterisation level	Study
Interferon regulatory factor 8 (IRF8)	AjIRF8	Aj	Up	Not tested (COE)	Promotes bacterial clearance and coelomocyte apoptosis	1, 2, 4,	[161]
Inhibitory kappa B kinase (IKK β)	HIKK β	HI	Up	TV, OE, COE, RM, BW, MU, CT, PV, INT, RT	Activates NF- κ B signalling; inhibits coelomocyte apoptosis	1, 2, 4, 6	[155]
Interleukin 1 receptor-associated kinase 4 (IRAK4)	AjIRAK4	Aj	Up	MU, RT, TT, INT, COE	Promotes TLR and BAX expression; inhibits coelomocyte apoptosis	1, 2, 4	[162]
LPS-induced TNF- α factor (LITAF)	AjLITAF	Aj	Up	COE, TT, MU, RT, INT	Putative cytokine involved in inflammation	1, 2	[139]
Major yolk protein (MYP)	AjMYP	Aj	Up	Not tested (COE)	Activates NF- κ B-mediated inflammation via MyD88	1, 2, 4	[160]
Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1)	AjMALT1	Aj	Up	COE, INT, MU, TT, RT	Activates NF- κ B and promotes pro-inflammatory factor expression	1, 2, 4	[158]
Myeloid differentiation (MyD88)	AjMyD88	Aj	Up	RT, INT, TT, MU, COE	Putative role in immune signalling following bacterial exposure	1, 2	[156]
Myeloid differentiation (MyD88)	HIMyD88	HI	Up	RT, COE, MU, TT, INT	Activates NF- κ B1; promotes cytokine expression and apoptosis	1, 2, 6	[157]
NF- κ B1 p105	Aj-p105	Aj	Up	Not tested (COE)	Participates in NF- κ B signalling	1, 2	[154]
NF- κ B1 p105	HIp105	HI	Up	INT, TV, RM, COE, RT, PV, CT, BW, OE, MU	Inhibits apoptosis and promotes pro-inflammatory factor expression	1, 2, 6	[153]
NF- κ B transcription factor Rel	Aj-rel	Aj	Up	Not tested (COE)	Participates in NF- κ B signalling	1, 2, 6	[154]
Pacifastin	Ajpacifastin	Aj	Up	Not tested (COE)	Antibacterial activity via immune gene regulation and inhibition of pathogen proteases	1, 2, 4, 7	[83]
p21-activated kinase 1 (PAK1)	AjPAK1	Aj	Up	COE, RT, INT, BW, TF, MU	Promotes lysozyme release via RAC1/MKK3/6 signalling	1, 2, 4	[166]
Peroxisome proliferator-activated receptor α (PPAR α)	AjPPAR α	Aj	Up	Not tested (COE)	Regulates IL-17 and NLRP3 via NF- κ B inhibition; reduces bacterial injury	1, 2, 4	[159]
TNF receptor-associated factor 3 (TRAF3)	AjTRAF3	Aj	Up	BW, TF, COE, MU, RT, INT	Regulates intracellular ROS; downregulation following bacterial exposure increases ROS	1, 2, 4	[164]
TNF receptor-associated factor 6 (TRAF6)	AjTRAF6	Aj	Up	COE, TT, MU INT, RT	Putative role in immune signalling following bacterial exposure	1, 2	[156]
β -Catenin	Aj β -Catenin	Aj	Up	INT, TT, RT MU, COE	Promotes inflammatory factor expression following bacterial exposure	1, 2, 4	[167]
Cell death regulation B-cell lymphoma 2 (Bcl-2)	AjBcl-2	Aj	Up	COE, RT, INT, TT, MU	Promotes coelomocyte apoptosis and downregulates Cyt c	1, 2, 4	[171]
B-cell lymphoma-2-associated X (BAX)	AjBAX	Aj	Down	COE, RT, INT, TT, MU	Inhibits apoptosis; downregulation following LPS/ bacterial exposure increases apoptosis	1, 2, 4, 8	[172]
Caspase 1 (CASP1)	AjCASP1	Aj	Up	RT, TT, COE, MU, INT	Binds LPS; proteolytic activity; promotes pyroptosis via IL-1 β	1, 2, 6, 7	[182]
Caspase 1 (CASPX1)	AjCASPX1	Aj	Up	MU, INT, RT, COE	Induces apoptosis via CASP3/7	1, 2, 7	[181]
Caspase 2 (CASPX2)	AjCASPX2	Aj	Up	COE, MU, INT, RT	Binds bacteria and inhibits bacterial growth	1, 2, 7	[181]
Caspase 2 (CASP2)	AjCASP	Aj	Up	COE, INT, RT, BW, TT	Putative role in apoptosis	1, 2	[180]
Caspase 2 (CASP2)	AjCASP2	Aj	Up	INT, COE, RT, TT, MU	Putatively modulates caspase 6 and promotes apoptosis	1, 2	[176]
Caspase 3 (CASP3)	AjCASP3	Aj	Up	COE, INT, RT, TT, MU	Putatively promotes apoptosis	1, 2	[176]
Caspase 6 (CASP6)	AjCASP6	Aj	Up	COE, MU, RT, INT, TT	Putatively promotes apoptosis	1, 2	[176]
Caspase 8 (CASP8)	AjCASP8	Aj	Up	INT, COE, RT, TT, MU	Putatively modulates caspase 3 and promotes apoptosis	1, 2	[176]
Caspase 6 (CASP6)	HI-CASP6	HI	Up	INT, COE, BW, RM, TV, RT, CT	Promotes apoptosis via p53 signalling	1, 2, 6	[179]
Caspase 8 (CASP8)	HI-CASP8	HI	Up	INT, COE, BW, RM, TV, CT, RT	Promotes apoptosis via TNF- α activation	1, 2, 6	[178]
Caspase 9 (CASP9)	HI-CASP9	HI	unchanged (up for abiotic stress)	COE, BW, PV, INT, RT, CT, TV, OE, MU, GO, RM	Promotes coelomocyte apoptosis following oxidative stress	1, 2, 4, 6	[175]

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Table 2 (continued)

Full name	Short name	Species	Stress expression	Tissue expression	Function	Characterisation level	Study
Cathepsin B	AjCTSB	Aj	Up	INT, RT, MU, TT, COE	Promotes coelomocyte apoptosis via caspase 3	1, 2, 4, 7	[174]
CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP)	AjCHOP	Aj	Up	Not tested (COE)	Promotes coelomocyte apoptosis via p38-MAPK signalling	1, 2, 4, 5	[184]
Death domain-containing Caspase (DD-CASP)	AjDD-CASP	Aj	Up	Not tested (COE)	Promotes lytic coelomocyte death following LPS/bacterial exposure	1, 2, 4, 5, 7	[189]
Death domain-containing NLR (DD-NLR)	AjDD-NLR	Aj	Up	TT, MU, RT, INT, COE	Promotes lytic coelomocyte death via AjDD-NLR	1, 2, 4, 5, 7	[189]
Fas-associated death domain (FADD)	HI-FADD	HI	Up	COE, INT, BW, TV, CT, RM, RT	Promotes apoptosis	1, 2, 6	[169]
Fas-associated death domain (FADD)	AjFADD	HI	Up	COE, INT, RT, TT, MU	Promotes apoptosis following LPS, poly(I:C) and bacterial exposure	1, 2, 4, 6	[170]
Fas-associated death domain (FADD-2)	AjFADD-2	Aj	Up	COE, INT, BW, TT, RT, MU	Promotes coelomocyte apoptosis following LPS/ bacterial exposure	1, 2, 4	[168]
Ferroportin (FPN)	AjFPN	Aj	Not tested	Not tested (COE)	Promotes bacterial ferroptosis in infected coelomocytes	4	[187]
Mixed-lineage kinase domain-like protein (MLKL)	HI MLK	HI	Down	OV, TT, COE, BW, MU, PV, TV, RM, CT, RT, TE	Putative role in necroptosis	1, 2	[185]
Mixed-lineage kinase domain-like protein (MLKL)	AjMLK	Aj	Up	Not tested (COE)	Activates antibacterial lytic cell death via CASP1/NLRC4	1, 2, 4, 5, 7	[186]
Mixed-lineage kinase domain-like protein (MLKL)	AjMLK	Aj	Up	Not tested (COE)	Activates antibacterial response via extracellular trap formation	1, 2, 4	[188]
Myelocytomatosis viral oncogene (MYC)	AjMYC	Aj	Not tested	No tested (COE)	Putatively inhibits coelomocyte apoptosis via BAX overexpression	1, 2, 5	[173]
Receptor-interacting protein kinases (RIPKs)	HI-RIPKs	HI	Down	COE, INT, BW, MU, TT, CT, RT, PV, TV, RM, OV, TE	Putative role in necroptosis	1, 2	[185]
β -Integrin	AjITGB	Aj	Down	MU, TT, INT, COE, RT	Binds PAMPs and inhibits apoptosis	1, 2, 4, 7	[183]
β -Integrin	AjITGB	Aj	Not tested	Not tested (COE)	Inhibits coelomocyte apoptosis via caspase-3 inhibition	1, 2, 4	[177]
REDOX regulation Betaine-homocysteine S-methyltransferase (BHMT)	AjBHMT	Aj	Down	Not tested (COE)	Regulates ROS via homocysteine-to-betaine conversion; downregulation increases ROS	1, 2, 4	[201]
Calmodulin (CaM)	Sm-CaM	Sm	Up	COE, INT, RT, BW, MU	Promotes NO production during bacterial challenge	1, 2, 4	[206]
Catalase (CAT)	AjCAT	Aj	Up	Not tested (INT)	Detoxifies H ₂ O ₂ and regulates intestinal microbiota	1, 2, 4	[197]
Catalase (CAT)	AjCAT	Aj	Up	INT, BW, TF, COE, RT, MU	Antioxidant response following bacterial challenge	1, 2	[196]
Glutathione peroxidase (GPX)*	Hs-GPX	Hs	Up	Not tested (COE)	Reduces peroxide; putative role in antioxidant response	1	[36]
Glutathione S-transferase (GST)	AjGST	Aj	Up	INT, RT, TT, MU, COE	Inhibits coelomocyte apoptosis and regulates microbiota	1, 2, 7	[200]
NADPH oxidase (NOX)	AjNOX	Aj	Up	INT, RT, BW, MU	Produces ROS and promotes inflammatory cytokines and apoptosis	1, 2, 4	[192]
Nitric oxide synthase (NOS)	AjNOS	Aj	Up	No tested (COE)	Promotes inflammation downstream of NF- κ B/Rel	1, 2	[193]
Nitric oxide synthase (NOS)	AjNOS	Aj	Up	No tested (COE)	Promotes NF- κ B-mediated inflammation and apoptosis via Bcl-2 downregulation	1, 2, 4	[194]
Superoxide dismutase (SOD)	AjSOD	Aj	Up	INT, COE, RT, BW, TT	Antioxidant response; inhibits coelomocyte apoptosis	1, 2, 4	[195]
Thioredoxin (TDX)	AjTDX	Aj	Up	MU, COE, TF, INT, RT, BW	Antioxidant response following bacterial challenge	1, 2, 7	[198]
Tissue inhibitors of metalloproteinases 1 (TIMP1)	AjTIMP1	Aj	Up	BW, COE, TT, MU, RT, INT	Inhibits ROS production via MMP1/MMP19 repression	1, 2, 4	[199]
Stress response proteins							
Heat shock protein 70*	AjHSP70	Aj	Up	Not tested (COE)	Putative DAMP	1	[76]
Heat shock protein 90*	AjHSP90	Aj	Up	Not tested (COE)	Putative DAMP	1	[76]
Heat shock protein 26*	Hs-HSP26	Hs	Up	Not tested (COE)	Putative DAMP	1	[36]

Non-self recognition

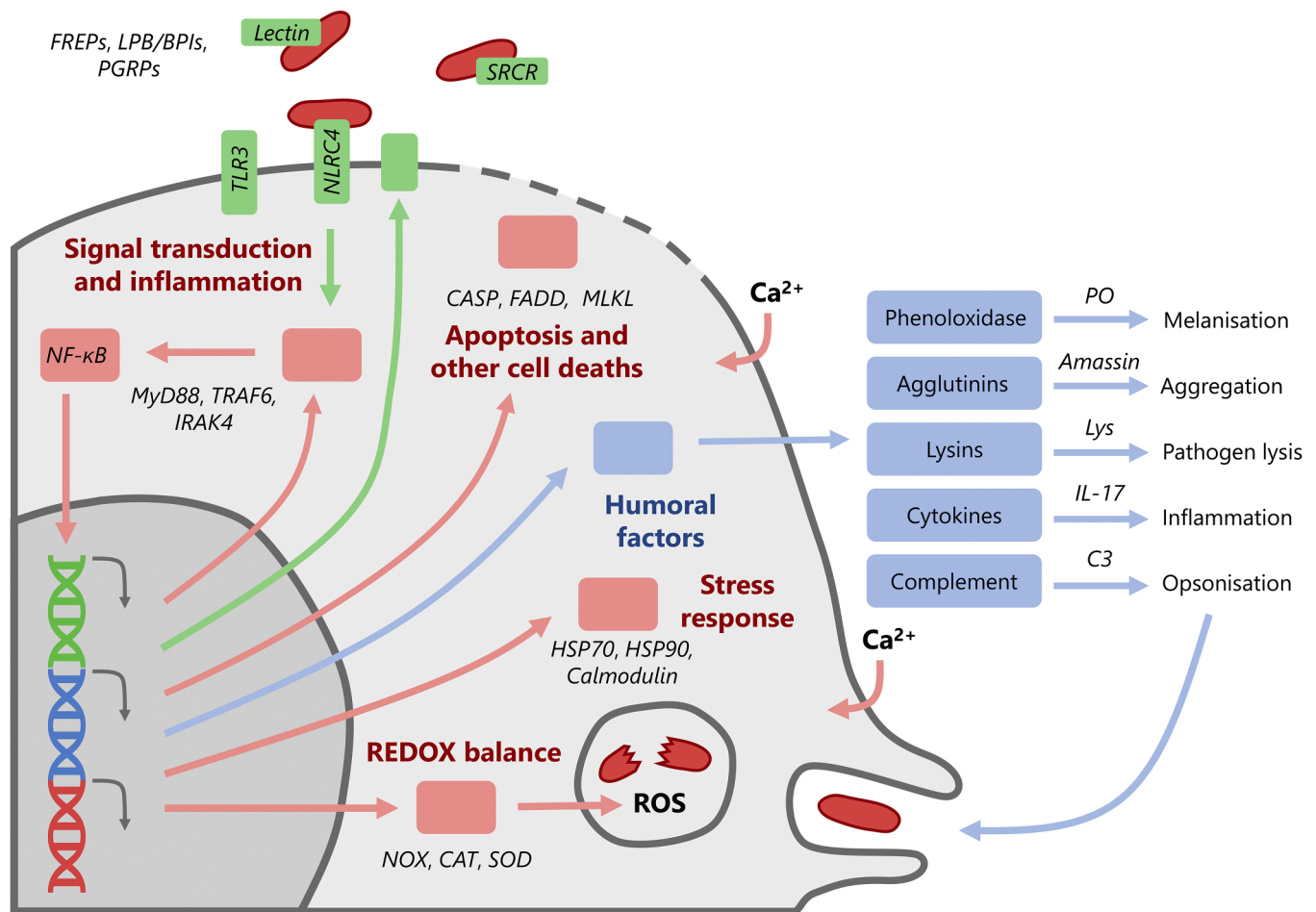


Fig. 3. Molecular actors of the immune system in holothuroids. The diagram is based on Table 2, dividing the molecular actors into three broad categories: non-self recognition in green, regulation of the immune response in red and humoral factors in blue. Note that for the readability of the diagram, only the main molecular actors were kept for each category. Upon recognition of pathogenic bacteria (in red) by pathogen recognition receptors, a transduction signal is activated, leading to the expression of many immune genes, notably via the NF- κ B transcription factor. Expressed genes include additional pathogen recognition receptors, genes involved in inflammation, apoptosis, redox balance and stress response. In addition, humoral factors are secreted in the extracellular environment, leading to different functions, notably the promotion of phagocytosis via the complement.

systematically include all tissues in the analysis. This means that caution should be exercised when interpreting these proportions for tissues that were included in only a few studies, such as gonads (only five studies; Fig. 2D).

The following section presents the function of different immune genes per broad functional category and gene family. It should be noted that we decided not to take into account miRNAs in this list, as these are rather involved in the regulation of the expression of effector genes and that they were recently reviewed [97].

Pathogen recognition receptors

In holothuroids, a high diversity of pathogen recognition receptors (PRRs) has been identified, many of which are highly conserved in metazoans. These include, among others, lectins, Toll-like receptors (TLRs), NOD-like receptors (NLRs), Scavenger receptor cysteine-rich domain-containing proteins (SRCRs) and Fibrinogen-related proteins (FREPs). Although genomics-wide analyses on the diversity of PRRs are currently lacking for most of these gene families in holothuroids, it should be noted that in sea urchins, a huge diversity of these genes has been highlighted, with 222 genes coding for TLRs, 208 genes for NLRs and 218 genes for SRCRs in the genome of *Strongylocentrotus purpuratus* [98]. This unique diversity of PRRs was proposed to result from a functional expansion of immune genes in echinoderms, leading to a

non-adaptive but highly complex immune system [6,99].

Lectins. Lectins are glycoproteins that are specialised to bind sugar motifs, which are often part of prokaryotic PAMPs [26]. Different types were identified, including C-type lectin [100–102], Mannan-binding C-type [103], Fucoselectin [76], Galactose-binding C-type lectin in *A. japonicus* [81] and T-antigen-specific lectin in *H. scabra* [104,105]. While these lectins preferentially bind different targets (i.e., different sugar residues such as mannose or galactose), they are secreted in body fluids and generally have a function in pathogen agglutination, which also confers them an agglutinin function. Mannan-binding C-type lectin was also proposed to have a function in the activation of complement through the lectin pathways [103,106]. More recently, lectin diversity was assessed at the genomic level in *H. leucospilota*, revealing 133 genes encoding lectins [96]. These lectins were classified into five different families based on their domains: C-type lectins (112 genes), S-type lectin (2 genes), F-type lectin (1 gene), SUEL-like lectin (17 genes) and finally malectin (1 gene). Note that in comparison to other echinoderm classes and holothuroid species, the number of genes encoding C-type lectins was particularly high in *H. glaberrima* and *H. leucospilota*, suggesting a gene expansion of this gene family in the *Holothuria* genus [96]. Interestingly, these different lectin families displayed distinct expression patterns, with C-type lectin being predominantly expressed in

coelomocytes, the two SUEL-like lectins being specifically expressed in the body wall, and one C-type lectin being predominantly expressed in the ovaries. These different patterns are suspected to reflect tissue-specific immune defence, respectively “internal” and “external” defence, with the ovary-specific lectin being putatively transmitted maternally to ensure immunity at early embryonic stages [96]. However, the involvement of all these lectin genes in immune defence remains to be functionally proven.

Toll-like receptors. Toll-like receptors (TLRs) are transmembrane glycoproteins known to be activators of many innate immune signalling pathways upon recognition of proper ligands. Two main TLRs were identified in *A. japonicus*, including TLR and TLR3 [107]. While their expression patterns differ between tissues, the highest expression levels of both genes were observed in the respiratory tree. In addition, their expression was upregulated in coelomocytes after lipopolysaccharides (LPS), peptidoglycan (PGN), Zymosan A and Polyinosinic: polycytidylic acid (poly(I:C)) challenges [76,107].

NOD-like receptors. NOD-like receptors (NLRs) are also highly conserved PRRs that are generally intracellular [108]. Three main types of NLRs have been identified in holothuroids: NLRP3, NLRP10, and NLRC4 [109–112]. While NLRP3 is intracellular and acts seemingly as in vertebrates, NLRP10 and NLRC4 both contain immunoglobulin and transmembrane domains. These last features appear to be specific to holothuroid NLRs [108,109]. While NLRP3 promotes inflammation [112], NLRC4 promotes phagocytosis [109], and NLRP10 functions as an immune response regulator [111]. NLRP3 and NLRP10 exhibited maximal expression in coelomocytes, whereas NLRC4 showed its highest expression in the intestine. Transcriptomic analyses on coelomocytes of *H. scabra* suggested that this family has undergone massive expansion in holothuroids, although further investigation at the genomic level is required to confirm this [36].

Scavenger receptors. Scavenger receptors (SRs) are a broad family of secreted proteins which can bind a wide spectrum of PAMPs [113]. SRs were initially described based on their function rather than their structure, resulting in a large, heterogeneous gene family. Among them, Scavenger receptor cysteine-rich domain proteins (SRCRs) are considered to be the most significant in the non-self recognition of echinoderms [113]. Although some of these proteins were found to be overexpressed following diverse immunological stress in holothuroids [76,78], still little is known about their underlying mechanism of action in these organisms. Several studies notably reported one gene coding for Deleted in malignant brain tumours 1 protein-like (DMBT1) in *A. japonicus* and *H. scabra*, which is known to contain an SRCR domain [36,114]. This gene was reported to be overexpressed following LPS challenge in coelomocytes of *H. scabra*. In addition to SRCRs, diverse SRs were described, including CD36 and Scavenger receptor B (SR-B) [76,115]. Both were also overexpressed in coelomocytes after immunological challenge. Furthermore, it was shown that SB-R is capable of binding LPS, PNG and Mannose and has the highest expression in the intestine [115].

Other pathogen recognition receptors. Some proteins are specialised to bind specific PAMPs. Two Lipopolysaccharide-binding protein/bactericidal permeability-increasing proteins (LBP/BPI1 and 2) were described in *A. japonicus*. Although these two were up-regulated in coelomocytes following immunological stimulation, they probably have distinct modes of action, as only silencing LBP/BPI1 resulted in a reduction of reactive oxygen species (ROS) production [116]. In addition, while the expression of LBP/BPI1 was maximal in the intestine, the expression of LPP/BPI2 was maximal in the tentacles [116]. Moreover, several Peptidoglycan recognition proteins (PGRPs) were identified in *A. japonicus* [117–119]. These proteins are secreted and have been

identified to bind PNG or bacteria, but not LPS. They have a function in the recognition of bacteria and agglutination [117]. Some were also reported to interact with other soluble humoral factors, such as complement systems or other PRRs, suggesting a potential function as immune response modulators [118,119].

Fibrinogen-related proteins (FREPs) were identified in *A. japonicus* to play a critical function in pathogen recognition and phagocytosis [76,114,120]. The expression of different FREPs seems to be tissue-specific, but particularly high in coelomocytes among the different tissues analysed [120,121]. This is consistent with the fact that a FREP was used to specifically target coelomocytes by fluorescent *in situ* hybridisation in *H. glaberrima* [122]. Recently, it was demonstrated that FREPs represent a large family of genes that has undergone gene expansion events in holothuroids [121]. Some of these were reported to contain a Til-domain (Trypsin-like inhibitor), which would confer on them their PRR function.

A Globular Complement component 1q Receptor (gC1qR) was described in *A. japonicus* as being able to bind LPS, PNG, and Mannose [123]. This PPR is thought to activate the complement system through the “classical pathway” [106]. An Apexrin C-terminal (ApeC)-containing protein (ACP) was identified for the first time as a pathogen recognition receptor in invertebrates using *A. japonicus* as a model [124]. This transmembrane protein recognises bacteria and participates in the subsequent activation of their internalisation for phagocytosis by coelomocytes. Finally, two mannose receptors, namely a C-type mannose receptor 2-like (MRC2) and a Macrophage mannose receptor 1-like (MMR1-like), were shown to be up-regulated in coelomocytes of *H. scabra* following LPS exposure *in vivo* [36]. However, these two receptors remain to be fully characterised.

Humoral factors

Humoral factors are molecules that are mainly secreted in the extracellular environment by immune cells to play diverse functions in the immune response [26]. These are generally classified according to their function. However, it should be noted that some can play different functions at the same time. Agglutinins are molecules which participate in the aggregation process of pathogens and between immune cells. Lysins generally bind the pathogen membrane in order to increase its permeability, leading to its lysis. Cytokines are the messengers of the immune response, promoting inflammation and recruiting immune cells. Opsonins contribute to the lysis of pathogens by marking them for elimination by immune cells, thus promoting phagocytosis. Finally, other humoral factors were reported to have specific functions, such as Phenoloxidase, participating in the melanisation process.

Agglutinins. Diverse proteins were suggested to have a function in pathogen aggregation associated with their pathogen recognition receptor function (e.g., lectins, PGRPs and FREPs). However, only a few proteins are known to be directly responsible for the aggregation process between coelomocytes in holothuroids. In echinoids, it was shown that two proteins, namely Amassin, an olfactomedin domain-containing protein, and arylsulfatase, were involved in cell-to-cell adhesion during the immune response [125,126]. Amassin is a secreted protein capable of forming disulfide bonds and polymerising to form bridges between coelomocytes [126]. Arylsulfatases are cross-linked to sulphated polysaccharides at the coelomocyte surface [125]. It was suggested that these two proteins interact with each other in the cell-to-cell adhesion system. Cross-links of arylsulfatase were shown to be calcium-dependent since adding chelating agents such as EDTA could inhibit their effect. In addition, reducing agents such as dithiothreitol have the same effect of aggregate dissociation by depolymerising disulfide bonds of amassin. Although these functions have not been demonstrated in holothuroids yet, homologues of the two proteins were reported to be overexpressed following LPS challenge in coelomocytes in *A. japonicus* and *H. scabra* [36,76]. In addition, a Galactose-binding

C-type lectin (GBCL) was identified in *A. japonicus* to play a crucial role in coelomocyte aggregation [81]. This lectin is mainly produced in the intestine and accelerates the coelomocyte aggregation. Adding galactose to the extracellular medium could inhibit this effect, suggesting that the activity of this enzyme depends on the recognition of this ligand at the cell surface [81].

Lysins and antimicrobial peptides. Three main types of lysins were identified in holothuroids: Antimicrobial peptides (AMPs), Lysozyme, Lysin motif-containing protein (LyM) and Whey acidic domain-containing protein (WAP). AMPs are short peptide chains of a dozen residues, highly conserved in the tree of life and with various actions [127]. In holothuroids, three AMPs were identified, and all are cationic AMPs. Two were identified in *Holothuria tubulosa*, namely holothuroidin 1 and 2 [22] and one in *A. japonicus*, AMP SCAK33 [128]. These positively charged amphipathic AMPs are capable of interacting with the negatively charged pathogen membrane, resulting in membrane instability and eventually cell lysis. Antimicrobial and anti-biofilm activities were experimentally demonstrated for Holothuroidin 1 and 2 [22,129].

Lysozymes are molecules that catalyse the hydrolysis of peptidoglycan contained in the bacterial cell walls and thus have a significant and highly conserved antimicrobial function. Different types of lysozyme were reported in holothuroids, including I-type and C-type lysozymes [130–134]. These two lysozymes were recombinantly produced and proved to have an effective growth-inhibitory effect on Gram-positive and Gram-negative bacteria, but a significant lytic activity only for Gram-positive bacteria [131,134]. An I-C-type lysozyme would also have a function in salinity stress response, notably by regulating the expression of NKA and NFCC1, two genes involved in ion transport [133,134]. In addition, Li et al. [132] reported a I-type lysozyme would also have an inhibitory effect on coelomocyte apoptosis following stimulation with PAMPs. While they were ubiquitously expressed across tested tissues, their maximum expression was recorded in the tentacle and tube feet, respectively [132–134]. However, it was recently shown that the genome of two holothuroid species, namely *H. leucospilota* and *H. scabra*, encodes a higher number of lysozymes, among which some showed tissue-specific expression, especially in the intestine, where they mediate bacterial digestion and shape intestinal microbiota [63]. Some were, however, specific to coelomocytes and showed an effective inhibitory effect against Vibrionaceae bacteria.

In addition to these types of lysins, a Lysin motif-containing protein (LyM) was identified in *A. japonicus* as an intracellular protein [135]. While this protein could bind PAMPs and had its maximum expression in coelomocytes, its lytic effect remains to be tested. Finally, a WAP-domain-containing protein was described in *A. japonicus*, which shows a higher expression following immunological challenge and has an antibacterial effect against several pathogenic bacteria [136].

Cytokines. Different types of cytokines were reported in holothuroids. In the species *H. leucospilota*, a transcriptomic analysis of coelomocytes revealed the presence of 21 genes classified into four main cytokine families, including Interleukin 17-like (IL-17), B-cell lymphokine-like (BCL/CCL), Erythroid differentiation-related factor 1-like (EPRF1) and Thrombospondin and Thrombopoietin-like (TSP and TPO, respectively) [80]. Among these, IL-17 were particularly overexpressed after immunological challenge with various PAMPs. Furthermore, Lv et al. [137] characterised IL-17 and its receptor in *A. japonicus* and showed that their interaction could activate coelomocyte migration and proliferation through the TRAF6 axis. Another type of cytokine, namely Transforming growth factor beta (TGF- β), was identified in *A. japonicus*, which regulates inflammation and promotes tissue repair [138]. Eventually, an LPS-induced TNF- α factor (LITAF) was overexpressed after immunological challenge in coelomocytes [76,139]. While further research is needed to uncover the diversity of cytokines in holothuroids, these few studies highlight the central function of IL-17 in immune response

activation.

Opsonins and complement. The complement system is an important molecular network that participates in opsonisation [5]. In holothuroids, many homologues of the complement were identified, including complement C3 [74,140], B factor [76,141,142] and H factor [76]. In addition, ficolin, which is known to participate in the initiation of this system in other organisms via the “lectin pathway” [106], was identified in sea cucumbers [143]. Opsonisation of the complement is thought to function partly as in vertebrates, in which the cleavage of the plasmatic element C3 leads to a self-amplifying loop via the “C3 convertase” activity of BF and C3b fragment complex, resulting in a massive and fast labelling of cells/pathogens to phagocytosis [106]. Different activation pathways were proposed in holothuroids, including the “classical pathway” via the Globular complement component 1q (via gC1qR; [123]), the “lectin pathway” through MBL [103] or the “alternative pathway” via ficolin and the Mannose-associated serine proteases (MASP) [106]. However, although C3, BF and HF, as well as gC1q receptor (gC1qR) and MBL were identified in holothuroids and were overexpressed following immunological challenge, to date, homologues of gC1q and MASP were not identified, as well as a C3 convertase activity [106]. Further research will thus be necessary to unravel the exact mechanisms of the complement in holothuroids.

Phenoloxidase. Phenoloxidase (PO) is the central enzyme of the melanisation process, which is highly conserved in the immune defence of invertebrate organisms [144]. This mechanism lies in the fact that melanin surrounds the foreign body and prevents, or at least limits, its growth and mobility. The entire signalling also leads to the production of reactive and toxic compounds that help fight the pathogen [144]. PO is critical in this process as it catalyses the conversion of phenols into quinone, which can then polymerise to form melanin [145]. This protein is initially secreted as prophenoloxidase, which then has to be activated by a serine protease cascade. PO activity was detected quite early in holothuroids [146]. However, the first molecular identification of protein sequences was only carried out in 2014 by Jiang et al. in *A. japonicus*, which revealed three different POs [147–150]. These were all reported to be laccase-type phenoloxidases, and their expression was significantly increased following immune challenges with various PAMPs [147,149]. However, these three proteins showed different domain architectures and different expression patterns across tissues; while POI had its maximal expression in coelomocytes, POII and POIII were more expressed in tube feet [147,149]. Their activity was also demonstrated to be calcium-dependent, and they were able to inhibit bacterial growth [150]. POI and POIII were reported to have a trans-membrane sequence, while all have a signal peptide [148,150]. Considering its high expression in coelomocytes, POI is considered the key phenoloxidase in the immune response of holothuroids [149]. In addition, a laccase-type PO was also reported in *H. scabra*, in which it was significantly overexpressed after LPS challenge [36]. Finally, PO was also reported to activate phagocytosis, as silencing its expression could decrease the coelomocyte phagocytic rate [151].

Immune response regulator

Immune response is a highly regulated mechanism because it sometimes has to respond quickly to an invader or a stress, but it must also not get carried away; for example, it can produce a large amount of ROS that can also be detrimental to the host organisms [152]. It thus relies on a large number of intermediate proteins to ensure this function of regulation.

Inflammation. Many different intermediates were described in the cascade of inflammation, which generally leads to the production of humoral factors. As in vertebrates, this cascade is mainly articulated around the nuclear factor kappa B (NF- κ B). In holothuroids, homologues

of NF- κ B rel and p105 were described in *A. japonicus* and *H. leucospilota* [153,154]. In this last species, it was demonstrated that its expression in HEK293T cells could inhibit apoptosis and promote the expression of IL-1 β and TNF- α , as it does in vertebrates, suggesting a conserved function across deuterostomes [153]. Some intermediate factors of the Toll signalling cascade, resulting in the activation of NF- κ B, could be identified, including the Myeloid differentiation factor 88 (MyD88), Inhibitory kappa kinase (IKK β) and TNF receptor-associated factor 6 (TRAF6) [155–157]. The expression of these genes led to the same result of apoptosis inhibition and production of cytokines [155,157]. In addition, Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) was identified as a promoter of NF- κ B-induced inflammation in *A. japonicus* after bacterial challenge [158]. Notably, histopathological analysis showed that silencing MALT1 could reduce *V. splendidus*-induced inflammatory damage. On the contrary, a Peroxisome proliferator-activated receptor α (PPAR α) was reported to inhibit the NF- κ B/Rel pathway [159]. This gene is particularly expressed during the late immune response and is supposed to stop inflammation. Its inhibition could notably reduce the expression of IL-17 and NLRP3 [159]. In NF- κ B signalling, two proteins, namely Major yolk protein (MYP) and Heat shock cognate protein 70 (HSC70), were described in *A. japonicus* to promote inflammation by binding MyD88 [160]. Another important transcription factor was recently described to have a function in the regulation of the immune response during bacterial infection, namely Interferon regulatory factor 8 (IRF8). In *A. japonicus*, silencing this gene could significantly increase the intracellular load of bacteria in coelomocytes and inhibit their apoptosis [161].

In addition to these transcription factors, several genes were described to have a function in the inflammation process in general. A homologue to Interleukin 1 receptor-associated kinase 4 (IRAK4) was identified in *A. japonicus*, and silencing its expression could promote apoptosis and inhibit the expression of TLRs [162]. A gamma-interferon-inducible lysosomal thiol reductase (GILT) was identified in *Stichopus monotuberculatus* and is known in vertebrates to have an antigen presentation function to naive T-cells [163]. It was significantly overexpressed following an immunological stress and could interact with NF- κ B. TNF receptor-associated factor 3 (TRAF3) was reported in *A. japonicus*, and it can inhibit the production of ROS during the immune response [164]. Interestingly, its expression was decreased after immunological challenge with *Vibrio* bacteria or LPS, suggesting a regulatory function in the respiratory (oxidative) burst [76,164].

Some genes were directly identified to regulate the production of some humoral factors. A double Ig interleukin-1 receptor-related (DIG-IRR) homologue was reported in *A. japonicus* to regulate inflammatory cell chemotaxis and vacuolisation and alleviate IL-17 expression [165]. A pacifastin-like gene was described in *A. japonicus* and regulates phagocytosis by inhibiting the melanisation cascade leading to the activation of PO [83,151]. A p21-activated kinase 1 (PAK1) was reported to regulate inflammation through Rac1 and MKK3/6 signalling and promote the release of lysozyme [166]. β -Catenin was described in *A. japonicus* to promote inflammation; more specifically, silencing β -Catenin reduced the expression of several inflammation factors [167]. Finally, the expression of other genes known to have a function in inflammation in vertebrates was reported; however, their precise function is still unclear in holothuroids. These include TANK-binding Kinase1-like (TBK1-like) and Baculoviral inhibitor of apoptosis repeat-containing 2 (BIRC2) [76,139].

Cell death regulation. Programmed cell deaths, such as apoptosis, are also highly regulated processes in the immune response. In holothuroids, numerous genes are involved in apoptosis regulation, with some highly conserved across metazoans, including FAS-associated Death Domain proteins (FADD), B-cell lymphoma 2 (BCL-2), Bcl-2-associated X (BAX), Cathepsin B and several types of Caspases. FADD was described in *H. leucospilota* and *A. japonicus*, in which it promotes apoptosis and is

ubiquitously expressed across tested tissues, but with a maximal magnitude in coelomocytes [168–170]. This gene was also overexpressed after immunological challenge with diverse PAMPs [169]. Bcl-2 and BAX were both described in *A. japonicus* and had antagonist activity: while Bcl-2 inhibits apoptosis, BAX promotes it and while the expression of BAX was increased following immunological challenge, the expression of Bcl-2 was decreased, resulting eventually in promoted apoptosis after the immunological challenge by these two genes [171,172]. In addition, it was shown that the protein Myelocytomatosis viral oncogene (MYC) could promote apoptosis by targeting BAX [173]. Cathepsin B was identified in *A. japonicus*, in which it was significantly overexpressed in coelomocytes after immunological challenge with *V. splendidus*, and silencing this protein significantly inhibits coelomocyte apoptosis [174].

As in vertebrates, diverse caspases were described to play a central function in apoptosis. These include caspase 1, 2, 3, 6, 8 and 9, which were identified in *A. japonicus* and *H. leucospilota* [76,175–181]. In *H. leucospilota*, it was shown that expression of caspase 6 and 8 could be induced by exposure to PAMPs [176,179], while expression of caspase 9 was rather induced by abiotic stress (i.e., heavy metal exposure [175]), all having the final effect of promoting apoptosis. In *A. japonicus*, caspases 2 and 8 were reported to have a correlated expression and were thought to respectively initiate the expression of effector caspases 6 and 3 [176]. Their expression was all increased following immunological challenge, but at different time points [176]. Finally, Caspase 1 was described in *A. japonicus*. This protein contained an LPS-binding site and would have a function in pyroptosis, i.e., a particular apoptosis type promoting the release of cytokines [182].

Other proteins were identified to have a function in apoptosis regulation. For instance, Silencing β -Integrin could promote apoptosis via the expression of caspase 3 [177,183]. CCAAT/enhancer-binding protein (C/EBP) homologue protein (CHOP) was reported to promote apoptosis via the p38-MAPK pathway [184]. Finally, two proteins, namely Mixed-lineage kinase domain-like protein (MLKL) and Receptor-interacting protein kinases (RIPKs), were described as being involved in apoptotic and necroptotic cell deaths [185]. However, it was shown that MLKL induces lytic coelomocyte death in a cleavage-dependent manner, but not dependent on RIPK phosphorylation as in mammals [186]. A ferroportin was reported to have a key function in ferroptosis within infected cell death, which mediates the iron-mediated lipid peroxidation leading to bacterial lysis [187,259]. Furthermore, Shao et al. [188] described that MLKL protein was necessary for inducing the formation of extracellular traps (ETs), a process also known as ETosis, which constitutes a distinctive cell death other than necroptosis and apoptosis (see dedicated Section Programmed cell deaths). Finally, two proteins containing Death domains, namely Death domain-containing NLR (DD-NLR) and Death domain-containing Caspase (DD-CASP), were reported to actively participate in *V. splendidus*-induced lytic cell death [189].

Redox balance. Redox balance plays a crucial role during the immune response, especially because oxidative compounds such as ROS are generally produced by immune cells in a process called respiratory burst to degrade pathogens [190]. This phenomenon occurs mainly within phagocytic cells, in which hydrogen peroxide (H₂O₂) and nitric oxide (NO) are generated [23]. These compounds, being highly reactive, alter other molecules such as DNA and are toxic to any organism at high concentrations, hence their antimicrobial properties [190]. However, when the respiratory burst is not adequately controlled, it can also create oxidative damage to the host tissues and thus needs to be finely regulated. Enzymes responsible for this regulation are highly conserved across organisms, with NADPH oxidase (NOX) and Dual oxidase (DUOX) responsible for the production of H₂O₂ and Nitric oxide synthase (NOS) responsible for the production of NO [191]. Other proteins are involved in the mitigation of ROS production, thus having an important

antioxidant function. Superoxide dismutase (SOD) converts superoxide radicals ($2O_2^-$) into H_2O_2 . This latter can be handled by ROS-detoxifying enzymes, namely Catalase (CAT) and Glutathione peroxidase (GPX) [191].

In *A. japonicus*, a NOX enzyme is required for the production of ROS, which promotes bacterial clearance by coelomocytes [192]. In addition, a NOS protein was reported in the same species, and its expression could be regulated by the NF- κ B transcription factor [193]. It was also shown that NOS can promote apoptosis through the NF- κ B signalling pathway [194]. Regarding SOD, a Cu/Zn SOD is known in *A. japonicus* to reduce H_2O_2 and LPS-promoted apoptosis [195]. Catalase was also identified in different tissues in *A. japonicus*, with the highest expression in the intestine [196]. Later, it was shown that knocking down its expression significantly increases ROS levels in the intestine, with the effect of altering the microbiota [197]. Finally, while GPX has never been finely characterised in holothuroids, it was reported to be up-regulated following LPS exposure in coelomocytes of *H. scabra* [36].

In addition to these central enzymes, a thioredoxin has been reported to have antioxidant activity during the immune response [198]. A Tissue inhibitor of metalloproteinases 1 (TIMP1) promotes ROS-mediated immune response against pathogenic bacteria [199]. A glutathione S-transferase (GST), known to be coupled with the GPX antioxidant response, was reported in *A. japonicus*, and it can reduce apoptosis during immunological challenge [200]. Finally, Betaine-homocysteine S-methyltransferase (BHMT) was reported to regulate ROS production during the immune response [201]. In normal conditions, this enzyme converts homocysteine, a metabolite promoting ROS production, into betaine. During an immune response, miRNA-mediated downregulation of BHMT results in a greater production of ROS [201].

Stress response proteins. Many molecules are known to interact with the immune system, although they are not properly considered as immune molecules. One big family of these molecules is Heat shock proteins (HSPs), which were initially described to be involved in response to thermal stress [202] but were then reported to be involved in the response to many other types of stress, including abiotic (e.g., salinity stress: [203] or immune stress [36,76]). For instance, Dong et al. [76] reported that cytosol-type HSP70 and HSP90 were both overexpressed in coelomocytes after LPS challenges at 4, 24, and 72 h post-injection. In addition to HSP70, HSP26 was also shown to be overexpressed 24 h post-LPS challenge in the coelomocytes of *H. scabra* [36]. In fact, it was shown in other lineages that HSPs, which are highly conserved proteins in metazoans, play, in addition to their chaperone function, the role of DAMPs when they are released in the extracellular environment, leading to the initiation of an immune response [204]. In addition to HSPs, some proteins are involved in calcium-mediated signalling, which directly impacts coelomocyte aggregation [205]. Indeed, during an immune response, an increase in intracellular calcium concentration leads to the activation of several processes, including activation of cell mobility and aggregation. In this way, proteins involved in this signalling can be considered as immune regulators. Among the most well-known, Calmodulin was described in *S. monotuberculatus* to play a role in the immune response by promoting the release of NO [206].

Cellular actors of immunity

Coelomocyte distribution and production

In echinoderms, immune cells are named coelomocytes because they occur in coeloms. These coelomocytes are primarily found suspended in the body fluids, lying in the lumen of three cavities: the perivisceral cavity, the hydrovascular system, and the haemal system [4]. In these fluids, coelomocyte concentrations generally exceed one million cells per millilitre [29,36,207,208], and under certain conditions can reach several dozens of millions of cells per millilitre [33]. However,

coelomocytes from these three compartments have not been studied equally: while the perivisceral fluid has been the primary focus of most studies investigating coelomocytes, the hydrovascular fluid has received comparatively less attention, with the haemal system coelomocytes being mainly reported in histological studies [209]. Importantly, distinctions among coelomocytes from different body fluids must be considered, as several comparative studies have revealed major differences in cell-type composition across compartments, especially between hydrovascular and perivisceral fluids [30,33,36,207]. This also underscores the risks associated with blind puncture for coelomocyte extraction, as such approaches may yield highly variable results depending on the compartment sampled [210]. Interestingly, growing evidence suggests that coelomocytes can transfer between compartments, a phenomenon thought to be involved in various physiological processes, including encapsulation [30]. For example, in *C. frondosa*, coal particles injected into the hydrovascular system were detected in the perivisceral cavity within a few hours [30]. More recently, antibody-labelled coelomocytes injected into the hydrovascular system of *A. japonicus* were shown to migrate into the perivisceral fluid [211]. However, the pathway by which these cells migrate from one compartment to another remains uncertain.

Beyond body fluids, coelomocytes have been found associated with all tissues, particularly the integument and intestine, notably during regeneration processes [45,46,122,212]. This widespread tissue association highlights their role as sentinel cells, functionally analogous to resident macrophages in vertebrates. However, despite extensive documentation of their distribution, little is known about coelomocyte production or the mechanisms governing their recruitment to different sites. It is widely accepted that coelomocytes originate from mesodermal tissue [4,13,62]. Based largely on morphological observations, several tissues have been proposed as potential hematopoietic sites, including the haemal system (mainly the rete mirabile), respiratory tree, Polian vesicle, coelomic epithelium, and stone canal [27,30,214–216]. However, these hypotheses were primarily inferred from the presence of poorly differentiated coelomocytes within these tissues, but functional evidence is still sparse. More recently, an EdU labelling assay for proliferative cell identification demonstrated active cell division in the rete mirabile, respiratory tree, and Polian vesicle, identifying these structures as the most likely hematopoietic tissues [217]. Notably, some circulating coelomocytes were also EdU-positive, suggesting a potential capacity for self-proliferation [217]. In addition to this study, transcriptomic analyses have further supported a role for the Polian vesicle in coelomocyte production [218–221]. This is consistent with the fact that, during evisceration, the Polian vesicle is preserved while other viscera are expelled, supporting its proposed function as an “immune stock” [220,221]. However, precise identification of hematopoietic tissues remains uncertain. Finally, it is worth noting that while coelomocyte ontogeny and the gene regulatory network leading to their differentiation have been extensively studied in early developmental stages of sea urchins [6,213], comparable studies on sea cucumbers are lacking.

Status and function of coelomocyte morphotypes

Attempts to classify coelomocytes were initiated more than a century ago (Cuénot, 1891 [214]; Kindred, 1924 [28]). Since then, many different authors have proposed their own classification [4,6,27,29,215,222–225]. The last review aiming at summarising the diversity of coelomocytes in holothuroid reports 9 morphotypes [29]. Nonetheless, most criteria used to distinguish cells remain morphological, partly because functional criteria are lacking. This makes coelomocyte classification of limited use, notably when interpreting results of coelomocyte count in stress response studies. For instance, many studies aim at evaluating different types of stress, including ecological ones, by a change in the concentration/proportion of the different coelomocyte populations [30,39,45,226]. But the lack of information about the

function of the different cell types makes these results difficult to interpret physiologically. Furthermore, as no consensus was established, the coelomocyte classification is currently confusing, resulting in a high heterogeneity of terminology across studies and species. An updated functional classification would therefore be of great interest not only for fundamental immunology but also for a better interpretation of stress response studies.

In recent years, different teams have sought to shed light on coelomocyte diversity and function, trying to adopt integrative approaches to characterise these cells. Overall, the main cell types that were reported in the literature can be classified in three categories according to the current status: (i) confirmed cell types – cell types consistently described across studies and species and for which the status of coelomocytes is not questioned; (ii) controversial cell types – cell types for which recent advances have led to a paradigm shift in the nature and/or the function of the cells; and (iii) uncertain cell types – inconsistently described across studies and for which additional investigations are needed to confirm their nature as coelomocytes (Table 3). Although this classification aims to provide a broad overview of the status of the different morphotypes, it is worth noting that almost none can be considered fully validated until robust criteria, such as molecular markers, are available

to define them. We also want to emphasise that the terminology used here reflects, to the best of our knowledge, the most up-to-date nomenclature. However, given the diversity of terms used in the literature, we have added **Table S1** to summarise the various synonyms, as well as the morphological and putative molecular criteria that we consider diagnostic for each morphotype.

Confirmed cell types

Phagocytes

Phagocytes, or (phagocytic) amoebocytes, are recognised in most investigated species ([29] for review of species distribution), where they are often one of the dominant cell types ([36,227,228]; Fig. 4A-F). This cell type is generally considered the biggest in size, at least when considering their pseudopodia, with a diameter ranging from 10 to 40 μm [4]. Several subtypes of phagocytes are described, including petaloid phagocytes (also called bladder-like or lamellipodial phagocytes; Fig. 4A-C) and filiform phagocytes (also called filapodial phagocytes; Fig. 4D-F). However, it was suggested that these two subtypes could be two forms of the same cell type, filiform phagocytes being the activated ones [36,229]. Phagocytes are thought to be a key coelomocyte type in

Table 3

Different coelomocyte types in sea cucumbers and their associated functions. Coelomocyte types are classified into three categories according to their status in the literature: confirmed cell types – cell types consistently described across studies and species; controversial cell types – cell types for which recent advances have led to a paradigm shift in the nature and/or the function of the cells; and finally uncertain cell type – inconsistently described across studies and for which additional investigation are needed to confirm their nature of coelomocytes. For abundance: fresh means in freshly collected samples, while late means in later cell cultures/punctured fluid samples (+: low, ++: middle, +++: high; variable: inconsistent between studies). For species distribution, “variable intra-sp.” and “inter-spp.” mean, respectively, variable between individuals within the same species and between different species. For body fluid distribution, “no spe.” means no specific distribution between the different body fluids; HF – hydrovascular fluid; PF – perivisceral fluid. Confirmed functions are those for which sufficient evidence has been provided to support them, while hypothetical ones are those for which further investigation is required to confirm them (n.d. means no data).

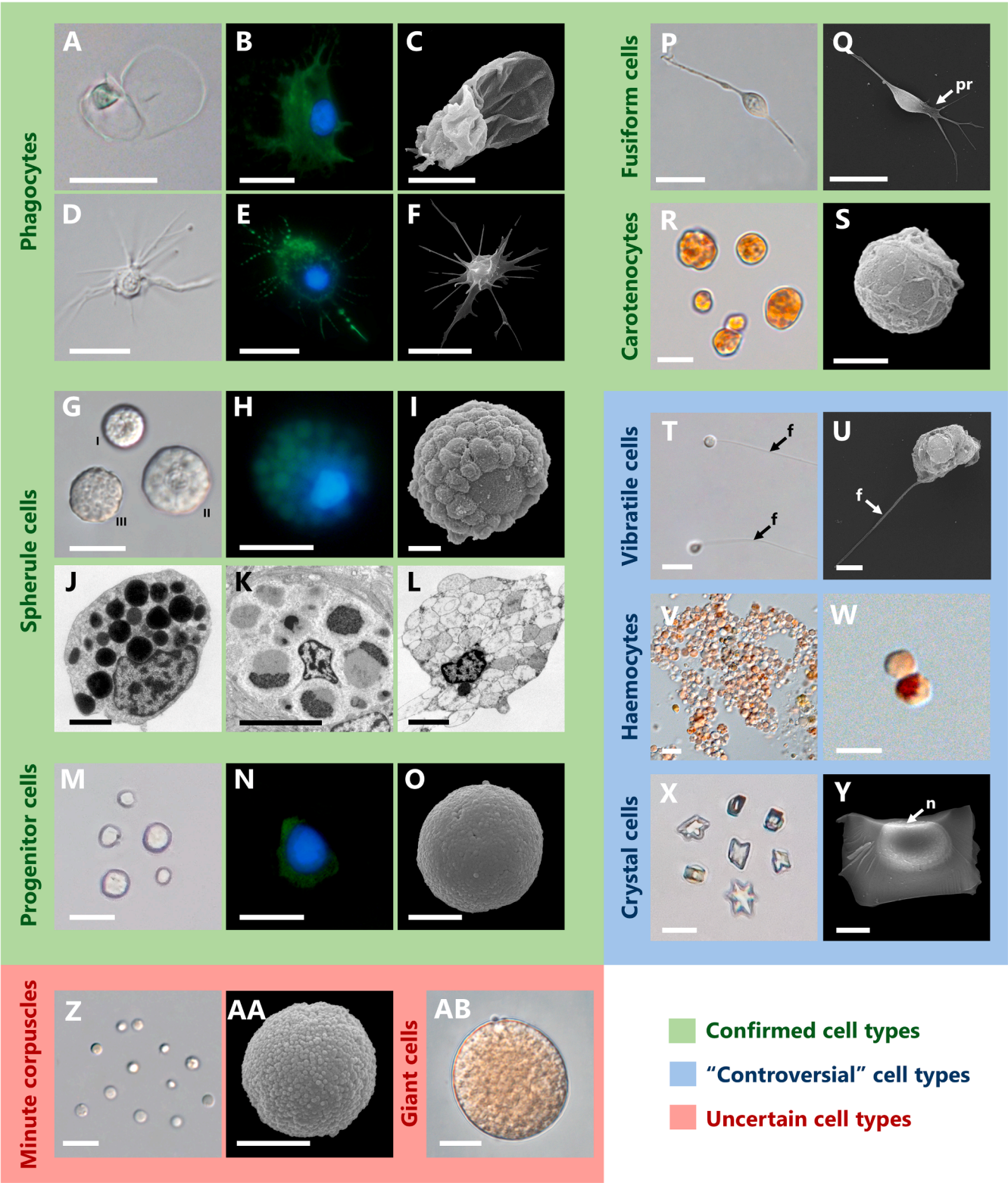
Coelomocyte type	Subtype	New hypothesis/name	Abundance		Species distribution	Body fluid dist.	Confirmed function(s)	Hypothetical function(s)
			Fresh	Late				
Confirmed cell types								
Phagocyte	Petaloid phagocytes		+++	++	Broadly distributed	No spe.	Phagocytosis [208], Aggregation [30], Encapsulation [30]	Wound healing [45], Cytotoxic response [239]
	Filiform phagocytes		++	+++	Broadly distributed	No spe.	Aggregation [229]	Wound healing [45], Cytotoxic response [239]
Spherule cell	Type I	Acidophilic spherulocytes	++	++	Broadly distributed	No spe.	Cytotoxic response [239], Wound healing [46], Encapsulation [257]	Production of humoral factors [237]
	Type II	Morula cells	++	++	Broadly distributed	No spe.	Production of glycosaminoglycans – elements of the extracellular matrix [234], Aggregation [33], Encapsulation [257]	Wound healing [45]
	Type III	Spherulocytes	++	++	Broadly distributed	No spe.	Wound healing [46]	Production of humoral factors [237], Encapsulation [257]
Progenitor cell	None	Small round cells	+++	+++	Broadly distributed	No spe.	Precursor of other coelomocyte types [94], Encapsulation [205]	Phagocytosis [208]
Fusiform cell	None		+	++	Variable – intra-sp.	PF > HF	Encapsulation [30]	Aggregation [33]
Carotenocyte	None		++	+++	Presumably broadly distributed	HF > PF	Encapsulation, storage of carotenoid, antioxidant response [33]	Antibacterial response [33]
Controversial cell types								
Vibratile cell	None	Spermatozoans	Variable		Variable – inter-spp., Variable – intra-sp.	No spe.	n.d.	Coelomic fluid circulation [225], Aggregation [29]
Haemocyte	None	Carotenocytes	Variable		Molpadida and Dendrochirotida	HF > PF	Encapsulation [30]	Oxygenation of tissues [248,249], ROS production [30]
Crystal cell	None	Biogenic microcrystals	+	+	Broadly distributed	PF > HF	Metabolism by-product [31,252]	Osmoregulation [240, 253]
Uncertain cell types								
Minute corpuscle	None		+	+++	Variable – inter-spp.	No spe.	n.d.	n.d.
Giant cell	None		+	+	Variable – inter-spp., Variable – intra-sp.	PF	n.d.	n.d.

the immune response, primarily due to their phagocytic capabilities [208,229,230]. Moreover, they are also reported as being responsible for the early stages of aggregation [30,33,36,205], encapsulation and formation of coloured bodies [30,33,231], ensuring the clearance of the coelomic fluids. Phagocytes were also described to be involved in graft rejection, suggesting a key function in non-self recognition [232]. These

cells are highly positive for ROS, suggesting a function in respiratory burst [33]. Finally, phagocytes were reported to be involved in wound healing [45,233].

Spherule cells

Spherule cells, sometimes referred to as spherulocytes, morula cells,



(caption on next page)

Fig. 4. Ten main coelomocyte morphotypes described in holothuroids and classified according to their status in the literature: confirmed cell types – cell types consistently described across studies and species; controversial cell types – cell types for which recent advances have led to a paradigm shift in the nature and/or the function of the cells; and finally, uncertain cell types – inconsistently described across studies and for which additional investigation is needed to confirm their nature of coelomocytes. **A–C.** Petaloid phagocytes by light microscopy, fluorescent microscopy with cytoskeleton (actin–green) and nucleus (DNA–blue) labelling and scanning electron microscopy (SEM), respectively. A and B are in *Holothuria forskali*, and C is in *Holothuria scabra*. **D–F.** Filiform phagocytes by light microscopy, fluorescent microscopy with cytoskeleton and nucleus labelling and SEM, respectively. In G, the different types of spherule cells can be distinguished based on their size and the size of their granules, as per Queiroz et al. [32]: (i) acidophilic spherulocyte; (ii) morula cell; (iii) spherulocyte. These are in *H. forskali*. **J–L.** Ultrastructure allowing the distinction of different types of spherule cells as per Canicatti et al. [227]: spherulocyte type I in J, spherulocyte type II in K, and spherulocyte type III in L. These are in *H. scabra*. **M–O.** Progenitor cells were observed by light microscopy, fluorescent microscopy with cytoskeleton and nucleus labelling and SEM, respectively. M is in *H. scabra*, and N and O are in *H. forskali*. **P–Q.** Fusiform cells observed by light microscopy and SEM in *H. forskali*. Note that the fusiform cell in Q shows pseudopod ramifications (pr). **R–S.** Carotenocytes by light microscopy and SEM in *H. forskali*. **T–U.** Vibratile cells by light microscopy and SEM in *H. forskali*; note the presence of the flagella (f). **V–W.** Haemocytes in light microscopy (large and close focus) in *Colochirus quadrangularis*. **X–Y.** Crystal cells by light microscopy in *H. scabra* and by SEM in *H. forskali*. Note the implementation of the nucleus (n) in Y. **Z–AA.** Minute corpuscles by light microscopy and SEM in *H. forskali*. **AB.** Giant cell by light microscopy in *H. forskali*. The scale bars represent 1 µm in AA; 2 µm in I, J, L, O, S, U and Y; 5 µm in C, H, K, N, R, W and Z; and 10 µm in A, B, D, E, F, G, M, P, Q, T, V, X, Z and AB.

granulocytes or amoebocytes with granules, are round cells of varying sizes (5 to 20 µm) characterised by the presence of numerous secretory granules – spherules in the cytoplasm (Fig. 4G–L) [27,32,227]. These cells are the most commonly reported coelomocytes in holothuroids, occurring in 79% of species investigated [29]. Some would contain mucopolysaccharides and proteins [227,234]. Canicatti et al. [227] proposed a classification into three subtypes based on the ultrastructure of the secretion granules. Type I spherulocytes have electron-dense, homogeneous granules (Fig. 4J). Type II spherulocytes have large heterogeneous granules with an electron-dense centre and a loose periphery (Fig. 4K). Type III spherulocytes have empty-like, irregularly shaped vacuoles that contain a few residual bodies (Fig. 4L). More recently, Vazzana et al. [235] and Queiroz et al. [32] proposed a new classification of spherule cells using different histological staining and morphometric criteria, facilitating the distinction between subtypes, which previously relied on ultrastructure studies. These included spherulocytes, acidophilic spherulocytes and morula cells, which may match the previous cell types described by Canicatti et al. [227]. Indeed, morula cells, being bigger with larger granules, would correspond to types II, acidophilic spherulocytes with dense granules to types I and spherulocytes to types III [32]. Note that for this last subtype, the empty-like aspect of the granule under transmission electron microscopy (TEM) may be a preparation artefact [227]. It is also important to highlight that while morula cells and spherulocytes were often used to refer to spherule cells in general, in this last classification they constitute different subtypes of spherule cells.

The presumed functions of spherule cells are varied: due to their morphology, it was postulated early that spherule cells degranulate during the immune response to release the content of their granules, including humoral factors [28,222,236]. In addition, spherule cells participate in the formation of coelomocyte aggregates and encapsulation [30,227,237]. Some of these contain glycosaminoglycans similar to those of the extracellular matrix, which would allow them to form an extracellular matrix within cell aggregates during encapsulation to structure them [234]. This observation was consistent with the observation of spherule cells undergoing cell lysis within aggregates and releasing fibrillar materials [212,237,238]. Spherule cells were also reported to have cytotoxic activity: it was suggested they could release prophenoloxidase, responsible for the melanisation process [227]. In addition, spherule cells contain different levels of ROS depending on the subtypes, with spherulocytes showing the highest level and morula cells displaying the lowest [33]. Finally, it was demonstrated that different types of spherule cells are recruited during wound healing and intestine regeneration [45,46,212]. Lacouth et al. [45] recently showed that among spherule cells, acidophilic spherulocytes were the first to be recruited. Owing to their cytotoxic activity [239], they would eliminate any microorganisms at the site of the wound, consistent with their higher ROS level [33]. Then, morula cells intervene and are supposed to release extracellular matrix elements that would participate in tissue

repair [212,234]. Morula cells would therefore have rather a mechanical function, whereas spherulocytes and acidophilic spherulocytes would ensure the cytotoxic response.

Progenitor cells

Progenitor cells are small, round cells (2 to 8 µm) with a large nucleus occupying most of the cell volume ([36,228,240]; Fig. 4M–O). These cells are mainly found in holothuroids, where they can sometimes constitute the predominant cell type [205,228]. They are generally considered to be a reserve of undifferentiated cells that can give rise to other types of coelomocytes rather than active immune cells, hence their name [228,241]. However, this hypothesis is still uncertain, and some authors prefer to use a different name to refer to them, including lymphocytes/lymphoid cells (due to their morphological resemblance to vertebrate lymphocytes [205,224]) or small round cells [33,36]. Taguchi et al. [205] showed that these cells are recruited late during aggregate formation in comparison to other cell types, suggesting an active function in the immune response. Moreover, it was suggested that progenitor cells can show a small phagocytic activity [208]. However, the authors attributed this activity to a subset of progenitor cells differentiating into phagocytes. Finally, recent transcriptomics analysis by scRNA-seq supports the presence of “undifferentiated cells” [93,94]. In particular, the cell population harbouring an undifferentiated appearance and being able to differentiate into other cell clusters based on scRNA-seq-based trajectory analysis was shown to express the PIWI stem cell marker in *A. japonicus* [94]. In addition, PIWI-positive coelomocytes were underscored by immunostaining in the species *Eupentacta fraudatrix* [242]. These molecular studies thus tend to support the hypothesis of progenitor function of undifferentiated cells among coelomocytes.

Fusiform cells

Fusiform cells are elongated cells with two thin opposing cytoplasmic projections (Fig. 4P–Q). They are 15–40 µm long with a relatively small cell body of 3–5 µm in diameter [27,30,33,36]. They are generally not observed in all individuals of the same species, and their number appears to increase with repeated manipulations [240]. Almost no information exists about their function, except that they may be involved in the maturation of aggregates leading to the formation of brown bodies [30]. In *C. frondosa*, they were predominantly found in the perivisceral fluid and were reported to participate in the compaction of aggregates by forming an external layer during the aggregate maturation process [30]. This is consistent with the observation of these cells forming ramifications (Fig. 4U), which could be useful for forming cellular bridges between cells and aggregates [33].

Carotenocytes

Carotenocytes were recently described in *H. forskali* as coelomocytes rich in carotenoids, conferring their characteristic reddish colour ([33]; Fig. 4R–S). Their size ranges from 1.5 to 8 µm, but most cells are around

2.5 µm. While carotenocytes are currently only reported in this species, the authors suggested that they may be more broadly distributed across holothuroids and may display variable colours according to species and the carotenoid they contain [33]. In *H. forskali*, this cell type benefited from an extensive functional and molecular characterisation, notably using proteomics, bulk and single-cell transcriptomics [33,93]. Interestingly, scRNA-seq analysis showed that carotenocytes were the most transcriptionally distinct cell types compared to all other coelomocyte types. Carotenocytes are predominantly found in the hydrovascular fluid, in which they are generally margined to the cell membrane or form small aggregates [33]. They would have an antioxidant function through the carotenoids they contain, participating in the regulation of ROS production during the immune response [33]. They may also have antibacterial activity, which is also characteristic of carotenoids [243].

Controversial cell types

Vibratile cells or spermatozoa

In holothuroids, vibratile cells or vibratile corpuscles were initially described as small cells (~ 5 µm) with a long flagellum measuring between 20 and 50 µm (Fig. 4T-U). These were reported in diverse holothuroid species [27,28,30,208,240]; however, they were more commonly found in echinoids [4,244–246]. Due to the mobility conferred by their flagellum, it was suggested that vibratile cells could have a function in coelomic fluid circulation [225]. However, it was recently demonstrated in holothuroids that these cells harbour a spermatozoon morphology, with a typical acrosome, when observed under SEM. In addition, systematic sexing of individuals in different sea cucumber species revealed that they were exclusively found in males [210]. Given these results, it was proposed that vibratile cells are contaminating spermatozoans that can be released during body fluid collection in holothuroids, and perhaps in all echinoderms. Since then, the true coelomocyte nature of vibratile cells could be confirmed in echinoids, but they are considered absent in other echinoderm classes [245]. Until proven otherwise, the occurrence of vibratile cells in holothuroids should be considered an artefact.

Haemocytes or carotenocytes

Haemocytes, historically referred to as red blood corpuscles, were described as pigmented spherical or biconcave cells ranging from 2 to 23 µm ([27]; e.g., haemocytes in *Colochirus quadrangularis*; Fig. 4V-W). Their red colour was attributed to a high intracellular concentration of haemoglobin [247]. These cells were primarily reported in Molpadida and Dendrochirotrida, but not in Aspirochirotrida (now separated into Holothuriida, Perciculida, and Synallactida) or Apodida [4]. In some species, haemocytes were considered dominant, representing up to 90% of coelomocytes, as observed in *Cucumaria miniata* [248].

Although their function remains unclear, haemocytes were proposed to participate in oxygen transport due to their haemoglobin content, thus having a limited role in immunity [27,28,248,249]. Notably, they were often found in higher abundance within the hydrovascular system [30,210,250,251]. Nonetheless, Caulier et al. [30] further showed that these cells can participate in coelomocyte aggregates in the hydrovascular fluid, producing large coloured bodies, suggesting an active function in immune response. Owing to their high haemoglobin concentration, the authors suggested that haemocytes could generate ROS involved in degrading encapsulated particles or pathogens. A more recent study indicates a broader distribution of haemocytes than previously assumed, with similar cells reported in apodids and several species in the genus *Holothuria* [210].

However, despite repeated detection of haemoglobin signatures [249,251], no study has conclusively demonstrated haemoglobin localisation within these cells by using modern molecular techniques such as fluorescent imaging or transcriptomic analysis. The recent identification of carotenocytes in *H. forskali*, which closely resemble previously described haemocytes, has raised doubts about the existence

of coelomocytes specialised for haemoglobin transport. Supporting this view, Wambreuse et al. [33] detected globin expression across all coelomocyte types as well as other tissues. This suggests that haemoglobin may be broadly expressed and released into body fluids rather than confined into specialised cells; hence, it could be detected in “blind” analysis of coelomocytes but with no specificity for these cells. Nonetheless, such specialised cells could exist in species adapted to low-oxygen environments, as proposed for *Paracaudina chiliensis* [249]. Further studies are therefore needed to clarify the presence of true oxygen-transporting coelomocytes and to confirm the broader distribution of carotenocytes in other holothuroid species. In the meantime, we recommend using the term “haemocyte” with caution and propose instead the terms “pigmented coelomocytes” or “haemocyte-like cells”, which do not necessarily imply the presence of haemoglobin.

Crystal cells or encapsulated biogenic crystals

Crystal cells were described as small (4 to 8 µm) prismatic cells, generally longer than wide (Fig. 4X-Y). Their name refers to the shape of the inclusion they contain, which resembles a crystal [4,27,252]. This inclusion is contained in a large vacuole that occupies most of the cell volume and restricts the nucleus to one side of the cell [228]. Crystal cells were mainly described in holothuroids, where they always represent a minority type of coelomocytes, with a proportion below 5% [252]. Hetzel [27] reports that crystal cells are not visible in fixed material due to osmotic shocks causing them to lose their crystalline inclusion. This observation leads some authors to believe that crystal cells may have a role in the osmoregulation of coelomic fluid [228,240]. More recently, different studies have shown contrasting results regarding this hypothesis: while hyposalinity stress had no impact on crystal cell concentration in *C. frondosa* [226], Chaar et al. [253] demonstrated that hypersalinity stress could affect crystal cell proportion in *H. grisea*, which may indicate a function in osmoregulation only during salinity increase.

Nonetheless, Ma et al. [252] have recently proposed a revision of crystal cells as phagocytes that have phagocytosed microcrystals corresponding to metabolic by-products. This revision is supported by a detailed morphological analysis of these cells combined with a chemical characterisation of their crystals, which revealed in *C. frondosa* that (i) many crystals are free from any membrane (i.e., outside a cell) and particularly present within some tissues, and (ii) the composition of the crystal could vary between uric acid, calcium carbonate and calcium oxalate dihydrate [31]. Thus, crystal cells would correspond to the encapsulated crystals (crystals within a cell) as a result of their phagocytosis by phagocytes to be excreted, similar to what is observed when foreign particles are injected into coelomic fluids [30]. Accordingly, crystal cells should not be considered as a proper coelomocyte type but rather as an excretion mechanism of metabolic by-products. However, as it was shown that their concentration can reflect some abiotic stress, such as air exposure or salinity increase [226,253], their evaluation remains relevant as a proxy of metabolic stress.

Uncertain cell types

Minute corpuscles

Minute corpuscles are reported as tiny spherical, possibly anucleate cells, measuring between 1 and 6 µm (Fig. 4Z-AA). They were only reported in a few holothuroid species [27,28,36,238,247]. While the function of these putative coelomocyte types remains totally understood, it was suggested that these could be debris artefacts, released by other cell types [27,33]. Hetzel [27] reported that some are suspected to be released by haemocytes, being coloured, while others are suspected to be released by phagocytes, and are colourless. Wambreuse et al. [33] also reported some small, pigmented corpuscles (~1.5 µm) suspected to be produced from bigger carotenocytes. The fact that these cellular elements could correspond to cell debris is supported by their increase in cell preparation over time post-cell collection [27,254]. However, a

recent study by Minakata et al. [255] on the sea star *Patiria pectinifera* described platelet-like cell fragments, some of which are very similar in size and morphology to sea cucumber minute corpuscles. In this study, platelet-like cell fragments are described as functionally analogous to vertebrate platelets, promoting aggregation notably at the site of the wound. In addition, the authors suggest that these produced extracellular vesicles may fuse to the coelomocyte membrane to deliver their contents into the coelomocyte cytoplasm, suggesting they could modulate the immune response of other coelomocyte types. These findings, therefore, warrant further investigation to validate or refute their status as a real immune component or cell debris in sea cucumbers.

Giant cells

Giant cells are large cells measuring between 20 and 54 μm and showing variable morphologies and a slightly granular cytoplasm [32, 208,256]. Some were also reported to have a light orange pigmentation ([33]; Fig. 4AB). These cells were only reported in a few species within the *Holothuria* genus [29]. Cytological staining reveals that these cells contain mucopolysaccharides [32]. However, the fact that this cell type is rarely observed, despite its relatively large size, suggests that it could also result from tissue contamination [33]. Queiroz et al. [32] also suggested that they could be dehiscent morula cells based on morphological observations. Currently, no hypothetical function has been proposed for this cell type.

Immune response in sea cucumbers

Non-self recognition

Although echinoderms cannot generate a new type of PRRs by somatic recombination as it occurs in gnathostomes, their genome appears to encode a large diversity of PRRs, which is thought to ensure a sufficient diversity to respond to a wide spectrum of invaders or even abiotic stress [6,99]. For example, it was shown early that coelomocytes in the Australian holothuroid species *Cucumaria tricolor* can promptly reject an allograft, while autografts were systematically accepted [232]. Later, coelomocyte response to a large diversity of non-self material was investigated, including biotic materials such as vertebrate erythrocytes or bacteria [238,257] or abiotic material such as coal particles or stain [30,216,258], and all could trigger a cellular response. The initiation of a cellular response could also be proven following exposure to diverse PAMPs, including some specific responses as a function of the coelomocyte population and the type of PAMPs [36,208]. This type of custom response, according to the stress, was eventually confirmed at the level of humoral response [76,80,87]. All these studies suggested that upon interaction with the proper ligand, coelomocyte PRRs, probably distinct between coelomocyte types, trigger a signalling cascade leading to the most adequate humoral and cellular response according to the encountered threat.

Phagocytosis

Phagocytosis plays a central role in echinoderm immunity [6,23]. This highly conserved process consists of entrapping a pathogen in a large vesicle called a phagosome, which then merges with lysosomes to form a mature phagolysosome in which the internal environment, comprising low pH and lytic enzymes, is prone to pathogen degradation [4] (Fig. 5A). This can also include a release of toxic reactive compounds like ROS during respiratory burst [23]. In echinoderms, this phenomenon is mainly ensured by phagocytes and is thought to be especially achieved by petaloid phagocytes [33,229], although some other cells were reported to achieve phagocytosis, including “lymphocytes” [208]. In addition, it is thought that when a phagocyte cannot perform phagocytosis anymore, it changes to the filopodial form and migrates toward the respiratory tree, where it would undergo an apoptotic process and be transferred to the cloaca to be expelled as dehiscent

coelomocyte aggregates [254,258]. Many molecular actors of this system are highly conserved, among which the complement system is known to play a role in opsonisation and promotes phagocytosis in holothuroids [106]. Other molecular actors could have been recently identified to play an important function in the phagocytosis of pathogens, such as NLCR4 and ApeC-containing protein [109,124].

Programmed cell deaths

Programmed cell deaths are essential processes in the immune response and are generally highly conserved mechanisms [259]. In holothuroids, four different types were reported, namely apoptosis, necroptosis, ferroptosis and ETosis [175,185,188], although their associated pathways are not fully resolved. Several studies have shown that apoptosis, i.e., self-programmed cell death, is finely regulated during immune response in sea cucumbers, with numerous genes involved in this process (see Section Cell death regulation). Coelomocyte apoptosis is thought to occur particularly in phagocytes during the immune response, once they have phagocytosed pathogens or foreign bodies [229]. However, other types of cell death have been described in holothuroids. A lytic cell death resembling vertebrate pyroptosis – consisting of a massive production of pro-inflammatory molecules – was recently described in *A. japonicus* and involved an MLKL/CASP1/NLRC4-like inflammasome [186]. Several genes involved in necroptosis were also reported, including RIPK and MLKL genes, although their exact activity is still uncertain [185]. Ferroptosis was recorded in coelomocytes during infection with pathogenic bacteria. This particular cell death implies an iron-mediated lipid peroxidation that disrupts the cell membrane. In *A. japonicus*, this type of cell death can be induced in intracellular bacteria through the action of ferroportin, an iron transporter, which has the effect of promoting bacterial lysis [187]. Finally, the formation of extracellular traps (ET), also referred to as ETosis, has been recently demonstrated in several echinoderm species [116,260,261] (Fig. 5B). This process, which may be common to all metazoans, involves the release of pre-decondensed chromatin, associated with antimicrobial molecules such as histones, into the extracellular environment, where it traps the pathogens in “DNA nets” to neutralise them [262,263]. In echinoderms, this process was reported in holothuroids *A. japonicus* and *H. forskali* [188,260] and in the echinoids *Strongylocentrotus intermedius* and *P. lividus* [260,261]. In *A. japonicus*, this process also appears to be dependent on the MLKL, as silencing its expression could inhibit ET formation [188]. In the two species, this process could be triggered by exposure to pathogenic bacteria, as well as diverse PAMPs and phorbol myristate (PMA) in holothuroids [188,261]. Although autophagy, i.e., the lysosome-mediated degradation of unnecessary or dysfunctional cellular components within the cell, does not constitute a form of cell death per se, it plays an important role in the cellular response to infection and may contribute to infection-mediated cell death [187]. Studies have demonstrated that sea cucumber coelomocytes are capable of autophagy [264,265], which is notably induced following bacterial infection and involves various enzymes and proteins, including transglutaminase 2-like (TGase) and calreticulin [264,265]. Despite the identification of diverse forms of programmed cell death in sea cucumbers, the underlying molecular pathways and their degree of conservation with those of vertebrates remain to be elucidated.

Aggregation

Aggregation, sometimes referred to as clotting, would serve to prevent the loss of body fluid by plugging the injury site and also prevent pathogen mobility by forming gel-like structures [266] (Fig. 5C). It seems that this process can occur simply by the collection of the body fluids, even by using sterile material [33,205]. However, this aggregation seemed to be calcium-dependent, as demonstrated by Taguchi et al. [205], who were able to dissociate the coelomocyte aggregate just by

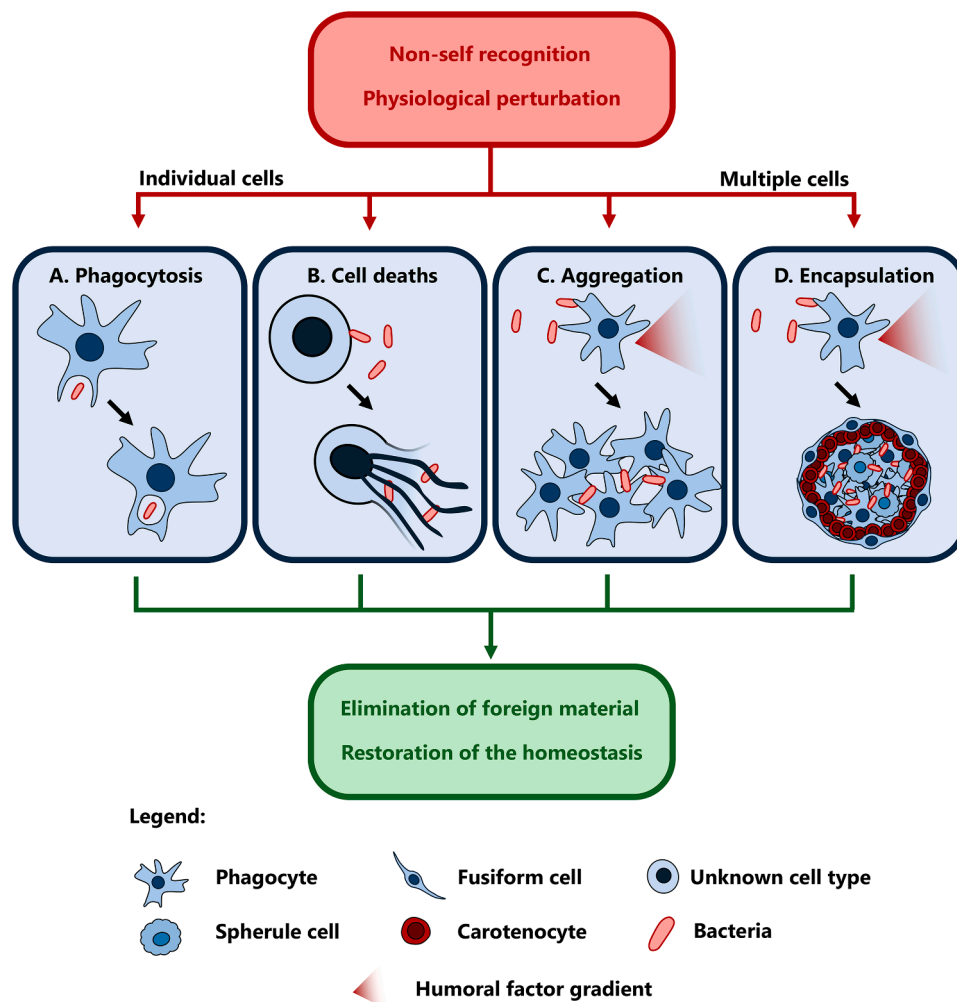


Fig. 5. Immune mechanisms in sea cucumbers. Interaction with pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) induces an immune response that can involve either single or multiple cells. Immune mechanisms involving single cells include phagocytosis and programmed cell deaths (e.g., apoptosis, necroptosis, ferroptosis and ETosis), while those involving multiple cells are aggregation and encapsulation. These are mediated by different cell types, each having its own functions. In optimal conditions, the set of these different mechanisms constitutes the sea cucumber immune response, which is supposed to eliminate foreign particles/pathogens and restore homeostasis.

adding EDTA – a calcium ion-sequestering molecule – in coelomocyte medium. Putative molecular actors of aggregation include extracellular or transmembrane proteins such as amassin, arylsulfatase and some lectins [81,125,126]. In echinoderms in general, cellular actors of the aggregation are considered to be mainly phagocytes, which, with their pseudopods, can form networks and capture other cell types [81,267]. However, a temporal study of the process revealed that other cell types are recruited at different time points, such as spherule cells or progenitor cells [33,205]. Overall, the aggregation mechanism is considered an essential mechanism, conserved among echinoderm classes [231].

Encapsulation and formation of coloured bodies

Encapsulation is an immune process involving aggregation of coelomocytes around a foreign body to entrap it, avoid its dissemination in the coelomic cavities and eventually eliminate it [4,216,258] (Fig. 5D). This process is thought to be reserved for larger foreign bodies or pathogens that cannot be phagocytosed. Thus, the phagocytes involved in encapsulation are sometimes referred to as “frustrated phagocytes” [4]. The process is initiated by phagocytes that, upon recognition of a foreign body, start to adhere to it. Phagocytes would then produce diverse cytokines to recruit other cells, including other phagocytes and spherulocytes. Notably, Wambreuse et al. [33] suggested that such a

signal could activate the mobility of morula cells to join the aggregate by chemotaxis so that they can release their fibrillar material. Other cell types may be involved in the production of cytotoxic compounds within the newly formed cell aggregate in order to degrade the encapsulated body. It was suggested that this could involve the melanisation process, similarly to the well-known encapsulation mechanism in arthropods [145]. Furthermore, a large amount of ROS would be released in these bodies to cause oxidative damage to the pathogen [30,33]. In some species, other cell types can be recruited: in particular, carotenocytes in *H. forskali* would form an “antioxidant shell”, thanks to the carotenoid they contain, which would prevent surrounding host tissues from being affected by oxidative damage [33]. Finally, it was suggested in *C. frondosa* that fusiform cells would form a last layer to compact the formed encapsulation body [30]. Nonetheless, the intervention of carotenocytes and fusiform cells in this process is yet to be confirmed in a larger number of species. These encapsulated bodies would then be eliminated via the anus, migrating from the coelomic cavity to the cloaca via the coelo-rectal canaliculi [258], consistent with the observation of such bodies in the environment surrounding the aboral extremity of some specimens [30].

The encapsulation mechanism would be responsible for the formation of coloured aggregates, which were initially called brown bodies according to their brownish colour [216]. However, it was shown later

that this colour can be highly variable, ranging from orange in *H. tubulosa* [257,258], to sharp red in *Psolus fabricii* and *H. forskali* [33, 231] and dark brown in *C. frondosa* [30]. Moreover, Caulier et al. [30] demonstrated that aggregate colouration ranges from red in the hydrovascular system to brown in the perivisceral fluid before being excreted out of *C. frondosa*. This transition was attributed to the deoxygenation of haemoglobin from haemocytes that join the aggregate, which may have a function in releasing ROS. However, recent observation of carotenocytes participating in aggregate formation suggests that aggregate colouration may also result from different types of carotenoids [33]. Pigmentation of coloured bodies is, therefore, still in debate; their colour could result from a melanisation process [257] or reflect the presence of pigmented coelomocytes such as haemocytes or carotenocytes [33,231]. In any case, it was demonstrated that these bodies are intrinsically linked to the immune response. Sections of brown bodies of specimens directly collected from the wild have

revealed the presence of sand grains, bacteria and parasitic gregarine eggs [237,268]. Furthermore, it is possible to induce their formation by injecting foreign particles into the echinoderm body, including coal particles, bacteria and mammalian erythrocytes [216,238,257].

New advances in molecular characterisation of coelomocytes

With regard to the current literature, it appears clear that we currently lack specific molecular data associated with the different coelomocyte types to have a clear idea about their respective functions. This gap in specific molecular characterisation in coelomocytes can be explained by the fact that coelomocytes are generally considered as a whole when molecular analyses are achieved, which could be attributed to the difficulty of isolating a pure coelomocyte population to carry out some subsequent targeted functional studies. In recent years, the advent of molecular biology, notably the development of single-cell RNA-

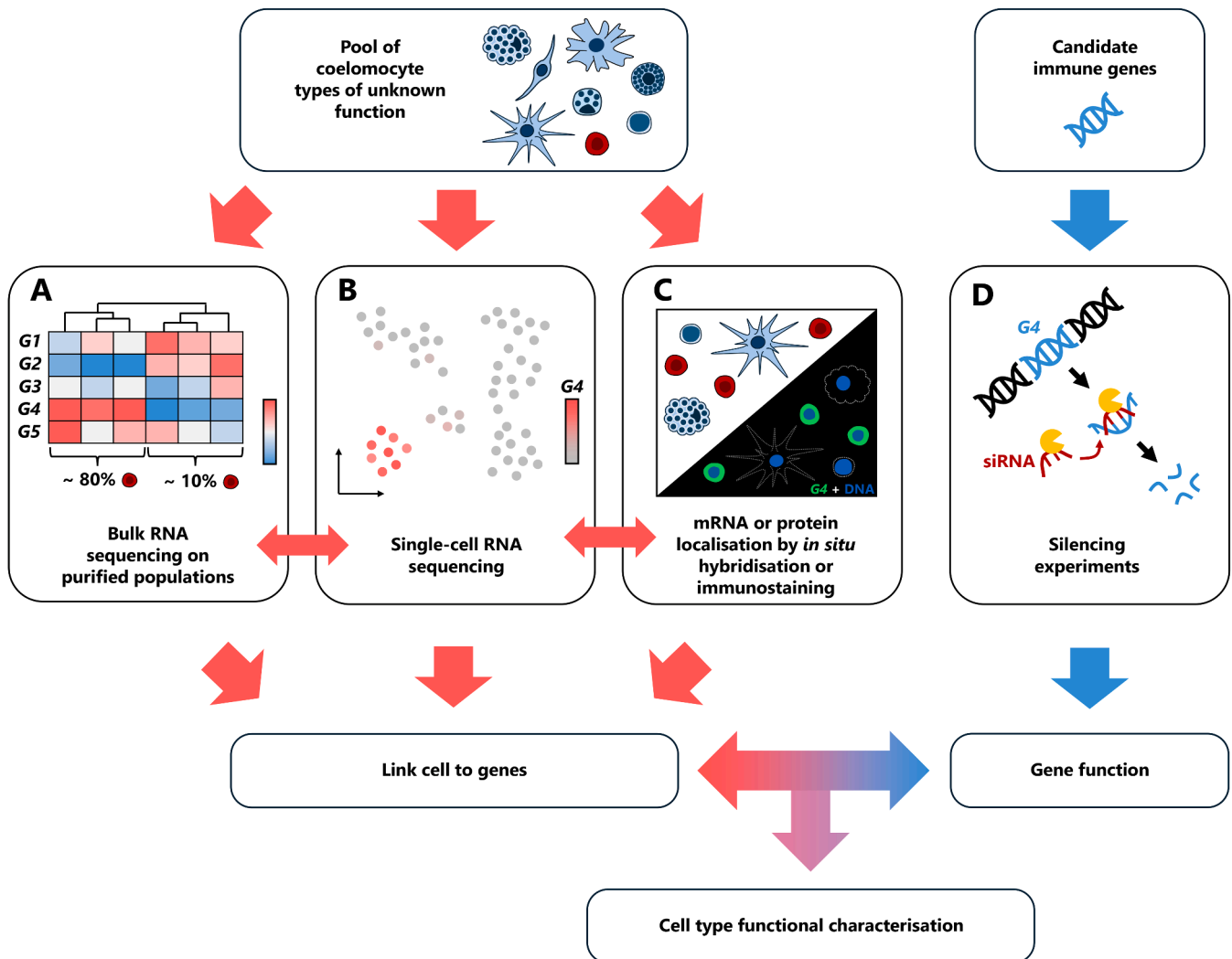


Fig. 6. Proposed framework for integrating molecular data into the functional characterisation of coelomocyte types. **A.** Comparison of different enriched cell populations enables the identification of population-specific gene expression. In particular, differential transcriptomic analysis using bulk RNA sequencing can reveal genes with population-specific expression; in this example, gene 4 (*G4*) is overexpressed in the enriched population of red cells (cell proportion is arbitrary in this example). **B.** Single-cell RNA sequencing provides gene expression profiles at single-cell resolution, allowing the identification of transcriptional clusters composed of cells with similar expression patterns. However, these datasets require validation; in this example, prior knowledge derived from panel A – specifically the expression of *G4* – enables the identification of the red cell cluster. **C.** Alternatively or complementarily, gene expression can be directly linked to cell morphology by localising marker gene transcripts (e.g., *G4*) or protein using fluorescent *in situ* hybridisation or immunocytochemistry, respectively. This approach requires prior imaging of live cells on slides or can be applied to enriched cell populations to provide a morphological context. **D.** Finally, to infer cell function from gene expression profiles, it is essential to characterise the function of marker genes, for instance, through gene silencing experiments (here using small interfering RNA – siRNA). Once gene expression is linked to cell morphotypes and marker gene function is established, a functional classification of coelomocyte cell types can be achieved.

sequencing (scRNA-seq), has allowed some pioneering studies to provide significant advances in our understanding of the expression of different subsets of coelomocytes in sea cucumbers. In addition, cutting-edge studies on other invertebrate models offer good perspectives for applying new approaches that allow for a comprehensive elucidation of the function of immune cells in these organisms. This section presents a framework to functionally characterise sea cucumber coelomocytes in the future and provides for each part key example studies carried out, if available, on sea cucumbers or in other invertebrates (Fig. 6).

A first approach to obtain specific molecular data on different coelomocyte populations, which was successfully employed in sea cucumbers, is performing functional or omics characterisation on an enriched coelomocyte subset (Fig. 6A). Yu et al. [35] performed RNA-seq analyses on two subsets of coelomocytes after their purification by centrifugation on Percoll density gradient, allowing them to separate “spherical cells” and “lymphocyte-like cells” (seemingly corresponding to a mix of phagocytes and spherule cells, and to progenitor cells, respectively). This revealed that spherical cells were more related to immune mechanisms per se, while lymphoid-like cells displayed a higher expression of genes involved in energy metabolism. In addition, lysosomal and phagocytic activity was tested by flow cytometry functional assays, revealing that spherical cells were predominantly involved in these activities, consistent with their gene expression [35]. Although it is difficult to attribute with certitude which cell types commonly described correspond to these two subsets, this article provided the first interesting transcriptomic dataset of coelomocyte subtypes. Recently, rather than purifying cell populations, Wambreuse et al. [33] took advantage of the natural enrichment of carotenocytes in the hydrovascular fluid to compare their expression with the cells of the perivisceral coelomic fluid, in which these carotenocytes are almost absent. This enabled performing differential expression analysis between the coelomocyte populations as well as carrying out some functional assays by flow cytometry, significantly advancing our knowledge on carotenocytes. These two studies demonstrate that comparing enriched coelomocyte populations are efficient in gaining expression knowledge on these, without the need to have pure populations.

In addition to this approach, many studies have recently employed scRNA-seq to characterise immune cells in invertebrates [269–271]. This powerful tool allows for the acquisition of gene expression profiles at the single-cell level without the need for prior cell purification (Fig. 6B). However, such tools require post-hoc validation to link the different “functional cell clusters” to different cell morphotypes. This can be provided by gene expression analysis on enriched populations for which morphology is known. For example, in the oyster *Crassostrea gigas*, scRNA-seq results were validated using a qPCR approach on different populations of hemocytes, enriched by gradient centrifugation [269].

Another way to validate scRNA-seq data is to localise the expression of some marker genes (Fig. 6C). To this end, antibodies can be employed to perform immunostaining, although this approach seems less suitable for a larger number of marker genes. In sea cucumbers, although some antibodies could be developed to target coelomocyte populations [207, 212, 272], they are still very few, and some show low degrees of specificity in comparison to other tissues. García-Arrarás et al. [212] used different monoclonal antibodies, namely Sph1-Sph4, to study the involvement of spherule cells in intestine regeneration. These antibodies were previously obtained from holothuroid immunogens, including homogenates of intestinal tissue (Sph1 and Sph4; [273]), collagen fraction (Sph2; [274]), and homeobox peptide (Sph3; [275]). All of these could successfully label spherule cells in *H. glaberrima*, but the signal of Sph2 was particularly localised in the granules. The monoclonal antibody mAb 3F6 could also recognise spherule cells in *A. japonicus*, but this antibody was also positive to the outer epidermis of the body wall and tentacles [272]. Finally, Li et al. [207] designed two other antibodies, namely Mab1C2 and Mab1E2, which reacted with all coelomocytes and lymphoid cells, respectively. Instead of using

immunostaining, *in situ* hybridisation offers a good alternative, especially with the recent advent of RNA *in situ* hybridisation by hybridisation chain reaction (RNA-HCR-FISH; [276]). This approach was, for example, used to link cell morphotypes to gene expression in the urochordate *Ciona robusta*, allowing the identification of 24 clusters out of the 33 clusters from blood cells, each corresponding to a cell morphotype [270]. This was allowed by imaging cells by light microscopy before performing the RNA-HCR-FISH, to then link the morphology to the expression of the marker genes.

In sea cucumbers, scRNA-seq data are still very limited on coelomocytes. A study aiming to describe the regenerative process of the intestine using scRNA-seq in *H. glaberrima* could identify four different clusters that were attributed to coelomocytes based on their markers, which are known as immune genes [122]. In addition, RNA-HCR-FISH on a marker gene coding for a FREP was employed to validate the expression of this gene in coelomocytes. Although which cluster corresponds to which morphotypes was not assessed in this study, it constitutes the first dataset presenting several coelomocyte clusters in an adult echinoderm. Another study performed scRNA-seq on coelomocytes from the perivisceral fluid, resulting in 10 different clusters in *H. forskali* [93]. Thanks to a list of specifically expressed genes previously obtained for carotenocytes [33], it was possible to confidently identify one cluster as the carotenocyte cluster. Finally, Wang et al. [94] have recently used scRNA-seq to characterise changes in cell composition following evisceration in hydrovascular and perivisceral fluids. Their dataset resulted in 19 cell clusters. Among these, they could identify a proliferative population of coelomocytes and used immunostaining on PIWI stem cell marker, which confirms an undifferentiated appearance of these cells. This study provides, therefore, key information on the process of recovering the coelomocyte population following evisceration. In these three pioneer studies, scRNA-seq has proven to greatly advance knowledge on specific gene expression between cell populations; however, a comprehensive study linking all cell morphotypes to all cell clusters is still lacking.

Complementary to the work done to link cell types with gene expression, it is important to characterise the function of immune genes beyond overexpression after an immunological challenge or solely based on sequence homology with characterised immune genes in model species (Fig. 6D). On this point, sea cucumbers can be considered a good model because extensive work has been done to characterise the function of genes individually (section Molecular actors of immunity; Fig. 2C). To this end, several approaches were adopted. The most common is silencing the expression of a gene of interest to see the impact on subsequent pathways or molecular functions, such as phagocytosis or ROS production. To do this, most studies have employed small interfering RNA (siRNA) to see how depletion of a gene of interest can influence the expression of other genes (e.g., IRAK4 regulates the expression of TLRs; [162]), the production of ROS (e.g., TRAF3; [164]) or phagocytic activity (e.g., ApeC-containing protein; [124]). In some other studies, the expression of sea cucumber immune genes was induced in heterologous vertebrate cellular models, such as in HEK293T cells, to see the effect on cellular metabolism, considering that these pathways are homologous to those of sea cucumbers (e.g., NF- κ B p105; [153]). Finally, some genes could be expressed recombinantly to obtain the protein and perform functional assays. For example, it was demonstrated that a recombinant lysozyme protein has significant antibacterial activity [132]. These different studies have been very informative and allow a better interpretation of data when, for example, differential omics tools are used and find that these genes are marker genes. However, it is noteworthy that a limitation of this was that some immune gene families in sea cucumbers, and overall in echinoderms, have undergone massive expansion in the number of genes, and it is sometimes difficult to appreciate whether genes with a similar sequence or annotation truly share the same function. In this way, genome-wide studies of the immune gene family are important to assess the diversity of genes within each family based on their structure. Such studies are still lacking

for most of the immune gene families; however, some recent advances have been carried out in FREPs or lectins, for example [96,121]. Gene functional characterisation is therefore crucial to better understand the function of cells expressing a set of genes of interest.

While the functional characterisation of the different coelomocyte subsets in sea cucumbers appears to be still ongoing, it can be seen that most of the framework presented in Fig. 6 has started to be addressed, and cross-linking all this information should enable the elucidation of the functional diversity of coelomocytes and establishing a functional cell classification, which will be of significant interest in many fields.

Outstanding questions and future directions in sea cucumber immunology

- Establishing a consensus on recognised coelomocyte morphotypes:

A crucial first step towards a better understanding of sea cucumber immunology is to establish uniform terminology for the different coelomocyte types. Despite the existence of several well-defined nomenclatures proposed in different reviews [4,27,29,224], these classification systems have often been developed independently by individual research groups and have not yet reached broad consensus within the scientific community. Consequently, they are applied inconsistently across studies. Broader efforts to establish a common classification at the echinoderm phylum level have also been undertaken [6], but these may sometimes have influenced cell recognition at the class level, such as the omission of fusiform cells from the classification proposed by Smith et al. [6]. To address this issue, a concise, consensus-based guideline developed collaboratively by researchers in the field would be valuable. Such a guideline should incorporate molecular markers to better reflect the functional diversity of coelomocytes, while remaining accessible to low-tech laboratories and applicable to ecological studies that use these cells as indicators of environmental stress. Establishing such a classification would improve reproducibility, facilitate comparisons between species and stress response studies, and enable larger-scale analyses of coelomocyte-type distribution and physiology.

- Increasing model diversity for molecular studies:

This review has highlighted the strong predominance of *A. japonicus* as a model species in immunological research on sea cucumbers in general, and particularly in studies characterising the molecular components of the immune system. This predominance is clearly driven by the species' high aquaculture interest and economic value. While having such an extensively studied model species is valuable for developing a broad picture of molecular immunity at the organismal level, it should not be considered representative of the entire class. Holothuroids currently comprise approximately 1800 species, some of which diverged millions of years ago and occupy highly distinct ecological niches [95, 277]. Such evolutionary and ecological divergence may have resulted in diversification of immune genes among different lineages. For example, Ren et al. [96] revealed substantial variation in the number of genes encoding C-type lectins in sea cucumbers, ranging from 49 in *A. japonicus* to 112 in *H. leucospilota*. The ecology of each species may also have shaped their immune system. *A. japonicus*, for instance, is a temperate species that experiences substantial seasonal changes in seawater temperature and salinity [278]. Several studies have investigated the effects of these environmental stressors on its immune gene expression [134,279]. However, these findings may not reflect the responses of tropical or deep-sea species, for which environmental conditions are generally more stable throughout their lifetime. Therefore, additional investigations of immune gene repertoire at the genomic level and their expression are needed across a more representative range of species spanning different taxa and ecological niches.

- Studying sea cucumber immunity at the holobiont level:

Recent advances suggest that the microbiota play an active role in the immune defence of sea cucumbers. Indeed, microbial communities have been shown to be highly specific to different body compartments of

sea cucumbers [63,280,281], suggesting active selection of bacterial strains and their associated functions. Notably, a large number of bacteria have been found to coexist with coelomocytes in the coelomic fluid, which likely reflects finely regulated interactions between the host and its microbiota [68,280]. In addition, several diseases have been associated with dysbiosis [282–285], and the use of probiotics has been shown to have beneficial effects on immune defence and disease prevention in *A. japonicus* [286–289]. Although many studies highlight the importance of the microbiota, the mechanisms underlying interactions between the host and its microbial community in the context of holobiont immunity remain largely unknown [62,290]. The present review has mainly focused on host immunity, reflecting the current state of the literature on sea cucumber immunology. However, future studies should address this knowledge gap by investigating immune responses at the level of the holobiont, i.e., by considering the host organism and its associated microbial community as an integrated entity [282].

Conclusion

This review highlights a fragmentation in sea cucumber immunology, where cellular and molecular approaches are often treated in isolation, limiting a truly integrative understanding of sea cucumber immunity as a whole. Current research remains heavily skewed toward molecular characterisation, with a primary focus on the commercially important species *A. japonicus*. While this body of work has substantially advanced our knowledge of holothuroid molecular immunity, its full potential remains underexploited at the cellular and functional levels.

The convergence of molecular insights with cellular biology represents a critical next step for the field. In this context, emerging single-cell technologies offer a transformative opportunity to resolve long-standing ambiguities and connect morphology to molecular functions. By synthesising existing knowledge and emphasising this integrative perspective, this review provides a framework for unifying molecular and cellular immunity in sea cucumbers. Such an approach is pivotal not only for improving the health management of farmed species but also for strengthening broader comparative immunology and for better interpreting ecological stress-response surveys in the context of one-health research.

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CRediT authorship contribution statement

Noé Wambreuse: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Guillaume Caulier:** Writing – review & editing, Conceptualization. **Jérôme Delroisse:** Writing – review & editing, Conceptualization. **Igor Eeckhaut:** Writing – review & editing, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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Data availability

No data was used for the research described in the article.

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